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(Especialidade: Regulação Bioquímica)

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Tese orientada pelo Doutor Carlos Alberto Alves Cordeiro e pelo

Prof. Doutor Alexandre Luís de Matos Botica Cortês Quintas

2009

De acordo com o disposto no artigo n°. 40 do Regulamento de Estudos Pós-Graduados da Universidade de Lisboa, Deliberação n° 961/2003, publicada no Diário da República – II Série n°. 153 – 5 de Julho de 2003, foram incluídos nesta dissertação os resultados dos seguintes artigos:

Gomes RA, Sousa Silva M, **Vicente Miranda H**, Ferreira AEN, Cordeiro C, Ponces Freire A. 2005. Protein glycation in *Saccharomyces cerevisiae*: Argpyrimidine formation and methylglyoxal catabolism. *FEBS J*. **272**: 4521-4531.

Gomes RA, **Vicente Miranda H**, Sousa Silva M, Graça G, Coelho AV, Ferreira AEN, Cordeiro C, Ponces Freire A. 2006. Yeast protein glycation *in vivo* by methylglyoxal: Molecular modification of glycolytic enzymes and heat shock proteins. *FEBS J*. **273**: 5273-5287.

Vicente Miranda H, Ferreira AEN, Cordeiro C, Ponces Freire A. 2006. Kinetic assay for measurement of enzyme concentration *in situ*. Anal Biochem **354**: 148-150.

Vicente Miranda H, Ferreira AEN, Quintas A, Cordeiro C, Ponces Freire A. 2008. Measuring intracellular enzyme concentrations. BAMBED **36**(2): 135-138.

Gomes RA, **Vicente Miranda H**, Sousa Silva M, Graça G, Coelho AV, Ferreira AEN, Cordeiro C, Ponces Freire A. 2008. Protein glycation and methylglyoxal metabolism in yeast: Finding peptide needles in protein haystacks. *FEMS Yeast Res.* **8**(1)*: 174-181.*

No cumprimento do disposto na referida deliberação, esclarece-se serem da minha responsabilidade a execução das experiências que estiveram na base dos resultados apresentados (excepto quando referido em contrário), assim como a interpretação e discussão dos mesmos.

Preface

Several neurodegenerative diseases, such as Parkinson's disease, are associated with specific proteins that undergo a misfolding process that occur by poorly understood mechanisms. Altough mutations in these proteins play an important role in this deleterious process, the majority of cases are not associated with genetic mutations. Other factors must contribute to the development of these misfolding diseases. The synthesis of the primary sequence of proteins, translated from the genetic instructions coded in the cell's DNA, is only the beginning of a protein life. An adequate folding and the correct post-translational modifications are vital for a protein to acquire its biologically active structure. It is then predictable that if the proteins are extensively modified in undesired ways, they may lose their structure and function. Throughout evolution, life has evolved systems able to cope with this phenomenon, including chaperones and a complex quality control mechanism to refold undesired misfolded protein, or as last resource, promoting protein degradation. However, these systems may be overcome and pathological conditions are likely to emerge. It is commonly accepted that ageing is the major risk factor for the development of neurodegenerative diseases. Nevertheless, all live forms are immersed in stressful environments that also contribute to disease onset and progression. Sugars, the main energy source of life, may play an important and unexpected role in this matter, since excessive glucose concentrations are known to be involved in diseases such as diabetes *mellitus*. Furthermore, reducing sugars react directly with proteins in non-enzymatic and uncontrolled processes, affecting their structure and function. Hence, it is not surprising that this post-translational modification is associated with several diseases including diabetes *mellitus* and amyloidotic neurodegenerative diseases. Beyond glucose, there are numerous other carbonyl-containing compounds that may irreversibly react with proteins. Methylglyoxal is believed to be the most important and reactive glycation agent in living cells and moreover, its formation is unavoidable, deriving from glycolysis, the most fundamental and universal energy producing pathway spanning all known life forms. Not surprisingly, cells evolved specific enzymatic systems to detoxify this compound.

The work hereby is primarily focused on the biochemical effects of methylglyoxal protein glycation *in vivo*, using yeast *Saccharomyces cerevisiae* as model eukaryote organism. Furthermore, the relationship between protein glycation and α-synuclein inclusion formation *in*

vivo was probed. This thesis is organized into six chapters and one appendix. The first chapter reviews and introduces the main concepts and the current understanding of protein glycation processes and amyloidogenesis, characteristic of neurodegenerative diseases, with special relevance to Parkinson's disease. Chapters II and III highlight the effects of protein glycation *in vivo*, providing the first evidences that this post-translational modification affects short lived proteins in preferential targets. Three glycolytic enzymes together with some chaperones were found to be modified and the activity of Hsp26, responsible for the prevention of protein misfolding and aggregation, was found to be modulated by methylglyoxal. These phenomena are dependent on the intracellular methylglyoxal concentration. Chapter IV establishes a link between protein glycation and amyloid disorders. It describes the effects of methylglyoxal protein glycation in yeast *Saccharomyces cerevisiae* cells expressing known mutations of *PARK1*, the gene encoding for α-synuclein. A clear-cut relationship between protein glycation and α-synuclein inclusion formation was identified, followed by an enhancement of toxicity. Differences between glycation of α-synuclein variants were observed and evaluated by peptide mass fingerprint of recombinant α-synuclein. In chapter V, a novel method for the characterization of α-synuclein post-translational modifications was developed. α-Synuclein from adult rat brain was found for the first time to be glycated. Moreover, among other post-translational modifications such as phosphorylation and acetylation, the glycated residues were identified and further suggest that glycation is likely to impair α-synuclein ubiquitylation and degradation by the ubiquitin proteasome system. The concluding remarks in chapter VI provide an overall framework of the findings presented in this research work. The relevance of the work and the perspectives for future research are also focused in this chapter. In parallel to the research on protein glycation effects *in vivo*, a novel method for the quantification of intracellular enzymes in living cells was developed and described in the appendix.

Aknowledgments/Agradecimentos

Concluída esta etapa, quero deixar uma palavra de agradecimento a todos os que, de alguma forma, contribuíram para a realização deste trabalho.

Primeiramente quero expressar o meu profundo agradecimento aos orientadores desta tese de doutoramento, Doutor Carlos Cordeiro e Doutor Alexandre Quintas.

O Carlos sempre me conseguiu surpreender com toda a sua calma, ponderação, compreensão e óptima disposição. O que mais tenho a agradecer foi a confiança que me atribuiu ao longo de todo o meu percurso de formação, acreditando sempre que eu conseguiria alcançar os objectivos, mesmo quando eu próprio estava céptico. Foi sempre capaz de dizer as palavras de incentivo chave para que eu pudesse avançar com sucesso. O seu elevado conhecimento em tantas matérias propiciou excelentes discussões científicas, transmitindo inúmeros conhecimentos que tiveram sem qualquer dúvida um papel preponderante no meu desenvolvimento como investigador. O Carlos não só contribuiu para uma excelente relação profissional, mas também para uma relação pessoal que me fez crescer como pessoa. Carlos, não tenho palavras para agradecer toda a ajuda que me deste, apenas te posso dizer que continuarei sempre a contar contigo.

Agradeço também ao Alexandre que principalmente no início da tese teve um papel imoportante para que eu pudesse dar os primeiros passos para este trabalho científico. Acolheu-me de braços abertos no seu laboratório e rapidamente abriu todas as portas para que o trabalho tivesse sempre a continuidade certa. O Alexandre sempre confiou inteiramente no meu trabalho e soube cultivar as minhas capacidades da melhor forma para que me pudesse tornar um investigador independente capaz de deliberar e executar as minhas próprias ideias. No momento certo do meu trabalho estabeleceu uma colaboração com o Doutor Tiago Outeiro, que se revelou preponderante para o desenrolar desta tese, e acompanhou sempre de perto todo o trabalho, transmitindo todo o seu profundo conhecimento nesta área de investigação. A sua experiência e domínio da biofísica foram cruciais para que eu pudesse ter uma perspectiva adequada ao trabalho que desenvolvemos. A nível pessoal, ganhamos uma amizade que guardo com muito respeito e em que aprecio a sua total abertura e sinceridade. Foi sem dúvida uma peça chave para

este trabalho. Alexandre, o meu mais sincero obrigado, e estou certo que vamos continuar juntos nesta aventura que é fazer ciência.

Tenho a agradecer ao Doutor Tiago Outeiro toda a ajuda que me deu na fase final deste trabalho. O Tiago recebeu-me no seu laboratório como se eu fizesse totalmente parte da sua equipa, fazendo-me sentir em "casa" desde o primeiro dia. Admiro profundamente a sua capacidade de trabalho, experiência e nível de conhecimento, que me têm inspirado a ser um melhor investigador. O Tiago esteve sempre disponível e ao meu lado incentivando-me sempre da melhor forma e ensinando-me sem quaisquer reservas tudo o que estava ao seu alcance. Posso afirmar que a minha cultura científica se deve bastante à minha presença no seu laboratório. Tiago, agradeço-te profundamente toda a tua ajuda e espero que possamos trabalhar durante muito mais tempo e alcançar todos os nossos objectivos.

Quero deixar uma enorme palavra de agradecimento à pessoa que me mostrou o que é a ciência! Falo da Professora Ana Ponces Freire. A Ana sempre foi uma fonte de inspiração desde os tempos da minha licenciatura em bioquímica. Sempre que me sentia desalentado ou precisava de energia para continuar em frente, apenas precisei de bater à sua porta. Fui sempre recebido de braços abertos e não eram precisos mais que alguns segundos para que eu me sentisse novamente motivado e apaixonado pela ciência. É uma pessoa que admiro profundamente por toda a sua serenidade, confiança, conhecimento e espírito inovador e sempre jovem. Ana, muito obrigado por toda a sua ajuda, será uma amiga que guardarei para sempre.

Ao Prof. António Ferreira agradeço todo o conhecimento que me transmitiu no maravilhoso mundo da simulação computacional. Aprendi a investigar de forma diferente e a pensar nos problemas de um ponto de vista teórico e fundamentado, o que me fez crescer como nunca. A sua sensatez, serenidade e amizade são os seus maiores atributos que contribuíram para me tornar um melhor investigador. António, muito obrigado pela tua ajuda, sei que poderei contar sempre contigo.

Não posso deixar de prestar o meu profundo reconhecimento aos meus colegas e amigos de bancada. Ao grupo de Enzimologia, quero agradecer todos os momentos divertidos por que passámos num ambiente excelente de total descontração e espírito de entre-ajuda. Quero

agradecer à Doutora Marta Sousa Silva toda a amizade e ajuda nos meus primeiros momentos na bancada, assim como todo o incentivo que me deu ao longo destes anos em que nos conhecemos. Ao Doutor Ricardo Gomes, não há palavras para expressar o meu agradecimento. Admiro a sua capacidade de trabalho e humildade que sempre demonstrou desde o primeiro dia em que o conheci. Era eu ainda um inexperiente aluno de licenciatura, quando tivemos as primeiras conversas sobre as experiências de laboratório e sempre me tratou como igual. Foi a pessoa que me ensinou a dar os primeiros passos no laboratório como investigador e é ainda um mentor que tanto me ajudou nesta tese de doutoramento. Ricardo, és o maior. Não posso deixar de agradecer à Lídia Barata, por toda a sua simpatia e por estar sempre com um sorriso nos lábios. Muito nos rimos na sua presença num verdadeiro espírito de conjunto e amizade. Ao Nuno Lages, colega de doutoramento, agradeço todas as discussões científicas e amizade que mostrou ao longo destes anos. Agradeço ao ex-membro Doutor Kirill, que esteve sempre disposto a ajudar-me e a incentivar o meu trabalho. Mais recentemente, o Doutor Gonçalo Costa tem sido um excelente colega de trabalho ajudando-me em tudo o que pode. A sua experiência e capacidade de trabalho, são inspiradoras. Obrigado, amigo. Ao Luís Oliveira, quero deixar um especial agradecimento. Fomos colegas desde os nossos tempos de licenciatura e a amizade tem sido um ponto fulcral ao longo de todos estes anos. Partilhamos a bancada não só no grupo de enzimologia, mas também no Instituto Egas Moniz, e juntos conseguimos pensar sempre melhor e avançar pelo melhor caminho. Purificámos ou não purificámos a proteína? Este foi sem dúvida um grande desafio neste projecto de doutoramento, mas vamos partilhar ainda muitos outros. Não quero apenas destacar a relação profissional, pois a nossa relação pessoal é a mais importante, não só pelos momentos de diversão fora do laboratório, mas também pela música, uma das grandes paixões da minha vida. Luís, ainda vamos tocar e cantar por esse mundo fora. Muito obrigado pela tua ajuda.
Tenho também de agradecer à Prof. Carla Ascenso. Embora nos bastidores deste trabalho, foi sempre capaz de me ajudar e incentivar. Carla, obrigado pela tua amizade.

Quero também deixar um enorme agradecimento a todos os colegas da Unidade de Neurociência Celular e Molecular no IMM (ou será antes, todas?). A Doutora Sandra foi a primeira pessoa a receber-me no laboratório onde me transmitiu muito conhecimento científico, principalmente sobre a nossa amiga “Levedura”. Muito obrigado pela tua ajuda, amizade e excelentes conselhos que sempre me deste. Doutora Rita, tem sido um enorme prazer trabalhar contigo, admiro todo o conhecimento que tens e humildade que sempre mostraste. Obrigado por

tudo e acima de tudo, por me ensinares juntamente com a Joana a trabalhar com linhas celulares de mamíferos! Doutora Joana, que diversão que é trabalhar contigo, sempre bem-disposta e sorridente, aqueles pulos de felicidade quando as experiências resultam e todo o carinho que tiveste sempre por mim são muito especiais. Muito obrigado (apenas como nota, se te casares uma quarta vez com o mesmo marido, lá estarei a tocar musica novamente). Sónia, que excelente colega de laboratório, estiveste sempre disponível para me ajudar em tudo e estou certo que a nossa amizade veio para ficar. Doutora Teresa, tem sido óptimo trabalhar contigo. Tens estado sempre disponível para me ajudar, principalmente nesta rampa final. Obrigado por tudo. À restante equipa, quero agradecer o excelente ambiente que partilhamos no nosso laboratório, por toda a boa disposição, amizade e entre-ajuda que nos tem propiciado tão bons momentos. O sorrizo da Ana, a humildade da Olga, a simpatida da Oli, a amizade da Susana, a juventude da Leonor, e a boa disposição da Zrinka e da Patricia, em conjunto com a Sandra, Rita, Joana e Sónia, fazem delas as 12 magníficas. Muito obrigado a todas.

Como a vida não é só trabalho, quero deixar um profundo agradecimento a todos os amigos que me rodeiam. Sejam antigos colegas, sejam elementos de uma banda de música (sim os Scramble) ou até moradores da mesma rua (viva o C.N.), ou até do outro lado do rio (os marqueses), muito obrigado.

Quero destacar a importância da querida Sara Mendes em todo este percurso. São já quase 4 anos de muito carinho e amizade. Os teus conselhos, a tua ajuda e a paciência para me aturar, principalmente nesta etapa final, não têm igual. És e serás sempre muito importante na minha vida.

Por fim, mas em primeiríssimo lugar, vem a minha excelente família. Sem vocês, não passo de um grão de areia nesta praia que é a vida. Vocês dão sentido à minha vida e ajudam-me em todos os momentos. Que bom que é sentir-me feliz na vossa companhia e saber que tenho sempre um coração amigo pronto para dar tudo o que eu precise. Tia Milú, não me esqueço da importância que sempre tiveste na minha vida. A tua força e dedicação em tudo o que acreditas, sempre foram uma inspiração. Obrigado pelo teu amor e amizade. Filipe, és o meu primo/irmão e sei que estarás sempre do meu lado.

Tenho de deixar um especial agradecimento aos meus pais, Jorge e Nanda e ao meu irmão e sua esposa, Rui e Vera. Não existem palavras no dicionário para expressar a importância que têm na minha vida. Quem me dera ter a bondade da minha mãe, o coração do meu pai, e a humildade do meu irmão. Vocês são a minha fonte de inspiração. Muito obrigado por tudo.

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Summary

Protein glycation, the non-enzymatic and irreversible modification of amino groups by carbonyl compounds, results in the formation of advanced glycation end products (AGE). This post-translational modification plays a key role in several human diseases, including diabetes mellitus and neurodegenerative diseases. However, the role of glycation in pathological conditions is still unknown.

In the work presented in this PhD thesis, a novel approach was developed to investigate protein glycation by methylglyoxal *in vivo*. Moreover, the role of glycation by methylglyoxal in protein amyloidogenesis was also investigated. It was found that argpyrimidine (methylglyoxal-derived AGE, MAGE) occurs in yeast in specific protein targets. Although protein glycation is believed to occur only in long-lived proteins in higher organisms, AGE modifications also affected proteins in short-lived organisms. The rate of methylglyoxal formation and protein glycation are directly associated, as determined by the use of yeast null mutant strains. A kinetic model of methylglyoxal metabolism was developed to investigate the relative importance of methylglyoxal catabolic pathways and methylglyoxal formation rate on its steady state concentration. It was found that both the glyoxalase system and aldose reductase enzyme were equally important in detoxifying methylgyoxal. A higher glycolytic flux is predicted to increase methylglyoxal concentration even in the presence of its catabolic routes. In fact, yeast cells recapitulate the predicted methylglyoxal increase. Challenging non growing yeast cells with D-glucose increased methylglyoxal concentration and induced MAGE formation. A relationship between the intracellular concentration of methylglyoxal and MAGE formation was then established and yeast cells were shown to form MAGE only when anti-glycation defenses were overcome.

Glycation targets were identified by mass spectrometry and the heat shock proteins Hsp71/72 and Hsp26 were found to be glycated *in vivo*. Interestingly, Hsp26, a critical element in the unfolding stress response, was only detected in the protein soluble fraction in highly glycated yeast cells. This finding suggests that Hsp26 is most likely activated upon methylglyoxal glycation. Three glycolytic enzymes were also modified in yeast, namely phosphoglycerate mutase, aldolase and enolase 2, which is the main glycation target. With the identification of enolase R414 residue modification, a critical residue for dimer stability, this modification may disrupt electrostatic

interactions with E20 on the other enolase chain that stabilize the enolase dimer. This may lead to its dissociation and consequent formation of inactive monomers. Enolase 2 has an arginine-rich region that appears to promote MAGE formation. Since enolase 2 glycation does not affect yeast metabolism, the results suggest that this protein may act as a methylglyoxal scavenger or signaling protein, assisting yeast cells to cope with methylglyoxal toxicity.

Yeast cells were established as a powerfull system in which to investigate both pretein glycation and the molecular basis of neurodegenerative disorders. Thus, we sought to investigate the link between protein glycation and amyloid disorders. Parkinson's disease, the second most prevalent neurodegenerative disease affecting 2% of the human population over the age of 65 years is characterized by the formation of proteinaceous intraneuronal cytoplasmic inclusions known as Lewy bodies, which are primarily composed of alpha-synuclein (a-syn). Here we studied the effect of protein glycation on the intracellular localization and toxicity of a-syn. Different a-syn variants were fused to GFP and expressed in *Saccharomyces cerevisiae* and the formation of intracellular *foci* were detected by fluorescence microscopy. The intracellular distribution of a-syn is not affected by glycation conditions.However, the percentage of cells containing a-syn *foci* increased upon glycation in cells expressing either WT or A53T a-syn, in contrast to cells expressing E46K. In parallel, we observed increased toxicity and decreased cell viability under glycating conditions in the reference strain used. The percentage of cells presenting a-syn *foci* was also higher in mutant yeast cells strain lacking the methylglyoxal catabolic glyoxalase I, reinforcing that this process is methylglyoxal concentration dependent. These results provide the first evidence that glycation *in vivo* induces the formation of a-syn inclusions.

To investigate if glycation of different amino acid residues in a-syn would explain the differences observed *in vivo*, the proteins were expressed and purified in *E. coli.* Methylglyoxal glycation was performed *in vitro* and mass spectrometry was employed to identify the specific MAGE location in a-syn. The glycation pattern of a-syn variants was found to be different. These results suggest that the exposure of the putative glycated residues might be different in the different a-syn variants. These structural differences may explain the singular behavior of each mutant form of a-syn *in vivo*. Glycation may occur in residues which are normally used for ubiquitylation and, in this case, it may block normal a-syn degradation. Thus, glycation may contribute to the accumulation of a-syn and all downstream effects such as aggregation and cytotoxicity.

To examine if a-syn is a glycated *in vivo*, a novel characterization method was developed. a-Syn is a thermostable protein, and we explored this property for the development of a method for enriching and characterizing a-syn in protein extracts. Rat brains were dissected and samples from different brain regions were prepared. Lysate heating led to an enrichment in the content of a-syn by up to 5 fold in the soluble fraction. Two-dimensional PAGE electrophoresis enabled further characterization of the post-translational modifications by mass spectrometry. In agreement with the glycation results obtained *in vitro*, a-syn was found to be AGE-modified at several ubiquitylation sites. This characterization method proved to be extremely effective for the analysis of post-translational modifications, revealing the occurrence of glycation, acetylation and phosphorylation.

The results and conclusions presented in this thesis contribute not only to the increase of our knowledge about protein glycation and its biochemical effects *in vivo*, but also to the assignment of a clear role for this non-enzymatic process in the development of amyloid disorders. In this context, yeast cells will certainly be a useful eukaryotic model to study these processes at a molecular level. Altogether, these findings may contribute to the design of novel and improved therapeutic strategies for neurodegenerative disorders associated with amyloid deposition.

Resumo

A modificação irreversível de grupos amina em proteínas por compostos carbonílicos é denominada de glicação de proteínas sendo os produtos finais desta reacção designados por produtos avançados de glicação (AGEs). Esta modificação pós-traducional desempenha um papel importante em várias doenças como a diabetes *mellitus* e doenças neurodegenerativas. No entanto, o papel da glicação no desenvolvimento de patologias é ainda um processo desconhecido.

Neste trabalho foi desenvolvida uma nova abordagem para investigar a glicação de proteínas e o metabolismo do metilglioxal *in vivo*, bem como o papel da glicação pelo metilglioxal no contexto das doenças neurodegenerativas. Observou-se que a formação de argpirimidina, um produto avançado de glicação derivado do metilglioxal (MAGE), ocorre em leveduras em proteínas específicas. Apesar da concepção de que a glicação de proteínas é um processo aleatório que ocorre em proteínas abundantes nos organismos superiores, observou-se que a formação de AGEs também ocorre em organismos com um curto período de vida. Através da análise de estirpes de leveduras com delecção de genes relacionados com o catabolismo do metilglioxal, foi identificada uma relação entre a velocidade de formação de metilglioxal e a glicação de proteínas. Para investigar a importância dos diferentes sistemas de catabolismo do metilglioxal, desenvolveu-se um modelo cinético do metabolismo do metilglioxal. Com base neste modelo, foi possível prever que o sistema dos glioxalases e o enzima aldose reductase são igualmente importantes na homeostase do metilglioxal. Previu-se também que a concentração intracelular de metilglioxal é proporcional ao fluxo da via glicolítica. Estas estimativas foram validadas em leveduras sujeitas a incubações com elevadas concentrações de D-glucose. Nestas condições o nível de glicação de proteínas pelo metilglioxal é claramente superior.

Por espectrometria de massa, foram identificadas as proteínas alvo de glicação pelo metilglioxal em levedura, nomeadamente proteínas de choque térmico Hsp71/72 e Hsp26, assim como alguns enzimas da via glicolítica. Curiosamente, a Hsp26 apenas foi detectada em extractos de proteínas solúveis de leveduras sujeitas a condições de glicação. Este resultado indicía que a Hsp26 pode ser activada pela glicação pelo metilglioxal. Os enzimas enolase 2, fosfoglicerato mutase e aldolase foram também identificados como proteínas modificadas com MAGE. Embora o enolase 2 seja o principal alvo de glicação em levedura e esta modificação reduza a sua actividade,

não se observou nenhum efeito da glicação deste enzima na viabilidade da levedura nem no seu metabolismo da glucose. A formação de MAGE especificamente no resíduo de arginina na posição 414, levou-nos a sugerir que esta modificação pode resultar na quebra das interacções electroestáticas que o resíduo R414 estabelece com o resíduo E20 da outra cadeia do enolase, levando à dissociação do dímero de enolase 2 e formação de monómeros inactivos. Curiosamente o enolase 2 apresenta uma cavidade rica em resíduos de arginina, localizada na interface do dímero, que poderá reagir com o metilglioxal que não é metabolizado pelo sistema dos glioxalases e aldose reductase e, dessa forma, sequestrá-lo.

Assim, conclui-se que a levedura *S. cerevisiae* é um bom modelo para investigar os efeitos bioquímicos da glicação na estrutura e função de proteínas *in vivo*. Desta forma, foi possível estudar o efeito da glicação no contexto da doença de Parkinson. Esta é uma doença neurodegenerativa que afecta cerca de 2% da população mundial com uma idade superior a 65 anos e é caracterizada pela formação de inclusões protéicas, maioritariamente compostas por α-sinucleína (a-syn), denominadas de corpos de Lewy (LBs). Neste trabalho foi investigado o efeito da glicação na localização intracelular e toxicidade da a-syn. As diferentes variantes de a-syn foram expressas como uma proteína de fusão com a GFP em *Saccharomyces cerevisiae* e a formação de inclusões foi observada por microscopia de fluorescência. Constatou-se que em condições de glicação a localização intracelular da a-syn não foi alterada. No entanto, nestas condições, a percentagem de células com inclusões de a-syn aumentou significativamente na expressão das variantes WT e A53T, em comparação com a expressão da variante E46K onde não se observaram diferenças. Paralelamente, a glicação é responsável por um aumento da toxicidade da a-syn e uma diminuiução da viabilidade das células da estirpe de referência BY4741. A estirpe com delecção do gene que codifica para o glioxalase I (enzima importante no catabolismo do metilglioxal) também apresenta uma maior percentagem de células com inclusões, reforçando a hipótese que a formação de inclusões pode estar relacionada com a concentração intracelular de metilglioxal. Este estudo demonstrou pela primeira vez que a glicação induz a formação de inclusões de a-syn *in vivo*.

De forma a investigar se a glicação afecta selectivamente diferentes aminoácidos entre as várias variantes de a-syn, as proteínas recombinantes foram produzidas em bactérias e recorrendo à técnica de espectrometria de massa, foram identificados os resíduos glicados *in vitro* pelo metilglioxal nas diferentes variantes de a-syn. Foi observado que os perfis de glicação são diferentes entre as variantes. Este resultado sugere que embora as variantes de a-syn apresentem

um nível de estrutura secundário semelhante que não as permite diferenciar, a exposição dos resíduos de lisina ao solvente poderá ser diferente. Desta forma, as diferenças estruturais entre as variantes de a-syn poderão estar relacionadas com o seu comportamento individual observado *in vivo*. Curiosamente, os resíduos que habitualmente são alvos de ubiquitinação foram glicados, o que sugere que a glicação poderá interferir com o processo de degradação da a-syn. Nesse caso, a glicação pode contribuir para a acumulação, agregação e citotoxicidade da a-syn.

Para confirmar se a glicação ocorre na a-syn *in vivo*, desenvolveu-se um novo método para a sua caracterização a partir de amostras de várias regiões cerebrais. Tendo por base que a a-syn é uma proteína termo estável, foi possível desenvolver um método que enriquece o conteúdo de a-syn em extractos proteicos e permite a sua posterior caracterização por espectrometria de massa. Nesse sentido, os extractos proteicos de diferentes regiões de cérebros de ratos foram aquecidos a 90 °C e posteriormente analisados. Com este tratamento, foi possível enriquecer a quantidade de a-syn, permitindo analisar a proteína por espectrometria de massa e identificar as suas modificações pós-traducionais. Tal como foi observado na a-syn glicada *in vitro*, os resíduos de aminoácidos de a-syn de cérebro de rato que são alvos de ubiquitinação também se apresentaram glicados. Este método mostrou-se extremamente eficaz na análise das modificações pós-traducionais de a-syn, tendo sido identificados *in vivo* resíduos de aminoácidos glicados, acetilados e fosforilados.

Os resultados e conclusões apresentados nesta tese contribuem não só para um maior conhecimento sobre a glicação de proteínas e os seus efeitos bioquímicos *in vivo*, mas também permitiram estabelecer uma relação entre a glicação e o desenvolvimento de doenças amiloidogénicas. Neste contexto, a levedura mostrou-se como um modelo celular que permite estudar estes processos a nível molecular. Desta forma, o trabalho realizado poderá contribuir para o desenho de novas estratégicas terapêuticas para doenças neurodegenerativas associadas com a deposição amilóide de proteínas.

Abbreviations

A	Alanine
A30P	Alanine to proline substitution in residue 30 of alpha-synuclein
A53T	Alanine to threonine substitution in residue 53 of alpha-synuclein
Aβ	Amyloid β peptide
ACN	Acetonitrile
AD	Alzheimer's disease
Ade	Adenine
ADP	Adenosine diphosphate
AGES	Advanced glycation end-products
ALR	Aldose reductase
ALS	Amyotrophic lateral sclerosis
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
APP	Amyloid β precursor protein
a-Syn	Alpha-synuclein
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
CD	Circular dichroism
CEL	N^{ε}-(carboxyethyl)lysine
CFU	Colony forming units
CID	Collision-induced dissociation
CJD	Creutzfeld Jakob disease
CK	Casein kinase
CML	N^{ε}-(carboxymethyl)lysine
CoA	Coenzyme A
CSF	Cerebrospinal fluid
CSPa	Cysteine-string protein alpha
C-terminal	Carboxyl-terminal
D	Aspartatic acid

Da	Dalton
DHAP	Dihydroxyacetone phosphate
DHB	2,5-Dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E	Glutamic acid
E. coli	*Escherichia coli*
E_0	Initial enzyme concentration
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
E46K	Glutamic acid to lysine substitution in residue 46 of alpha-synuclein
E_{add}	Added enzyme concentration
ECD	Electron capture dissociation
EDTA	Ethylenediamine tetraacetic acid
E_{in}	Assayed enzyme concentration
ENO2	Yeast enolase2 gene
Eq	Equation
ER	Endoplasmic reticulum
ESI	Electrospray ionization
FAP	Familial amyloidotic polyneurophaty
FRDA	Friedreich's ataxia
FTICR	Fourier transform ion cyclotron resonance
G	Glycine
GAP	D-Glyceraldehyde 3-phosphate
GAPDH	D-Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GLO1	Yeast glyoxalase I gene
GLO2	Yeast glyoxalase II gene coding for the cytosolic isoform
GLO4	Yeast glyoxalase II gene coding for the mitochondrial isoform
GLR	Glutathione oxidoreductase

GLXI	Glyoxalase I
GLXII	Glyoxalase II
GPX	Glutathione peroxidase
GRE3	Yeast aldose reductase gene
GRKs	G-protein-coupled receptor kinases
GSH	Glutathione (L-γ-glutamyl-L-cysteinylglycine)
Gsh	Glutathione synthase gene
GST	Glutathione S-transferase
GSSG	Oxidized glutathione
H	Histidine
HbA_1	Haemoglobin glycated by glucose
HOG	High osmolarity glycerol
HPLC	High performance liquid chromatography
HSA	Human serum albumin
Hsp	Heat shock protein
HT	Huntington's disease
ICAM-1	Intercellular adhesion molecule 1
IGF-I	Insulin growth factor-1
IL-1	Interleukin-1
IL-6	Interleukin-6
ImageJ	Image processing and analysis in Java
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRMPD	Infrared multiphoton dissociation
K	Lysine
k_{cat}	Catalytic constant
K_m	Michaelis-Menten constant
K_s	Enzyme specific constant
LB	Luria Bertani
LBs	Lewy bodies
Lc	Loading control
LC-MS	Liquid cromathography-mass spectrometry
LDL	Low-density lipoprotein

Lys	Lysine
M	Methionine
MAGE	Methylglyoxal advanced glycation end products
MALDI	Matrix assisted laser desorption ionization
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MES	2-(*N*-morpholino)ethanesulfonic acid
MG-H	Hydroimidazolone
min	Minutes
MODIC	Lysine-arginine methylglyoxal-derived cross-link
MOLD	Methylglyoxal-lysine dimer
mRNA	Messenger RNA
MS	Mass spectrometry
N	Asparagine
NAC	Non-amyloid beta peptide component of Alzheimer's disease amyloid
NAD^+	Nicotinamide adenine dinucleotide, oxidized form
NADH	Nicotinamide adenine dinucleotide, reduced form
$NADP^+$	Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NF-κB	Nuclear factor-κB
NFTs	Neurofibrillary tangles
NMR	Nuclear magnetic ressonance
P	Proline
PAGE	Polyacrilamide gel electrophoresis
PARK1	Alpha-synuclein gene
PBS	Phosphate buffer saline
PD	Parkinson's disease
PDB	Protein data bank
PepMix1	Peptide mixture for mass spectrometers calibration
P_i	Inorganic phosphate
PK	Proteinase K

PLAS	Power law analysis and simulation software
PLD2	Phospholipase D2
PLK	Polo-like kinase
PMF	Peptide mass fingerprint
PMSF	Phenylmethylsulfonyl fluoride
PolyQ	Polyglutamine domain
Pro	Proline
PrP	Prion protein
PrP^{c}	Cellular prion protein
PrP^{res}	Proteinase K resistant prion protein
PTM	Post-translational modifications
PVDF	Polyvinylidene difluoride
Q	Glutamine
R	Arginine
RAGE	Receptor for advanced glycation end-products
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Serine
S. cerevisiae	*Saccharomyces cerevisiae*
S129A	Serine to alanine substitution in residue 129 of alpha-synuclein
S129D	Serine to aspartic acid substitution in residue 129 of alpha-synuclein
SD	Standard deviation
SDLGSH	*S*-D-Lactoylglutathione
SDS	Sodium dodecyl sulphate
Ser	Serine
SOD	Superoxide dismutase
Sod1	Copper-zinc superoxide dismutase gene
sRAGE	Soluble receptor for advanced glycation end-products
SSAO	Semicarbazide-sensitive amine oxidase
T	Threonine
TAE	Tris/acetate/EDTA buffer
TBS	Tris-buffer saline

TFA	Trifluoroacetic acid
TH	Tyrosine hydroxilase
THP	Tetrahydropyrimidine
TIM	Triosephosphate isomerase
TNF-α	Tumor necrosis factor-α
TOF	Time of flight
Tris	Trishydroxymethylaminomethane
Trp	Tryptophan
TTR	Transthyretin
Tyr	Tyrosin
Tween-20	Polysorbate 20 sorbitan monolaurate
UBP	Ubiquitin specific proteases
UCH	Ubiquitin carboxy terminal hydrolase
UPS	Ubiquitin-proteasome system
Ura	Uracil
UV	Ultra violet
UV-vis	Ultra violet - visible
V	Valine
VEGF	Vascular endothelial growth factor
WT	Wild-type
Yap1	Yeast transcription factor of the AP-1 family
YFH1	Yeast frataxin homologue gene
Yhb1	Yeast flavohemoglobin gene
YNB	Yeast nitrogen base growth medium
YPGlu	Yeast extract, peptone and D-glucose growth medium
α-CHCA	α-Cyano-4-hydroxicinamic acid
% v/v	Percentage expressed in volume/volume
% w/v	Percentage expressed in weight/volume
2D-PAGE	Bi-dimensional polyacrilamide gel electrophoresis

Chapter I

Introduction

1. Protein Folding

The three dimensional structure of a protein has long been recognized to determine its functional state. In 1951 Linus Pauling and co-workers discovered two simple arrangements of amino acids residues in a polypeptide chain, the α-helix and the β-sheet (Pauling*, et al.*, 1951). Protein folding requires Van der Walls, hydrogen bonds, persulfide bounds, electrostatic forces, and hydrophobic forces. Evidences show that the hydrophobic effect is the most dominant forces (Dobson, 2004). The pioneer work of Christian Anfinsen showed that proteins actually can fold back into their functional structure after being denatured and clearly revealed an univocal relationship between the primary amino acid sequence and protein structure (Anfinsen, 1973).

Protein folding *in vivo* is an achievement, coping with temperature and the high intracellular concentrations of molecules in the intracellular milieu. Thus, it is not surprising that proteins may fold into abnormal structures. Today it is known that cells developed complex systems to deal with protein synthesis, regulation and folding. Specific molecules interact with nascent polypeptides and with proteins avoiding them to assume an incorrect shape, these include molecular chaperones, folding catalysts and quality control systems capable of targeting incorrectly folded proteins to refolding, reactivation or degradation pathways. However, it may happen that the cell is no longer able to refold proteins. At this point, if they overload the cells protein quality control systems, they may accumulate and establish undesired interactions with other macromolecules causing cytotoxicity or other pathological phenotypes. In some cases, they may form insoluble aggregates in the cells, typical of some known pathologies as neurodegenerative disorders. This can happen both spontaneously or due to genetic mutations.

1.1 Cellular protein quality control systems

A nascent polypeptide chain may fold in the cytosol or in specific compartments such as the endoplasmic reticulum (ER) or mitochondria as soon as it emerges from the ribosome (co translational folding), or after translation (post-translational folding). Other proteins may require specific microenvironments such as membranes. In any of the above cases, the molecular environment is crucial for a proper folding, namely the molecular crowding and the presence of molecular chaperones, which play a major role in efficiently correcting folding errors. Macromolecular crowding is the total amount of macromolecules within a given compartment,

typically an average of 300-400 mg/mL (Minton, 1994), and this concentration may disturb the folding and binding of proteins. One slight increase in macromolecular crowding concentration may result in an order of magnitude increase in molecular binding affinities, that may lead to a more compact structure of the proteins and ultimately promoting aggregation (Ellis, 2001).

The main physiological role of chaperones both in the cytosol (heat shock proteins, crystallins, prefoldin) and in the ER (Bip, Grp94, calnexin) is to assist and promote the folding of the nascent polypeptide chains or refolding of abnormal folded proteins, to guide later stages of the folding process and to avoid inappropriate interactions of misfolded or incompletely folded peptides (Bukau & Horwich, 1998, Hartl & Hayer-Hartl, 2002). When these tasks are not fulfilled, molecular chaperones promote degradation of polypeptides with non-native conformations. Chaperones do not add further structural information to nascent polypeptides, they provide a microenvironment suitable for a polypeptide to fold, rather than interacting improperly with other molecules or biological structures, thus escaping competing reactions such as aggregation (Bukau & Horwich, 1998, Hartl & Hayer-Hartl, 2002).

Knowledge of protein folding mechanisms and the effectsof environmental and chemical factors on protein folding is of great importance to understand misfolding in living organisms. A protein spends little time in non-native conformations, hindering the occurrence of inappropriate inter or intra-molecular interactions resulting in the appearance of aggregates. However, in misfolding conditions such as over-expression of proteins, post-translational modifications or the presence of specific mutations may favour aggregation when the action of chaperones are overwhelmed (Calloni*, et al.*, 2005). The importance of chaperones is unquestionable, as the concentrations of several chaperones are increased when cells are exposed to these environments (Ellis, 1987, Parsell & Lindquist, 1993). Several human pathologies share this feature like neurodegenerative diseases (Parkinson's and Alzheimer's), systemic amyloidoses and cystic fibrosis.

When molecular chaperones are not able to cope with inappropriate proteins, misfolded and damaged proteins are targeted by the ubiquitin-proteasome system (UPS) to avoid accumulation and the potentially deleterious effects on cells. The UPS is the major player in extralysosomal cytosolic and nuclear protein degradation system. The 26S proteasome, a large multi-catalytic subunit protease complex, composes the main core of UPS, responsible for degradation of proteins sometimes involved in the regulation of several cell functions as development, differentiation, proliferation, cell cycling, apoptosis and many others (Baumeister*, et*

al., 1998, Hershko & Ciechanover, 1998, Glickman & Ciechanover, 2002, Naujokat & Hoffmann, 2002, Goldberg, 2003, Wolf & Hilt, 2004).

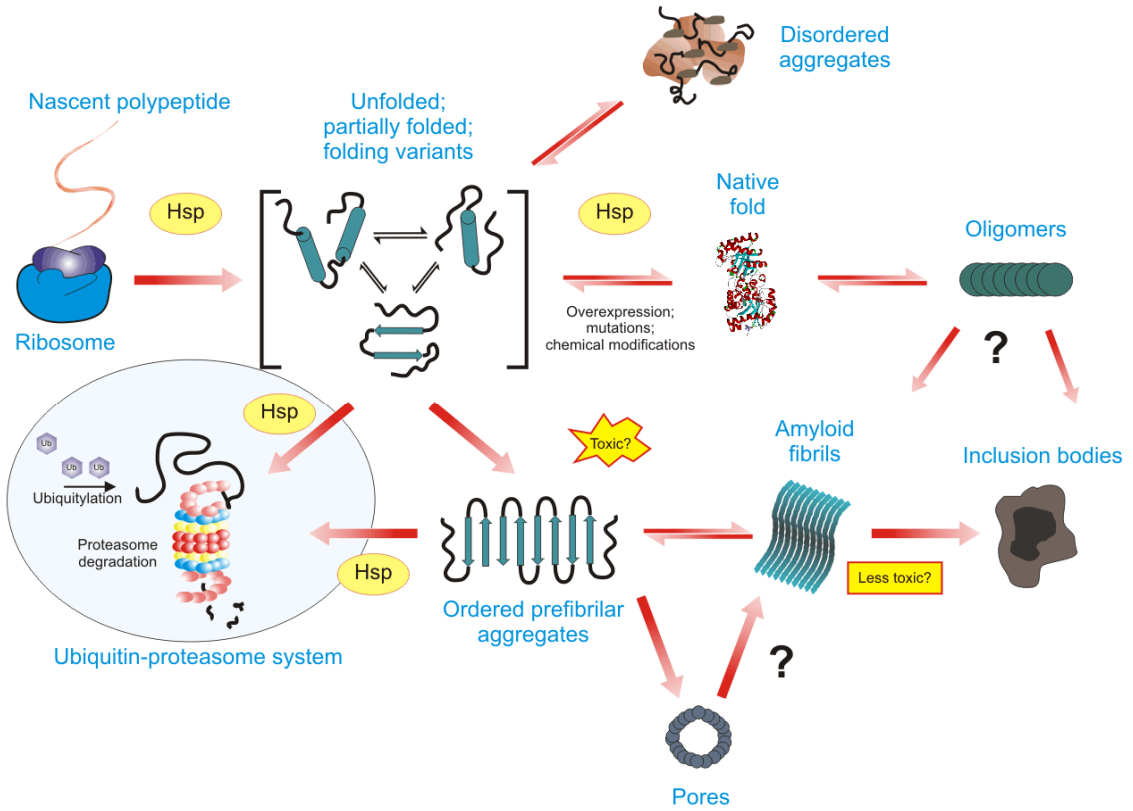


Figure I.1 Possible conformation states of a nascent protein. In physiological environments, the equilibrium between a native fold and the partially folded or misfolded species is shift to the folded states. Under unfavourable situations, such as over expression, mutations, post-translational modifications or loss of the quality control systems of protein folding, the concentration of misfolded species may increase. The result is the nucleation of oligomers and later, the formation of fibrils and aggregates. Adapted from (Savitt, *et al.*, 2006).

Most proteasome degradation is ubiquitin-dependent, a protein modification by covalent attachment of multiple monomers of the 76 highly conserved amino acid sequence of ubiquitin (Hershko & Ciechanover, 1998, Pickart & Eddins, 2004). This process, ubiquitylation, is a multistep reaction involving three classes of enzymes: ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3). E1 activates ubiquitin by forming a high-energy thiol ester bound between E1 and the ubiquitin C-terminal in an ATP dependent reaction. The ubiquitin activated moiety is then transferred and bound to E2, which serves as a carrier. E3 catalyzes the covalent attachment of ubiquitin to the target protein by the formation of isopeptide bounds. After multiple cycles, it results in the attachment of a polyubiquitin chain that serves at a recognition signal for the degradation by the 26S proteasome (Hershko & Ciechanover, 1998, Glickman & Ciechanover, 2002). The proteasome complex consists

of the 20S catalytic core complex and two 19S regulatory complexes capping the 20S complex at both ends. The 20S complex is formed by four stacked rings, two non-proteolytic and the other inner two with three distinct proteolytic catalytic activities: chymotryptic-like, tryptic-like and peptidylglutamyl peptide hydrolyzing activities (Arendt & Hochstrasser, 1997, Heinemeyer*, et al.*, 1997). Although 20S complexes are incapable of degrading ubiquitin-conjugated and folded substrates, 19S regulatory complexes capping the 20S complex at both ends are responsible for this step. The poliubiquitylated target protein enters the 19S complex, is recognized, deubiquitylated, unfolded and translocated into the narrow catalytic chamber of the 20S core (Glickman*, et al.*, 1998, Yao & Cohen, 2002).

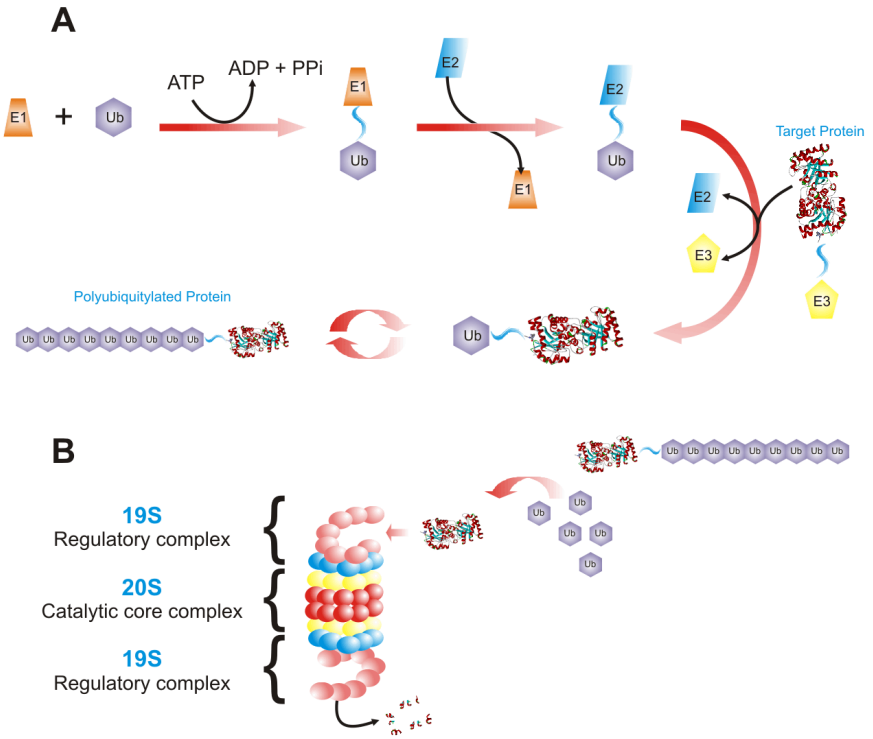


Figure I.2 The ubiquitin-proteasome system. (A): Attachment of ubiquitin to the target protein, involving a cycling three step enzymatic reaction involving E1 (Ubiquitin-activating enzyme), E2 (Ubiquitin-conjugating enzyme) and E3 (Ubiquitin-protein ligase). (B) Schematic representation of the 26S proteasome: a 20S catalytic core complex capped by two 19S regulatory complexes at both ends. Polyubiquitylated target protein is recognized be 19S complex, which then recycles the polyubiquitin chain, unfold the protein and delievers it to the 20S catalytic complex. The protein is hydrolysed and the resulting peptides are released to the cytosol and further degraded to single amino acids by cytosolic peptidases or used for major histocompatibility class I antigen presentation. Adapted from (Goldberg, 2003).

Ubiquitin is recycled by the ubiquitin carboxy terminal hydrolase (UCH) and ubiquitin specific proteases (UBP), and the resulting peptides are released from the 26S proteasome by

diffusion and further degrade to single amino acids by cytosolic peptidases or are used for major histocompatibility class I antigen presentation (Wilkinson & Hochstrasser, 1998).

Deregulation, as well as mutations in the genes involved with UPS, may affect cell protein homeostasis which contributes to the pathogenesis of several diseases as cancer, neurodegenerative, autoimmune, genetic and metabolic disorders. Inhibition of this system induces apoptosis in cancer cells, being a molecular target for therapy (Ciechanover, 2003, Golab*, et al.*, 2004).

2. Protein Glycation

In 1912, Louis Camille Maillard studied the reaction between reducing sugars and amine-containing compounds (Maillard, 1912), also referred as browning reaction, since it develops a brown coloration. This was associated with the attractive colours and flavours of cooked food, which began as soon as mankind invented fire and cooked their meals. However, scientists started studying this process in more detail since they have found it *in vivo* either in diabetic or healthy individuals (Bunn*, et al.*, 1975). The process involves multi-step non-enzymatic reactions between carbonyl-containing groups and amino groups leading to the formation of irreversible products named advanced glycation end-products (AGEs) (Brownlee*, et al.*, 1984). The main targets of this modification are biomolecules with free amine groups like proteins, nucleotides and also some phospholipids. Side chains of arginine and lysine residues, the protein n-terminal amino group and the thiol groups of cysteine residues, are the main targets of glycation in proteins. In contrast, with cysteine thiol group, reversible and unstable adducts are formed. Since the n-terminal is usually modified, the main glycation targets in proteins are lysine and arginine side chains (Driessen*, et al.*, 1985, Lo*, et al.*, 1994, Westwood & Thornalley, 1995, Westwood & Thornalley, 1997).

The first step of the Maillard reaction involves the nucleophilic attack by the nitrogen atom of the amino group to the electrophilic carbonyl group of an aldehyde or ketone. After elimination of a water molecule, an unstable Schiff's base is generated which undergoes a spontaneous rearrangement to form a ketoamine named Amadori product (Amadori rearrangement) (Hodge, 1955, Westwood & Thornalley, 1997). Specific reaction between glucose and lysine residues are named fructosamines (Njoroge & Monnier, 1989, Westwood & Thornalley, 1997). The Amadori product further reacts to yield irreversible bound adducts, the AGEs. This involves intramolecular

rearrangements, oxidative and non-oxidative fragmentation and dehydratation reactions (Bucala & Cerami, 1992, Vlassara, 1994, Westwood & Thornalley, 1997). Glycation, depends on several conditions such as the concentration and reactivity of the glycation agent (Acharya & Manning, 1980, Eble*, et al.*, 1983, McPherson*, et al.*, 1988), the presence of catalytic factors (metals, buffer ions and oxygen), pH and temperature (Watkins*, et al.*, 1987, Smith & Thornalley, 1992, Fu*, et al.*, 1996) and the protein half-life (Schleicher & Wieland, 1986). The solvent exposure of the amino acid residue in the folded protein also influences the rate of glycation, either because the neighbouring amino acids influence the pKa value of the residue or they spatially hinder the residue (Westwood & Thornalley, 1997, Ahmed, 2005).

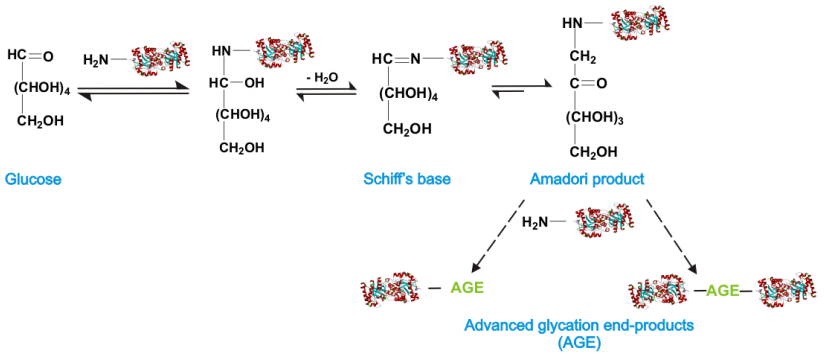


Figure I.3 Reactions involved in protein glycation by glucose. Initial formation of the Schiff's base by the nucleophylic attack of the nitrogen from protein amino acid side chains amino group to the carbonyl group of glucose. Subsequent formation of fructosamine by an Amadori rearrangement and the formation of irreversible adducts, AGEs. Cross-links between two amine groups may also be formed. Adapted from (O'Brien, 1997, Westwood & Thornalley, 1997)

There are several characterized AGEs. The first ones were derived from lysine amino acid residues, N^{ε}-(carboxymethyl)lysine (CML), a degradation product from fructosamines, and pentosidine, a fluorescent lysine-arginine cross-link (Ahmed*, et al.*, 1986, Sell & Monnier, 1989). These AGEs were identified *in vivo* and accumulate with age in tissue collagens and lens proteins and require oxidation to occur, which led to the concept where that Amadori product autoxidation is required for the formation of AGEs, a process named glicoxidation (Ahmed*, et al.*, 1986, Sell & Monnier, 1989, Baynes, 1991, Dyer*, et al.*, 1993). This is in agreement with the original concept where the Amadori product is the central precursor to AGEs formation (Hodge, 1955). Nevertheless, it was found that Schiff's base may also fragment prior to the Amadori rearrangement (Namiki & Hayashi, 1975, Wolff & Dean, 1987, Wolff*, et al.*, 1991). Furthermore, it was discovered that metal-catalysed autoxidation of glucose may be an important player in

triggering the reaction (Wolff & Dean, 1987, Wolff*, et al.*, 1991). It is now accepted that there are multiple mechanisms leading to AGEs formation *in vivo*.

Glucose autoxidation, Shiff's base fragmentation and Amadori product autoxidation form highly reactive dicarbonyl compounds, like glyoxal, 3-deoxyglucosone and methylglyoxal (Hayashi*, et al.*, 1986, Kato*, et al.*, 1987, Wells-knecht*, et al.*, 1995, Thornalley*, et al.*, 1999). As glucose, these compounds also react with amine groups forming ketoimines, similar to fructosamines, but they are much more reactive (Hunt*, et al.*, 1993, Lo*, et al.*, 1994, Westwood & Thornalley, 1997). Since the discovery of glycated haemoglobin *in vivo* by glucose, currently used as a biomarker of diabetes disease, protein glycation by glucose has been intensively investigated (Bunn*, et al.*, 1975). However, glucose is indeed the least reactive of all sugars and its intracellular concentration is negligible, while reactive dicarbonyl compounds are far more reactive. These observations focused the attention on methylglyoxal, since this α-oxoaldehyde is present in all cells and is considered to be the most reactive glycation agent *in vivo* (Thornalley, 1994, Thornalley, 1996, Chang & Wu, 2006).

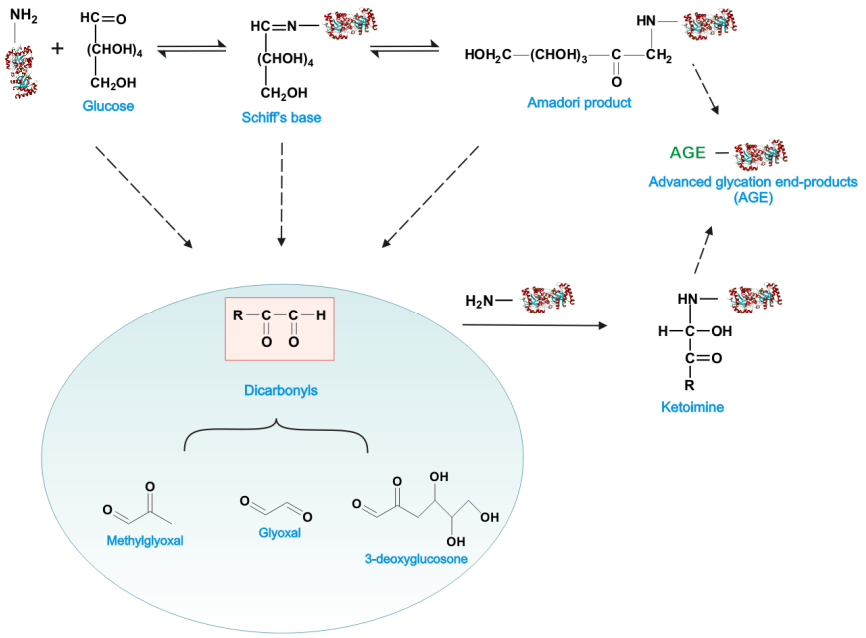


Figure I.4 Formation of dycarbonyl species in the first steps of protein glycation. These may arise from glucose autoxidation, Schiff's base fragmentation and/or Amadori product autoxidation. Methylglyoxal as other dycarbonyls may initiate protein glycation by forming a ketoamine that further forms AGEs.

2.1 Methylglyoxal

Methylglyoxal is an unavoidable product of metabolism and is present in all healthy cells. The first studies started with the discovery of an enzymatic system which converts α-oxoaldehydes such as methylglyoxal, into α-hidroxyacids (Dakin & Dudley, 1913, Dakin & Dudley, 1913, Neuberg, 1913). It was assumed for about two decades that methylglyoxal was a key glycolytic intermediate. However, this idea was dropped out with the discovery of glutathione (GSH) as an essential cofactor of the glyoxalase system as well as the final product of reaction being D-Lactate rather than L-Lactate, which is formed by glycolysis (Neuberg & Kobel, 1928, Lohmann, 1932, Racker, 1951). Nevertheless, glyoxalase activity, methylglyoxal and D-lactate were found *in vivo* in several models, raising questions about their function (Hopkins & Morgan, 1945). Szent-Györgi presented a hypothesis where methylglyoxal and the glyoxalase system played a key role in the control of cell division and could be involved in cancerogenesis (Szent-Gyorgyi, 1965). It stimulated scientific research in this field leading to the discovery of novel pathways involving methylglyoxal metabolism and its biochemical effects.

2.1.1 Production of Methylglyoxal

Methylgyoxal may arise from different metabolic pathways involving enzymatic or non-enzymatic reactions. It is produced in L-threonine metabolism (Ray & Ray, 1987, Lyles & Chalmers, 1992); catabolism of the ketone bodies acetoacetate and acetone (Casazza*, et al.*, 1984, Koop & Casazza, 1985, Aleksandrovskii, 1992); as a by-product of glycolysis (Richard, 1993); enzymatically by methylglyoxal synthase (only in bacteria) (Cooper & Anderson, 1970); Maillard and lipoperoxidation reactions (Esterbauer*, et al.*, 1982, Thornalley*, et al.*, 1999).

Methylglyoxal synthase (glycerine-phosphate phospho-lyase, EC. 4.2.3.3.) catalyses the formation of methylglyoxal from the triose phosphate dihydroxyacetone phosphate (DHAP). In bacteria, the formation of methylglyoxal may represent a by-pass to glycolysis since the glyoxalase system produces D-lactate from methylglyoxal, which later may be converted to pyruvate by the D-lactate dehydrogenase (D-lactate: NAD^+ oxidoreductase EC. 1.1.1.28) (Cooper & Anderson, 1970). Inorganic phosphate is a known allosteric inhibitor of methylglyoxal synthase, thus, in high concentrations of inorganic phosphate, glycolysis is more prone to occur, where glyceraldehyde 3-phosphate dehydrogenase (GAPDH, D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC. 1.2.1.12) activity is higher than methylglyoxal synthase (Hopper & Cooper,

1971, Hopper & Cooper, 1972). If inorganic phosphate concentrations decrease, since GAPDH activity is inorganic phosphate dependent, methylglyoxal synthase activity rises and methylglyoxal is produced from DHAP, which represents a compensator regulator mechanism that may restore the inorganic phosphate concentration required for glycolysis, while still producing pyruvate (Cooper, 1984). Methylglyoxal synthase activity was found in prokaryotes (Cooper, 1984); but its presence in eukaryotes is controversial. In *Saccharomyces cerevisiae*, its activity was reported in some mutant strains, nevertheless no activity is detected in wild-type strain and no significant gene sequence homology is revealed (Penninckx*, et al.*, 1983, Murata*, et al.*, 1985, Phillips & Thornalley, 1993, Phillips & Thornalley, 1993, Hodges*, et al.*, 1999).

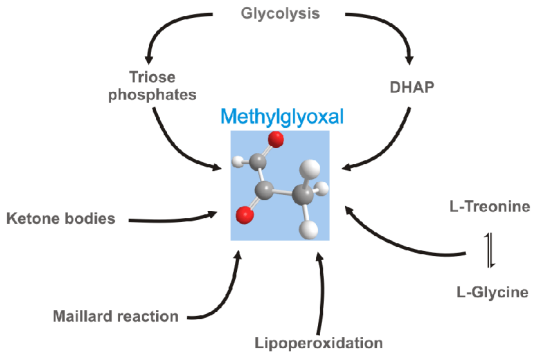


Figure I.5 Routes of methylglyoxal formation in biological systems. This α-oxoaldehyde is an unavoidable product of cell metabolism. The β-elimination of the phosphate group from DHAP and GAP produces this metabolite and represents the most important pathway of methylglyoxal formation.

Methylglyoxal may be formed by the catabolism of L-threonine, via aminoacetone by the enzyme semicarbazyde-sensitive amine oxidase (SSAO, amine:oxygen oxidoreductase (deaminating) (copper-containing), EC. 1.4.3.6) (Lyles & Chalmers, 1992). This pathway forms glycine and acetyl-CoA using aminoacetone as intermediate metabolite; in low CoA conditions, such as diabetic ketoacidosis, where most CoA is in the form of acetyl-CoA, the formation of aminoacetone increases from L-threonine catabolism, enhancing the production of methylgyoxal (Tressel*, et al.*, 1986). The enzymatic oxidation of acetoacetate by myeloperoxidase (donor:hydrogen-peroxide oxidoreductase, EC. 1.11.1.7) (Aleksandrovskii, 1992) as well as the enzymatic oxidation of acetone by cytochrome P450 IIE1 (substrate, reduced-flavoprotein:oxygen oxidoreductase (RH-hydroxylating or –epoxidizing), EC. 1.14.14.1) in a NADPH-dependent two-step reaction, with acetol as intermediate, also produces methylglyoxal (Casazza*, et al.*, 1984, Koop &

Casazza, 1985). In pathological conditions such as ketosis and diabetic ketoacidosis, ketone bodies may represent an important source of methylglyoxal (Turk*, et al.*, 2006).

In eukaryotic cells, the main pathway of methylglyoxal formation is the β-elimination of the phosphate group from the triose phosphate intermediates glyceraldehyde 3-phosphate (GAP) and DHAP. At physiological pH, phosphorylated trioses are much more reactive towards the loss of α-carbonyl protons than the corresponding triose, producing an enediolate phosphate, which has a low energy barrier for the phosphate group expulsion. In summary, the substrate, enediolate phosphate intermediate formation and phosphate group cleavage leads to the formation of methylglyoxal (Richard, 1993). The role of triose phosphate isomerase (TIM, D-glyceraldehyde-3-phosphate aldose-ketose-isomerase, EC. 5.3.1.1) in stabilizing the enzyme-bound enediolate phosphate intermediate is crucial to avoid substrate degradation into methylglyoxal, being the protonation of the enediol 10^6 faster than it expels the phosphate group (Richard, 1991). Nevertheless, an estimative for the methylglyoxal non-enzymatic formation rate is given as 0.1 mM per day (Richard, 1993).

The rate of formation of methylglyoxal depends on the organism, tissue, cell, metabolism and physiological conditions. It appears to be related to the glycolytic rate, confirming that triose phosphate degradation is the main pathway for the production of methylglyoxal (Fareleira*, et al.*, 1997, Martins*, et al.*, 2001, Altenberg & Greulich, 2004).

2.1.2 Catabolism of Methylglyoxal

Methylgyoxal damages proteins and other biomolecules through the Maillard reaction and it is known that in high concentrations it may cause cell death (Kalapos, 1999, Maeta*, et al.*, 2005). Although methylglyoxal formation is unavoidable in living systems, protective enzymatic mechanisms are involved in the degradation of this metabolite. The glyoxalase system is the most extensively studied catabolic route of methylglyoxal, even so, some oxide-reductases and dehydrogenases are also able to catabolize methylglyoxal in its oxidized or reduced form (Kalapos, 1999): α-oxoaldehyde dehydrogenase (2-oxoaldehyde:NAD(P)$^+$ 2-oxidoreductase, EC. 1.2.1.23) (Monder, 1967), aldehyde dehydrogenase (aldehyde:NAD$^+$ oxidoreductase, EC. 1.2.1.3) (Izaguirre*, et al.*, 1998), aldose reductase (alditol:NAD(P)$^+$ 1-oxidoreductase, EC. 1.1.1.21) (Vander Jagt*, et al.*, 1992), methylglyoxal reductase (D-lactaldehyde;NAD$^+$ oxidoreducatase, EC. 1.1.1.78) (Ray & Ray, 1984) and pyruvate dehydrogenase (pyruvate: dihydrolipoyllysin-residue acetyltransferase-

lopoyllysine 2-oxidoreductase (decarboxylating, acceptor-acetylating), EC. 1.2.4.1) (Baggetto & Lehninger, 1987) are examples of these. The actual contribution of each of these enzymes *in vivo* is still controversial, nevertheless, it is commonly agreed that the glyoxalase system and aldose reductase are the most relevant methylglyoxal catabolic routes.

2.1.2.1 The glyoxalase system

In 1913, two independent studies discovered the reaction of production of lactic acid from methylglyoxal. Initially it was believed to be composed by a single enzyme, named glyoxalase (Dakin & Dudley, 1913, Dakin & Dudley, 1913). Afterwards Racker showed that in fact the system was composed by two enzymes, glyoxalase I and II (Racker, 1951). Methylglyoxal is one substrate of the glyoxalase system, present in the cytosol of all mammalian species and in most microorganisms. It is then formed of two enzymes: glyoxalase I (GLXI) (*S*-D-lactoylglutathione methylglyoxal-lyase, EC. 4.4.1.5) and glyoxalase II (GLXII) (*S*-2-hydroxyacylglutathione hydrolase, EC. 3.1.2.6) and catalytic amounts of GSH. Glyoxalase I can catalyze the formation of *S*-D-lactoylglutathione in two different ways. First, by using the non enzymatic conjunction of methylglyoxal with GSH, which forms a hemi-thioacetal, or second by using both methylglyoxal and GSH directly to produce *S*-D-lactoylglutathione as final product. Gyoxalase II then catalyses the thioester hydrolysis to D-lactate while GSH is reformed. D-Lactate may further be metabolized to pyruvate.

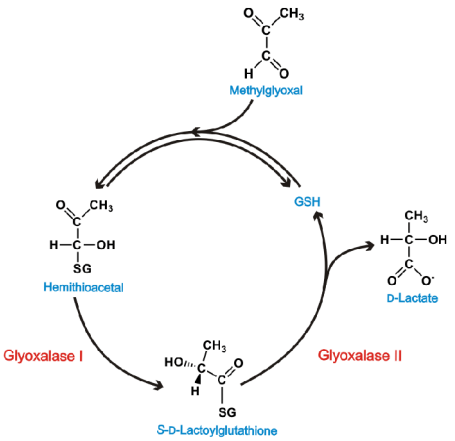


Figure I.6 The glyoxalase system. Methylglyoxal main catabolic pathway, comprising two enzymes (glyoxalase I and glyoxalase II) in a GSH-dependent reaction, producing D-lactate. The single substrate mechanism for glyoxalase I is shown.

Glyoxalase I was purified and characterized at the molecular and kinetic level from various sources, including mammalian tissues. Human GLXI is a homodimer, each monomer composed by two similar domains. The enzyme contains two active sites located at the dimer interface and residues from both subunits contribute to each of the binding pockets. Two zinc (Zn^{++}) are found to be essential to glyoxalase activity and are located in each active site (Thornalley, 2003). The major substrate of glyoxalase I is methylglyoxal, although glyoxal, phenylglyoxal, hydroxypiruvaldehyde and 4,5-dioxovalerate are also substrates (Vander Jagt*, et al.*, 1972, Jerzykowski*, et al.*, 1973, Vander Jagt*, et al.*, 1975). Overexpression of this enzyme has shown to prevent the increase of AGEs and increases the concentration of D-lactate *in vivo* (Thornalley, 2003). Decreasing concentrations of GLXI, due to the aging process and oxidative stress are associated with increased glycation and tissue damage. Its role has been implicated also in Alzheimer's disease (AD). GLXI is upregulated in AD probably in a compensatory mechanism to prevent increased concentrations of methylglyoxal and glyoxal (Wong*, et al.*, 2001, Kuhla*, et al.*, 2005, Kuhla*, et al.*, 2007).

There is considerable controversy on how the enzymatic activity of GLXI is regulated. It is known that GLXI sequence contains several potential phosphorylation sites (Thornalley, 1996) and it has been associated with protein kinase A mediated phosphorylation, where GLXI is phosphorylated in cells exposed to tumor necrosis factor (TNF) (Van Herreweghe*, et al.*, 2002). High osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway also is involved in the expression of GLXI induced by osmotic stress (Inoue*, et al.*, 1998), plus, Hog1p phosphorylation and translocation to the nucleus induces GLXI expression in stress response conditions (Maeta*, et al.*, 2005).

Human glyoxalase II is a metalloprotein with two Zn^{++} per molecule. Also purified from different sources, it contains two domains in which the first folds into a four-layered β-sheet, similar to metallo-β-lactamases, and the second is predominantly α-helical (Cameron*, et al.*, 1999). In contrast to GLXI, it is present also in mitochondria (Talesa*, et al.*, 1988, Bito*, et al.*, 1997, Cordell*, et al.*, 2004). In mammals, both isoforms are coded by a single gene, being produced by alternative translation initiation of the gene transcripts (Cordell*, et al.*, 2004). In *S. cerevisiae*, two different genes code for the mitochondrial (GLO4, YOR040W) and the cytosolic glyoxalase II (GLO2, YDR272W) (Bito*, et al.*, 1997). Mitochondrial glyoxalase is only expressed under glycerol-containing medium, while the cytosolic isoform is expressed also under glucose containing-medium (Bito*, et al.*, 1997). The role of the mitochondrial isoform is not clear. It may be associated

to GSH import to the mitochondria, since GLXII may hydrolyse *S*-D-lactoylglutathione transported from the cytosol and regenerate GSH (Scire*, et al.*, 2000). Another possible role is to hydrolyse other thioesters formed in this organelle. In fact, although *S*-D-lactoylglutathione is the major substrate, GLXII shows a broad specificity for GSH thioesters such as *S*-D-manedolylglutathione, *S*-D-acetylglutathione, *S*-D-acetoacetylglutathione and *S*-D-glycolylglutathione (Thornalley, 1990, Thornalley, 1993, Vander Jagt, 1993). Even though it is highly specific to glutathione moiety, in trypanosomatids, where GSH is functionally replaced by trypanothione [N^1, N^8-bis(glutathionyl)spermidine], GLXII is specific for trypanothione thioesters (Irsch & Krauth-Siegel, 2004, Sousa Silva*, et al.*, 2005).

Glyoxalase II has been implicated in Huntington's and Parkinson's disease and as an inhibitor of apoptosis (Willingham*, et al.*, 2003, Xu & Chen, 2006).

2.1.2.2 Aldose reductase

Aldose reductase (ALR2; alditol:NAD(P)$^+$ oxidoreductase, EC. 1.1.1.21), the first enzyme of the polyol pathway, catalyses the NADPH-dependent reduction of glucose to sorbitol. Beyond glucose, this enzyme has a broad specificity for aldehydes, playing an important role in the etiology of diabetic complications. Endogenous aldehydes, present in higher amounts in this disease, are shown to induce advanced glycation endproducts formation, altering the expression of growth factors through pathways associated with oxidative stress (Vander Jagt*, et al.*, 1992, Murata*, et al.*, 1997, Tomlinson*, et al.*, 1997). Since the polyol pathway is dependent of NADPH, in hyperglycaemia, a NADPH/NAD$^+$ depletion is also observed, leading to cellular damages (Gonzalez*, et al.*, 1984, Gonzalez*, et al.*, 1984, Yabe-Nishimura, 1998). All these aldehydes, present in diabetes, are substrates of aldose reductase. Nevertheless, methylglyoxal is one of the best substrates of the enzyme, in contrast to glucose that is actually a poor substrate, suggesting that ALR2 plays an important role in methylglyoxal detoxification (Vander Jagt*, et al.*, 1992). ALR2 catalyses the NADPH dependent reduction of methylglyoxal to acetol. Acetol is also a substrate of the enzyme, being reduced in a NADPH dependent reduction yielding L-1,2-propanediol. D-Lactaldehyde can also be reduced to D-1,2-propanediol (Vander Jagt*, et al.*, 1992).

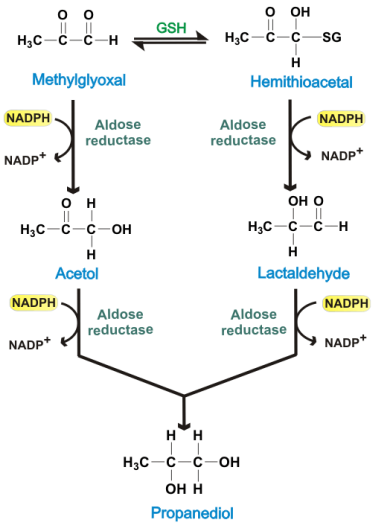


Figure I.7 Methylglyoxal catabolism by aldose reductase. In the presence of low GSH concentrations, the enzyme reacts as an aldehyde reductase (left) in contrast to high GSH concentrations reacting as a ketone reductase (right) with methylglyoxal as substrate in a NADPH depending catalysis. A further reduction reaction may take place forming propanediol. Adapted from (Vander Jagt & Hunsaker, 2003).

In vivo, the concentration of GSH is in the milimolar range in contrast to methylglyoxal, in the micromolar range. Since methylglyoxal reacts with glutathione forming a hemithioacetal with a dissociation constant at pH 7 of 3 x 10^{-3} M, the percentage of methylglyoxal that is free (most of which is actually hydrated) is very low, compared to the hemithioacetal form (Cappiello*, et al.*, 1996, Vander Jagt*, et al.*, 2001). Interestingly, human ALR2 contains a putative glutathione binding site near the active site, which may have a major role in methylglyoxal catabolism. In the presence of high glutathione concentrations, the efficiency of reduction of methylglyoxal by ALR2 increases and the site of reduction switches from the aldehyde to the ketone carbonyl. Thus, GSH converts ALR2 from an aldehyde reductase to a ketone reductase with methylglyoxal as substrate and D-lactaldehyde as product. In lower concentrations of GSH, ALR2 mainly produces acetol, a poor substrate for the second reduction reaction (Vander Jagt*, et al.*, 2001). In fact, acetol accumulation was observed in diabetic patients, and the reaction is reversible, therefore, acetol may be converted back to methylglyoxal (Reichard*, et al.*, 1986, Vander Jagt*, et al.*, 2001). Therefore, the reduction of methylglyoxal to acetol may represent an undesirable reaction, although ALR2 is involved in the detoxification of this α-oxoaldehyde, the accumulation of acetol, together with NADPH depletion and sorbitol concentration increase, could explain the beneficial effects of aldose reductase inhibition in the context of diabetic complications.

2.1.3 Methylglyoxal Biochemical Effects

Methylglyoxal presents two functional groups, a highly reactive aldehyde group and an electron acceptor ketone group. Due to the electronic interaction between the two adjacent C=O double bonds and higher polarization, the aldehyde is more reactive than the ketone group. Thus, methylglyoxal is an electrophile involved in the nucleophilic addition to the carbonyl group (Abdulnur, 1976, Szent-Gyorgyi, 1976, Jencks, 1987). In living cells, the main nucleophiles are amine groups of proteins, nucleic acids and basic phospholipids, which may be irreversibly modified by the Maillard reaction.

Basic phospholipids such as phosphatidylethanolamine and phosphatidylserine are targets of methylglyoxal glycation forming lipid-linked AGEs (Bucala*, et al.*, 1993). This reaction is followed by the oxidation of unsaturated fatty acid side chains, which was observed in the rat liver of streptozotocin-induced diabetic animals and also in human plasma of diabetic patients (Bucala*, et al.*, 1993, Pamplona*, et al.*, 1995).

Methylglyoxal is also able to modify nucleic acids by reacting with guanine nucleotides (Shapiro*, et al.*, 1969). This reaction is faster with RNA and single strain DNA than with native duplex DNA (Krymkiewicz, 1973). Nevertheless, glycated DNA is detected *in vivo* either in human cells and cultured human smooth muscle cells and bovine aorta endothelium cells (Bucala*, et al.*, 1984, Seidel & Pischetsrieder, 1998, Schneider*, et al.*, 2006). DNA glycation causes loss of genomic integrity associated with genetoxic effects. Thus, methylglyoxal is thought to be mutagenic and genotoxic (Migliore*, et al.*, 1990, Rahman*, et al.*, 1990, Pischetsrieder*, et al.*, 1999).

Arginine and lysine amino acid residues are the methylglyoxal main targets, although it may also react reversibly with cysteine residues. The irreversible products of reaction with arginine and lysine residues are named MAGE (methylglyoxal-derived advanced glycation end-products) (Gomes*, et al.*, 2005). BSA (McLaughlin*, et al.*, 1980, Vander Jagt*, et al.*, 1992, Lo*, et al.*, 1994), HSA (Ahmed*, et al.*, 2005), aspartate aminotransferase, collagens (Bowes & Cater, 1968), lens proteins (Riley & Harding, 1995), among others, are examples of *in vivo* protein targets of MAGE. The reaction between methylglyoxal and lysine residues forms N^{ε}-(carboxyethyl)lysine (CEL), a MAGE found in human lens proteins with increased concentrations over ageing (Ahmed*, et al.*, 1997).

However, the methylglyoxal preferred targets are arginine residues (Lo*, et al.*, 1994, Oya*, et al.*, 1999). 5-Hydroimidazolones (N^{δ}-(5-methyl-imidazonole-2-yl)-ornithine) (Henle*, et al.*, 1994) is a MAGE found *in vivo* at high levels in the kidneys of streptozotocin-induced diabetic mice (Niwa*, et al.*, 1997, Niwa*, et al.*, 1997, Uchida*, et al.*, 1997, Ahmed*, et al.*, 2003). These

non-fluorescent hydroimidazolones exist as three structural isomers (Ahmed*, et al.*, 2002, Ahmed & Thornalley, 2002). Another non-fluorescent MAGE tetrahydropyrimidine (THP, Nd-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)ornithine) is also derived from reaction of methylglyoxal with arginine residues (Oya*, et al.*, 1999). BSA and lens proteins, as *intima* and media small artery walls of diabetic kidneys modified by methylglyoxal display fluorescent properties (Lo*, et al.*, 1994, Riley & Harding, 1995, Oya*, et al.*, 1999). The MAGE responsible for this property was identified as argpyrimidine (N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine) (Shipanova*, et al.*, 1997, Oya*, et al.*, 1999).

As mentioned, with cysteine residues, methylglyoxal reacts reversibly forming hemithioacetals (Lo*, et al.*, 1994). This reaction is believed to represent an important role in the activation of transcription factors. In *S. cerevisiae* transcriptional factor Yap1, a functional homologue of mammalian AP-1, is activated by methylglyoxal by a non-disulfide bonds mechanism (Maeta*, et al.*, 2004). In mammals, the formation of a specific cysteine glycation adduct seems to be involved in TNF-induced cell death. It was proposed that phosphorylated glyoxalase I could convert the normally reversible hemithioacetal into irreversible adducts, playing a major role in cell physiology, regulating protein activity while extensive unregulated irreversible modifications that could result in cell dysfunction (Van Herreweghe*, et al.*, 2002).

Methylglyoxal is also responsible for protein cross-linking. With lysine residues, MOLD (methylglyoxal-lysine dimers) is found in an age dependent concentration in lens proteins, as well as in diabetic patients (Nagaraj*, et al.*, 1996, Frye*, et al.*, 1998). A lysine-arginine methylglyoxal-derived cross-link (MODIC) was also described (Lederer & Klaiber, 1999). Methylglyoxal was found to cause characteristic dissectional findings in mice, namely, decrease of liver weight in a dose-dependent methylglyoxal concentration, plus pulmonary hyperaemia and intestine degeneration (Kalapos*, et al.*, 1991, Kalapos*, et al.*, 1994, Choudhary*, et al.*, 1997). Methylglyoxal-treated mice display movement slowness and ataxia (Kalapos*, et al.*, 1991). In murines, a time-dependent diminishing of superoxide dismutase, glutathione S-transferase (GST), catalase, glyoxalase I and II was observed (Choudhary*, et al.*, 1997). This α-oxoaldehyde also exerts an anti-tumour activity *in vivo*. When supplied intraperitoneally or intravenously, the arrest of tumour growth and reduction of tumour size were observed (Apple & Greenberg, 1967, Conroy, 1978, Dianzani, 1978). However, problems of selective toxicity against tumour cells, necessary condition to any clinical application, have not yet been solved.

Methylgyloxal is considered to be an apoptosis inducer by various mechanisms. Generating reactive oxygen species that may modify glutathione reductase (Du*, et al.*, 2001, Amicarelli*, et al.*, 2003); as well as activating p38 MAPK, a signalling intermediate inducing apoptosis in kidney epithelial cells and Schwann cells (Liu*, et al.*, 2003, Fukunaga*, et al.*, 2004); moreover, the JNK pathway is also important for MGO-induced apoptosis (Chan*, et al.*, 2007).

2.2 Biochemical Effects of AGEs

Proteins are made of amino acids linked by peptide bonds in a linear chain that may present secondary and tertiary levels of structure. However, even with the full human DNA sequenced, it is still not possible to determine accurately protein 3D structures from the primary amino acid sequence. Post-translational modifications, the chemical modification of some amino acid residues in a protein after its translation, play a major role in proteins structure, function, biological half-life and protein-protein interactions, after attaching biochemical functional groups such as phosphorylation, glycosylation, acetylation, alkylation, among others. However, extensive and non-programmed modifications, such as glycation, may result in deleterious effects on protein structure and functions which ultimately may cause cellular and tissue damage, observed in several pathological conditions and aging. This non-enzymatic formation of the advanced glycation end-products is observed endogenously inside as well as outside cells by the Maillard reaction from carbonyl containing compounds, such as glucose, fructose, glyoxal, methylglyoxal, glucosone, among others, with free amine groups of proteins, nucleotides and phospholipids. It has been observed that increased AGEs formation alters the structure and function of tissue proteins, contributing to the development of diabetes *mellitus* (Bucala & Cerami, 1992, Vlassara, 1994). CML And pentosidine (glyoxal and glucose-derived AGEs) accumulation is found in retinophaty, nephrophaty, neurophaty, vascular disorders, diabetic cataracts and diabetic atherosclerosis, and its formation is accelerated in high glucose concentration environments (Dyer*, et al.*, 1991, Dyer*, et al.*, 1993, Ahmed, 2005). Since glucose is the least reactive sugar (Bunn & Higgins, 1981), highly reactive compounds as methylglyoxal are more relevant glycation agents. It is noteworthy that the stimulation of glucose metabolism observed in diabetes complications causes an increase of methylglyoxal levels and consequent increase in AGEs content (McLellan*, et al.*, 1994), as in other diseases like atherosclerosis in non-diabetic patients (Kume*, et al.*, 1995). There are also reports of glycated short-lived plasma proteins (Makita*, et al.*, 1992) as well as intracellular proteins

(Giardino, *et al.*, 1994, Gugliucci & Allard, 1996). Alzheimer's disease (Vitek, *et al.*, 1994, Yan, *et al.*, 1994, Du Yan, *et al.*, 1997) and Parkinson's disease (Castellani, *et al.*, 1996, Munch, *et al.*, 2000) display AGEs accumulation in amyloid deposits. These reports lead to the introduction of a new type of cellular stress, carbonyl stress, characterized by the accumulation of glycation agents due to a higher formation of these species or a diminished activity of their catabolic pathways.

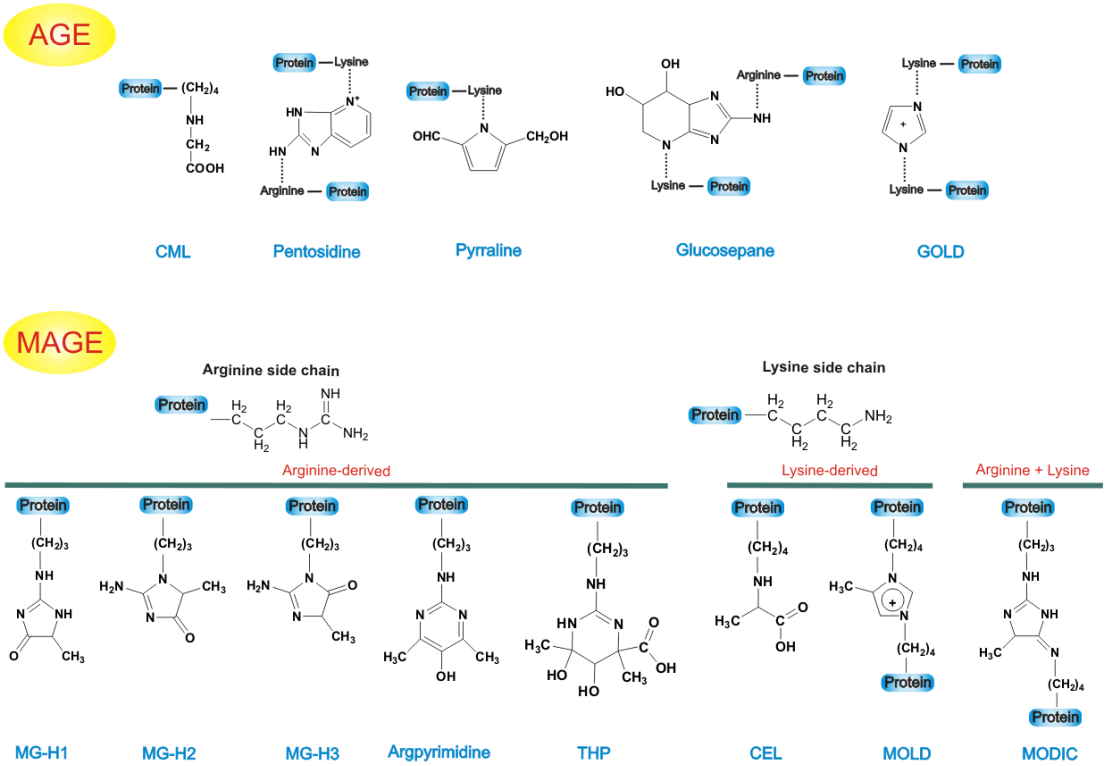


Figure I.8 Chemical structure of advanced glycation end-products (AGEs) including methylglyoxal-derived AGEs (MAGE). AGEs include N^{ε}-(carboxymethyl)lysine (CML), pentosidine, pyrraline, glucosepane and glyoxal-lysine dimer (GOLD). MAGE include, hydroimidazonoles structural isomers (MG-H1, MG-H2 and MG-H3), argpyrimidine, tetrahydropyrimidine (THP), N^{ε}-(carboxyethyl)lysine (CEL), methylglyoxal-lysine dimer (MOLD) and lysine-arginine methylglyoxal-derived cross-link (MODIC). To a better understanding of the modification caused by methylglyoxal, arginine and lysine side chains are also shown. Adapted from (Nemet, *et al.*, 2006).

2.2.1 The Effects of Glycation on Protein Structure and Function

Since AGEs accumulate in several human pathologies, it is essential to address the importance of this modification on structure and protein function.

Arginine, lysine and cysteine residues (putative targets of protein glycation) are usually present in enzymes active sites. Almost all glycolytic enzymes, for example, contain arginine residues in their active site (Riordan, *et al.*, 1977). One example of the drastic effect of MAGE is

serum albumin, whose esterase activity is seriously compromised (Ahmed*, et al.*, 2005). Cysteine proteases (Zeng & Davies, 2005), Cu,Zn-superoxide dismutase (Kang, 2003), GAPDH, glutathione reductase, lactate dehydrogenase (Morgan*, et al.*, 2002), aspartate aminotrasferase (Seidler & Seibel, 2000), catalase (Yan & Harding, 1997),enolase 2 (Gomes*, et al.*, 2008), among others, are also inhibited by MAGE. MAGE can also improve enzymatic activity, as observed in the esterase activity of haemoglobin and myoglobin (Sen*, et al.*, 2007); it also may increase HSP27 and α-crystallins chaperone activity (Nagaraj*, et al.*, 2003, Oya-Ito*, et al.*, 2006). Another example showing that glycation is not always deleterious is the accumulation of AGEs in embryonic stem cells that are rapidly eliminated during cellular differentiation (Hernebring*, et al.*, 2006), which suggests a role in cell cycle and differentiation. It was also observed that a high glycolytic rate caused an accumulation of MAGE in the transcriptional corepressor mSIN3a, revealing an association of glycolytic activity with Angiopoetin-2 (Yao*, et al.*, 2006). Thus, the formation of AGEs modifications may display regulatory functions dependent on the carbonylic stress.

Different cellular functions may be altered upon glycation. Several proteins are translated as inactive precursors requiring further cleavage, such as hormones, receptors, proteases and growth factors. Proteases cleave specific sequences in which arginine-lysine or arginine-arginine are essential for their recognition (Rehemtulla*, et al.*, 1992). Thus, glycation may impair the final processing of many proteins, resulting in their degradation or aggregation. For example, trypsin only cleaves the carboxyl side of lysine and arginine residues, and thrombin hydrolyses arginine-glycine bonds on specific peptide chains. Glycated proteins are in fact highly resistant to proteolysis (Fu*, et al.*, 1992, Fu*, et al.*, 1994). Another deleterious effect of is the impairment of signal sequences containing lysine and arginine residues (Pohlschroder*, et al.*, 2005). An example of signal impairment is the case of low-density lipoproteins (LDL). When glycated, the LDL receptor displays a diminished recognition of the lipoprotein due to the modification of a lysine residue within the putative receptor binding domain, essential for the recognition by the LDL receptor (Bucala*, et al.*, 1994, Bucala*, et al.*, 1995). Other studies involving signalling impairment by glycation, include the inhibition of the epidermal growth factor receptor that regulate multiple cellular processes such as cell growth, mobility, differentiation, survival and death (Portero-Otin*, et al.*, 2002).

Protein misfolding can also be related to AGEs modification of some proteins in the context of amyloidotic neurodegenerative disorders (Bouma*, et al.*, 2003).

2.2.2 RAGE and Oxidative Stress

Advanced glycation end-products may directly affect protein structure and functions, nevertheless, AGEs-modified proteins also exert cellular effects mediated by specific receptors including the macrophage scavenger receptor, MSR type II, OST-48, 80K-H, galectin-3, CD36 and the receptor for AGEs (RAGE) (Neeper, *et al.*, 1992, el Khoury, *et al.*, 1994, Vlassara, *et al.*, 1995, Li, *et al.*, 1996, Ohgami, *et al.*, 2002). RAGE belongs to the immunoglobulin superfamily of receptors with a broad repertoire of ligands that can be generated endogenously, such as AGEs, β-sheet fibrils composed of amyloid β-peptide, transthyretin amyloid fibrils, amphoterin and proinflammatory cytokine-like mediators of the S100/calgranulin family. It was first identified from tissues based on its affinity to bind a glycated form of albumin (Schmidt, *et al.*, 1992). It is composed of one extracellular domain, a single transmembrane spanning domain and a cytosolic tail (Neeper, *et al.*, 1992, Schmidt, *et al.*, 1992). The extracellular domain, V-type, is known to be responsible for the binding of ligands to the receptor, while the 43-amino acid cytosolic tail is critical for RAGE-mediated intracellular signalling (Neeper, *et al.*, 1992, Schmidt, *et al.*, 1992, Schmidt, *et al.*, 2001). The receptor is expressed at low levels in normal tissues, but whenever the ligands accumulate, it becomes upregulated (Li & Schmidt, 1997, Li, *et al.*, 1998, Tanaka, *et al.*, 2000). Diabetes complications and hyperglycaemia are one of the most striking examples of RAGE overexpression, where mediated intracellular responses increase in the presence of high concentrations of AGEs-modified proteins. Engagement of RAGE by AGEs results in VCAM-1, tissue factor and IL-6 expression and in changes of endothelial permeability to macromolecules (Schmidt, *et al.*, 1994, Schmidt, *et al.*, 1995, Wautier, *et al.*, 1996). It may also elicit other cellular responses, namely monocyte chemotaxis stimulation followed by mononuclear infiltration (Kirstein, *et al.*, 1990), increased angiogenesis through production of vascular endothelial growth factor (VEGF) (Hirata, *et al.*, 1997, Yamagishi, *et al.*, 2002), cell proliferation (Kirstein, *et al.*, 1990, Kirstein, *et al.*, 1992, Seki, *et al.*, 2003) and has a central role in the inflammatory response (Schmidt, *et al.*, 2001, Basta, *et al.*, 2002). RAGE may induce inflammatory response by two mechanisms: interaction on leukocytes or endothelial cells activate NF-κB (Kislinger, *et al.*, 1999, Bierhaus, *et al.*, 2001); or directly interacting with leukocyte B2-integrins on endothelial cells, recruiting inflammatory cells (Sligh, *et al.*, 1993, Chavakis, *et al.*, 2003).

RAGE-AGEs interaction enhances cellular oxidative stress, by activating the transcriptional factor NF-κB, probably by p21ras activation that will subsequently activate MAP kinases and therefore NF-κB, ultimately leading to its target genes transcription (Yan, *et al.*, 1994, Schmidt, *et*

al., 1995, Lander*, et al.*, 1997). Interestingly, the receptor has a putative NF-κB binding site, which may be responsible for positive feedback of activation, receptor upregulation and further activation (Li & Schmidt, 1997).

RAGE may be found in several tissues as blood cells, lung, liver, spleen, and also in brain, including microglia, neurons and astrocytes of the hippocampus, entorhinal cortex, cerebellum and superior frontal gyrus (Lue*, et al.*, 2001, Mruthinti*, et al.*, 2003). It is associated to white matter damage in regions of myelination and is implicated in neuronal cell death by ischemic stress, being highly expressed in dying neurons (Toth*, et al.*, 2006). As mentioned, besides AGEs, RAGE may also be activated by amyloid β-peptides, promoting NF-κB activation and macrophage-colony stimulating factor production (Du Yan*, et al.*, 1997). Curiously, enhanced expression of RAGE co-localizes with β-amyloid deposition, and transport of amyloid β across the blood brain barrier was reported, revealing an important association with Alzheimer's disease (Lue*, et al.*, 2001, Deane*, et al.*, 2003).

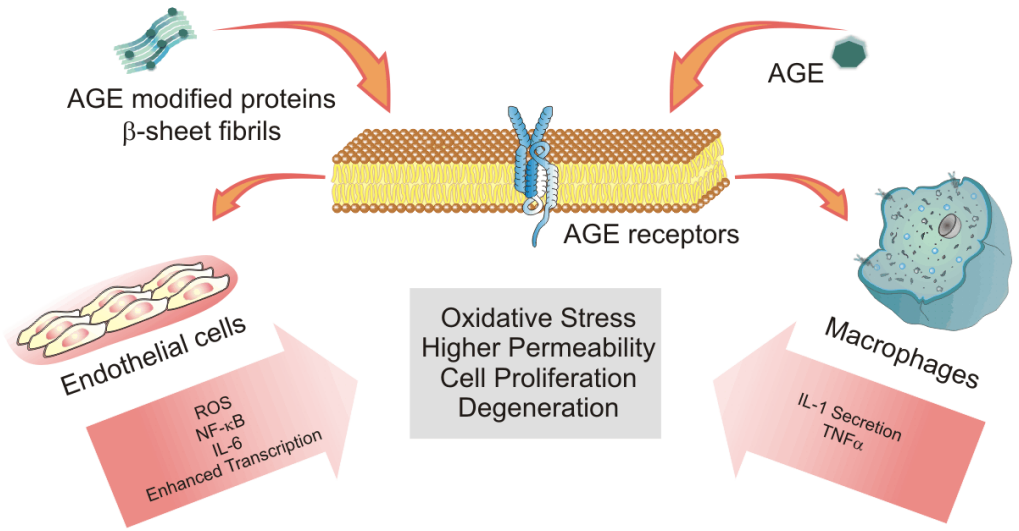


Figure I.9 RAGE is expressed in several cell types in various tissues. Downstream signalling pathways are activated by a broad range of ligands as AGEs and β-sheet fibrils including secretion of pro-inflammatory cytokines such as TNFα, IL-1, IL-6, NF-κB which will lead to oxidative stress, higher permeability to macromolecules, cell proliferation and degeneration. Adapted from (Gugliucci, 2000).

A c-truncated isoform of RAGE, named soluble RAGE, lacks the transmembrane-anchoring domain, being present in plasma, smooth muscle cells, monocyte-derived macrophages, endothelial cells and neurons (Brett*, et al.*, 1993, Malherbe*, et al.*, 1999, Schmidt*, et al.*, 2001). This

form is found to alleviate the deleterious effects of RAGE by scavenging its ligands without further RAGE mediated processes activation (Sakaguchi*, et al.*, 2003, Wendt*, et al.*, 2003). RAGE inhibition may be a putative therapeutic strategy for diabetic vascular disease and Alzheimer's disease, in which sustained chronic elevated NF-κB concentration plays a major role in inflammation (Bierhaus*, et al.*, 2001).

Although RAGE may be involved in oxidative stress, AGEs *per se* may contribute to this process (Elgawish*, et al.*, 1996). Arterial wall AGEs deposits may generate radicals able to oxidize vascular wall lipids and accelerate atherogenesis in hyperglycaemic diabetic patients (Mullarkey*, et al.*, 1990); glycated LDL increases oxidative damage susceptibility (Bucala*, et al.*, 1993); glycated BSA is able to catalyse oxidative modification of macromolecules (Lee*, et al.*, 1998); glycated proteins in mitochondria are implicated in mitochondria-induced oxidative stress (Rosca*, et al.*, 2005).

2.3 Anti-AGEs Systems

An AGEs defense comprises systems able to cope with the pre-existent forms of AGEs or inhibiting its formation by scavenging or degrading glycation agents. Beyond specific enzymatic systems, some specific glycation inhibitors may also prevent cross-linking of proteins due to glycation. Aminoguanidine, via its fast reaction with α-dicarbonyl compounds, forms 3-amino-1,2,4-triazine derivatives (Thornalley*, et al.*, 2000), whereas tenilsetam is a known AGEs cross-linking inhibitor (Munch*, et al.*, 1994). Interestingly, both compounds were found to protect the neurotoxic effects of methylglyoxal (Webster*, et al.*, 2005). Some evidences also point to the presence of specific enzymes able to degrade glycated proteins (deglycases or amadoriases). These include fructosamine 3-kinase, one enzyme that phosphorylates protein-bound fructosamines and break down spontaneously the Amadori rearrangement into inorganic phosphate along with 3-deoxyglucosone and the amino compound (Delpierre*, et al.*, 2000, Szwergold*, et al.*, 2001). This process was observed *in vivo* leading to deglycation of haemoglobin (Delpierre*, et al.*, 2002) and protein-bound psicosamines and ribulosamines in erythrocytes (Collard*, et al.*, 2004). Fructoselysine oxidase (Takahashi*, et al.*, 1997) and fructoselysin 6-kinase (Wiame*, et al.*, 2002) are also believed to play a role in protein deglycation. The importance of deglycases is still controversial, since the resulting degraded compounds are able to react once

more with amino groups of proteins, lipids and nucleic acids. Nevertheless, the inhibition of AGEs formation may represent a therapeutic approach to neurodegenerative diseases.

3. Glycation in Neurodegenerative Diseases

The increase in life expectancy, observed over the last century, has led to the emergence of a series of age-related diseases that pose novel challenges to modern societies (Oeppen & Vaupel, 2002). Neurodegenerative disorders, like Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and prion diseases, are debilitating and yet incurable disorders that demand intensive research. These diseases are characterized by a slow and progressive loss of neuronal cells, and by the deposition of misfolded and aggregated proteins in the brain, either intra- or extracellularly. In Alzheimer's disease (AD), extracellular amyloid β-peptide deposits (amyloid plaques) and intracellular tau protein deposits (neurofibrillary tangles) are the key pathological hallmarks (Ghiso*, et al.*, 1994). In Parkinson's disease (PD), cytoplasmic proteinaceous inclusions mainly composed of the protein α-synuclein (a-syn), named Lewy bodies (LBs) are the pathognomonic inclusions (Spillantini*, et al.*, 1997).

While the vast majority of cases of neurodegenerative disorders are sporadic, familial forms are also known. Several mutations are associated with the amyloidogenic behavior of proteins such as Aβ (Haass*, et al.*, 1994), a-syn (Polymeropoulos*, et al.*, 1997, Kruger*, et al.*, 1998, Zarranz*, et al.*, 2004) or transthyretin (Saraiva, 2001).

Endogenous chemical agents such as oxygen, sugars, oxidative and carbonyl stress, are important players in the damage of biomolecules, leading to the loss of their normal function and, ultimately, to cell dysfunction and death.

3.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease affecting ~5% of people aged 65-75 and almost 50% of people over the age of 85 (Grossman*, et al.*, 2006). One of the most recognized symptoms is the gradual difficulty in memorizing new information followed by confusion, disorientation and disordered thinking. Neurodegeneration occurs in several areas like the hippocampus, amygdala, nuclei basalis and entorhinal cortex. Most AD cases are sporadic, whereas only 6% has a known genetic origin (Campion*, et al.*, 1999). Genetic cases are, in great

majority, related to the presence of the ε4 allele of the apolipoprotein E (ApoEε4) and also to mutations in the amyloid β precursor protein (APP) (Rademakers & Rovelet-Lecrux, 2009).

The diagnosis of AD can only be confirmed upon autopsy, where the presence of amyloid β plaques and neurofibrillary tangles (NFTs) are the hallmark pathological signs (Cummings*, et al.*, 1998). Overexpression of tau protein, hyperphosphorylation and mutations are known aggregation promoting factors (Wittmann*, et al.*, 2001, Sato*, et al.*, 2002, Andorfer*, et al.*, 2005). Nevertheless, glycation is also believed to play an important role in NFTs formation, as in the development of senile plaques (Smith*, et al.*, 1994).

In AD, there is an increased AGEs content in the Aβ plaques (Vitek*, et al.*, 1994). In a parallel study, AGEs were identified in both plaques and NFTs (Smith*, et al.*, 1994). Recently, multiple efforts were deployed to better understand the contribution of glycation to AD pathogenesis. Tau glycation enhances the formation of paired helical filaments in AD and reduces its ability to bind microtubules *in vitro* (Ledesma*, et al.*, 1994). In addition, AGEs modification of tau is responsible for a higher fibrillization state of the protein, although it does not affect the lag time of the equilibrium. These results suggest that glycation, together with pseudophosphorylation, do not promote fibrillization *per se* but, instead, promote the stabilization of the filaments once they nucleate (Necula & Kuret, 2004). In addition, AGEs-modified tau leads to an increase in the production and secretion of Aβ, followed by reactive oxygen species formation (Yan*, et al.*, 1995). Interestingly, glycation agents such as methylglyoxal are able to activate the p38 MAP kinase which is able to phosphorylate tau (Reynolds*, et al.*, 2000, Liu*, et al.*, 2003), a process believed to occur in neurons in AD (Zhu*, et al.*, 2000). These results suggest that glycation may play a role on tau phosphorylation.

In Aβ, glycation by methylglyoxal promotes the formation of β-sheets, oligomers and protofibrils and also increases the size of the aggregates (Chen*, et al.*, 2006). Interestingly, the expression levels of glyoxalase I in the brains of AD patients is lower in later stages of the disease, as confirmed both at the level of mRNA and protein (Kuhla*, et al.*, 2007). Since this is the main methylglyoxal catabolic pathway, a higher carbonyl stress is expected and an increase in AGEs content, oxidative stress, sustained inflammation, plaques and tangles formation and, ultimately, apoptosis are likely to occur.

Hyperglycemia is a major player in carbonyl stress, which is observed in AD, and there is also a link between diabetes *mellitus* and AD. Diabetic patients have a 2 to 5 fold higher susceptibility of developing AD when comparing to non-diabetic control patients (Haan, 2006).

Another factor eliciting carbonyl stress are the AGEs derived from glyceraldehydes, which are present in the cytosol of neurons in the hippocampus and parahippocampal gyrus (Choei*, et al.*, 2004). These may inhibit the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) causing an increase in glyceraldehydes and, consequently, methylglyoxal, forming a vicious cycle leading to neurodegeneration (Takeuchi*, et al.*, 2000, Takeuchi*, et al.*, 2004, Sato*, et al.*, 2006). Remarkably, glycation levels in AD are three fold higher than normal subjects (Vitek*, et al.*, 1994).

Whether glycation is a trigger or a late stage event remains controversial. Some data suggests that AGEs formation is a late secondary event in AD, since Aβ alone induces free radical generation that can promote cross-linking between peptides and sugars (Mattson*, et al.*, 1995). On the other hand, glycation of paired helical filaments of A68 proteins, a known precursor of NFTs, suggests that it could be an early event (Takeda*, et al.*, 2000). A clear increase in the AGEs content upon ageing is observed (25%). Nevertheless, in early and late stages of AD the levels of AGEs were clearly increased (38% and 73%, respectively) (Luth*, et al.*, 2005). Oxidative stress and carbonyl stress were also shown to precede NFTs and plaque formation in AD [83]. ApoE Also seems to be modified by AGEs since staining patterns of AGEs and ApoE are similar in AD (Dickson*, et al.*, 1996). RAGE is an important player in AD since Aβ is also recognized by this receptor (Li*, et al.*, 1998). RAGE is expressed in astrocytes and recruits monocytes across the blood-brain barrier activating NF-κB and, consequently, reducing oxidative stress (Yan*, et al.*, 1998, Giri*, et al.*, 2000). In fact, AGEs, Aβ, and RAGE, colocalize in astrocytes suggesting that Aβ may be taken via RAGE to be degraded by the lysosomal pathway in these cells (Sasaki*, et al.*, 2001).

AGEs are also found in the cerebrospinal fluid (CSF) of AD patients suggesting it may represent a diagnostic tool for AD (Shuvaev*, et al.*, 2001).

3.2 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with a low prevalence, nevertheless its incidence increases with age (Choonara*, et al.*, 2009). Intracellular accumulation and perikaryal inclusions of neurofilament and Lewy body-like cytoplasmic inclusions are hallmarks of the disease. Degeneration of motor neurons in the brain and spinal cord results in muscle weakness, affects voluntary movements and also respiratory muscles, leading to muscle atrophy, paralysis and breathing complications (Dickson, 2003). The cause of ALS is still unknown and 90% of the cases are sporadic. Several inherited cases of disease are related to *Sod1* copper-zinc

superoxide dismutase gene (Cu, Zn-SOD) missense mutations, suggesting that oxidative-stress plays an important role in the disease development (Kikuchi*, et al.*, 2003). Both familial and sporadic cases display extensive neurofilament conglomeration in motor neurons, and in extreme conditions, axonal spheroids formation (Chou, 1997).

Glycation was first detected in sporadic and inherited ALS patients both in spinal cord and brain samples. Chou and co-workers postulated that glycation could be involved in the time-dependent cross-linking of neurofilament protein (Chou*, et al.*, 1998). Neurofilament protein subunits contain multiple Lys-Ser-Pro sequences and serine and threonine residues may require phophorylation and/or glycosylation to normally assemble neurofilaments. If the lysine residue is glycated, the assembly process may be impaired (Chou*, et al.*, 1998). Further studies revealed that AGEs levels were higher in the presence of *Sod1* mutation, while control human and mouse cases did not display AGEs immunoreactivity (Shibata*, et al.*, 2002). The authors state that the AGEs presence in astrocytes in the spinal cord suggest that carbonyl stress occur mainly in the cytoplasm in *Sod1* mutant subjects inducing the potentially deleterious effect of AGEs in cross-linking, superoxide and other radicals formation and local inflammatory responses mediated by RAGE. Strikingly, sRAGE levels, a c-truncated isoform of RAGE, named soluble RAGE, are significantly lower in the serum of ALS patients (Ilzecka, 2008). sRAGE, lacking the transmembrane-anchoring domain, is found to ameliorate the deleterious effects of RAGE by scavenging its ligands without further RAGE mediated processes activation (Sakaguchi*, et al.*, 2003, Wendt*, et al.*, 2003). Thus, sRAGE may function as an endogenous protection factor in ALS, indicating that the low sRAGE levels may pose a risk factor in the disease.

One interesting finding is that glycation, thought to be a random process, modifies SOD1 specifically at Lys3, Lys9, Lys39, Lys36, Lys122 and Lys128 residues (Arai*, et al.*, 1987), although 15 possible lysine and arginine residues glycation targets are present. Diminished activity occurs when Lys122 and Lys128 (Arai*, et al.*, 1987) are modified and a higher susceptibility of mutated *sod1* towards glycation was observed (Takamiya*, et al.*, 2003). These results lead to the idea that glycation could be responsible for the observed oxidative stress in ALS. Recently, it was established that N^{ε}-(carboxymethyl)lysine (CML) concentration significantly increases in serum and cerebrospinal fluid of ALS patients, evidences which may represent a diagnostic tool (Kaufmann*, et al.*, 2004).

3.3 Familial Amyloidotic Polyneuropathy

Familial amyloidotic polyneuropathy (FAP) is a systemic amyloid disease, characterized by the extracellular deposition of transthyretin (TTR) particularly associated to the peripheral nervous system. Andrade in 1952 reported the clinical symptoms of the disease as a sensorimotor peripheral polyneuropathy also affecting autosomal nervous systems (Andrade, 1952). TTR is normally an innocuous protein responsible for the transport of thyroxine hormone and retinol, with a tetrameric structure, present in the plasma and cerebrospinal fluid (Kanai*, et al.*, 1968). TTR is the main constituent of the typical amyloid fibrils (Costa*, et al.*, 1978) present in the disease and its misfolding and aggregation mechanisms are far from being understood. The best established model for TTR fibril formation postulates that under certain unknown conditions, TTR undergoes structural changes and dissociates into partially unfolded monomers. These may associate into high molecular mass soluble aggregates that might progress to insoluble amyloid β-fibril deposits (Quintas*, et al.*, 1997, Quintas*, et al.*, 1999). In fact, mutant forms of TTR, responsible for some FAP cases, display higher ability to form partially unfolded monomers and soluble aggregates (Quintas*, et al.*, 2001). Although mutations in TTR are responsible for the development of FAP, non-mutated TTR may also form amyloid fibrils in patients with senile systemic amyloidosis, a disease that affects around 25% of the population aged above 80 years (Westermark*, et al.*, 1990).

The intrinsic amyloidogenic behavior of TTR indicates that non-genetic factors may contribute to the disease progression. Since glycation promotes cross-linking and insoluble aggregate formation, it is a good candidate to impair the reversible process of soluble aggregates formation, thus contributing to the fibril formation. This hypothesis was advanced by Gomes and co-workers who reported and quantified the presence of AGEs in TTR (Gomes*, et al.*, 2005) a few years after the first report of glycation in the amyloid deposits of FAP patients (Nyhlin*, et al.*, 2000). Glycated TTR may contribute to cytotoxicity via oxidative stress directly by the AGEs or by interaction with RAGE (Yim*, et al.*, 1995, Huttunen*, et al.*, 1999). A clear association between transthyretin amyloid deposits and RAGE was shown (Matsunaga*, et al.*, 2002), where RAGE induces inflammatory and oxidative stress pathways (Sousa*, et al.*, 2000, Sousa*, et al.*, 2001). The interaction of TTR fibrils with RAGE result in a NF-κB nuclear translocation (Sousa*, et al.*, 2000), induction of tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) (Sousa*, et al.*, 2001). Although some evidences suggest that glycation may display a deleterious effect in the disease, more experiments on glycation effects in TTR aggregation are required. Nevertheless, since

blockage of RAGE by anti-RAGE antibodies results in a lower cytokine induction (Sousa*, et al.*, 2001), RAGE may represent a good therapeutical target for FAP disease.

3.4 Prion Diseases

Prion diseases are characterized by the accumulation and aggregation of abnormally folded cellular prion protein (PrP^{c}). Neuronal loss, vacuolation, microgliosis, astrogliosis and spongiform change are hallmarks of this group of neurodegenerative diseases (El Khoury*, et al.*, 1996, Knight & Will, 2004), including bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep. It also occurs in humans as sporadic Creutzfeld Jakob disease (CJD) or through inherited genetic mutations within the prion protein (Prusiner, 1998). Apparently, these diseases may be transmissible through ingestion of abnormal proteins. The "protein only" hypothesis state that the disease is caused by the conversion of a normally folded prion protein (PrP^{c}) into an abnormal isoform (PrP^{res}), however, the mechanism is still controversial and there are arguments against this hypothesis (Soto & Castilla, 2004). The transformation of a α-helical to a β-sheet structure occurs, and it may arise from either the cell surface or along the endocytic pathway via a mechanism of seeded polymerization (Saborio*, et al.*, 2001). PrP^{res} aggregates may bind to PrP^{c} and promote its transformation into PrP^{res} by unknown mechanisms. PrP^{c} is a glycoprotein which exhibits SOD like activity, whose transformation results in a lower antioxidance response (Klamt*, et al.*, 2001, Sakudo*, et al.*, 2003). The abnormal isoform is proteinase K (PK) resistant and may accumulate intra or extracellularly, one feature considered of diagnostic value for these diseases, together with specific antibodies towards PrP^{res} (Saborio*, et al.*, 2001). Since glycation promotes cross-links, increases protease resistance and may induce oxidative stress, AGEs formation has been studied in these diseases.

Nobuyuki Sasaki and coworkers (Sasaki*, et al.*, 2002) observed for the first time that AGEs and the receptor for advanced glycation end products (RAGE) were present in the occipital lobe of CJD patients in higher amounts than in control individuals. They used an anti-AGEs-modified ribonuclease antibody and reported colocalization between PrP-positive granules and AGEs, moreover RAGE were also detected in these PrP granules. The authors hypothesized that glycation would advance over time and like Aβ, PrP would be degraded through lysosomal pathway, mediated by RAGE. Actually, RAGE recruits microglia that adheres to surfaces coated with PrP peptides (El Khoury*, et al.*, 1996). The form of AGEs in PrP plaques is different from the astrocyte

PrP granules, since AGEs imunoreactivity in plaques was observed only after autoclaving treatment (one method proven to improve AGEs detection). These findings suggest that astrocytes, RAGE mediated, may scavenge glycated proteins to degrade abnormal form of PrP (Sasaki*, et al.*, 2002).

It was reported that glycation occurs in the N-terminus of the protein, since upon PK digestion, which cleaves ~90 amino acid residues from the PrP^{res} n-terminal, no glycation was detectable (Choi*, et al.*, 2004). Moreover, PrP glycation is not a random process, although containing 21 arginine and lysine residues (in the cleaved and processed PrP human protein), only lysines at residues 23, 24, and 27 and the arginine at residue 37 of PrP^{res} may be glycated (Choi*, et al.*, 2004). Glycation was only reported in the abnormal form in several models of mouse brains infected with 139A, ME7, 22L and 87V scrapie strains; hamster-adapted 263K and 139H scrapie strains; and both sporadic and genetic CJD. Other interesting report is the higher occurrence of glycation in diglycosylated PrP^{res}, although these glysolyated residues are not targets of glycation (Choi*, et al.*, 2004), they might alter the protein lysine residue solvent exposure thus enhancing their susceptibility to glycation. Other important observation is the oligomerization of glycated PrP as seen in SDS-PAGE analysis, where imunoreactivity was observed with molecular weights corresponding to monomer and dimer forms of PrP (Choi*, et al.*, 2004). Therefore, the role of glycation in the formation and aggregation of PrP^{res} may not be excluded. However some answers need to be addressed. Is glycation a triggering or a late event on PrP^{res} conversion? The answer is still controversial, while PrP^{res} is observed before AGEs formation, glycation could act as a late event stabilizing PrP^{res} and protecting its cellular degradation (Choi*, et al.*, 2004). Nevertheless, this observation can be the result of a misdetection of the AGEs by limited sensibility of the antibodies deployed. In contrast, astrocytes are believed to be the first affected PrP^{res} cells (Diedrich*, et al.*, 1991), and the majority of PrP^{res} positive astrocytes, are also positive for AGEs (Choi*, et al.*, 2004), suggesting that glycation could be an early event.

More efforts have to be deployed to better understand the role of glycation in prion aggregation and disease development.

3.5 Parkinson's Disease

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder with a prevalence of 2% in people over the age of 60 years (Van Den Eeden*, et al.*, 2003). Muscle rigidity, resting tremor, bradykinesia, rigidity and postural instability are the most common

symptoms of this disease (Forno, 1996, Burke, 1999). Loss of nigrostriatal dopaminergic neurons and the accumulation of proteinaceous intraneuronal cytoplasmic inclusions called Lewy bodies (LBs) are the main features (Gai*, et al.*, 2000, Braak*, et al.*, 2003). The majority of PD cases are sporadic, although several genetic loci have been associated to familial cases of the disease. Three mutant forms of *PARK1*, the gene encoding for alpha-synuclein (a-syn), are associated with familial cases (Polymeropoulos*, et al.*, 1997, Kruger*, et al.*, 1998, Zarranz*, et al.*, 2004). All three mutations, A30P (alanine to proline substitution in residue 30), E46K (glutamic acid to lysine substitution in residue 46) and A53T (alanine to threonine substitution in residue 53) occur within the N-terminal side of the protein and are able to accelerate the oligomerization of a-syn (Brandis*, et al.*, 2006). Genetic polymorphisms in the intron 1 appear to confer a risk for sporadic PD (Kobayashi*, et al.*, 2006), furthermore, duplication or triplication of *PARK1* has been reported in familial forms of PD (Singleton*, et al.*, 2003), suggesting that a concentration dependent effect may underlie the pathogenesis of a-syn in PD.

While the function of a-syn is still poorly understood, it is thought to be associated with vesicular trafficking, modulation of synaptic function and plasticity modulation, and the regulation of dopamine neurotransmission (Klein & Lohmann-Hedrich, 2007). a-Syn is a target of several post-translational modifications. In LBs, oxidized and phosphorylated forms of the protein have been reported (Hirsch, 1993, Fujiwara*, et al.*, 2002, Hasegawa*, et al.*, 2002). In the test tube, under physiological conditions, a-syn exhibits a natively unfolded conformation. If associated with membranes, it can adopt different conformations rich in α-helical content (Zhu & Fink, 2003). In other situations, it may also form aggregates or oligomers, with higher β-sheet content, which are believed to represent the most cytotoxic forms (Lansbury & Brice, 2002, El-Agnaf*, et al.*, 2003, Cookson, 2005, Outeiro*, et al.*, 2007). Nevertheless, the process leading to aggregation and LBs formation is unclear and, in addition to oxidation and phosphorylation, glycation might constitute another factor affecting the aggregation process.

Glycation was first reported in the substantia nigra and locus ceruleus displaying higher immunoreactivity at the periphery of LBs of PD patients (Castellani*, et al.*, 1996). In agreement with this report, colocalization of AGEs with a-syn in very early LBs was observed, where a-syn present also at the periphery of the LBs (Munch*, et al.*, 2000). These results suggest that glycation may be involved in the chemical crosslinking and proteolytic resistance of the protein deposits. While glycation was also detected in the cerebral cortex, amygdale, and substantia nigra of older control individuals, the number and levels of glycated proteins were significantly higher in PD

patients (Dalfo*, et al.*, 2005). RAGE were also highly expressed in PD patients when compared to age-matched controls (Dalfo*, et al.*, 2005), suggesting a role for AGEs in the disease.

a-Syn is a lysine-rich protein with 15 putative glycation targets. Thus, it is likely that glycation occurs influencing the nucleation of pathologic aggregates. One important feature of PD is the acute decrease in the levels of cellular reduced glutathione (GSH) in early stages of the disease which results in a lower activity of the glyoxalase system, the main catabolic pathway of the most important glycation agents *in vivo, such as* methylglyoxal (Thornalley, 1998). The carbonyl stress will then be responsible for an increase in AGEs concentrations that will raise oxidative stress that further induce AGEs formation. This deleterious cycle might contribute to the cell damage reported in dopaminergic neurons where, moreover, dopamine autoxidation and its degradation by monoamine oxidase is likely to contribute to an increase in oxidative stress. Strikingly, methylglyoxal and glyoxal are able to induce oligomerization of a-syn *in vitro* (Lee*, et al.*, 2009). The membrane-binding ability of glycated a-syn was also decreased (Lee*, et al.*, 2009).

4. Alpha-Synuclein

4.1 Biochemical Properties and Interactome

a-Syn is a member of the synuclein family which includes β-syn and γ-syn (von Bohlen Und Halbach, 2004). A-Syn is an acidic, thermostable and natively unfolded small protein composed of 140 amino acids (Lavedan, 1998). Upon interaction with lipids it acquires α-helical content (Li*, et al.*, 2001). Nevertheless, its unfolded conformation may assist vesicle fusion in all eukaryotes (Fiebig*, et al.*, 1999).

a-Syn sequence may be subdivided in three domains. The N-terminal domain (residues 1-65), unordered in solution, includes seven imperfect repetitions of an unusual 11 amino acid residues sequence of a KTKEGV consensus that may shift to an α-helical structure upon interaction with phospholipidic vesicles. This suggests that the protein may be membrane-associated (Davidson*, et al.*, 1998, George, 2002, Kahle*, et al.*, 2002, Lee*, et al.*, 2002, Bussell & Eliezer, 2003). The mutations do not affect the overall structure of a-syn, remaining intrinsically unfolded. Nevertheless, the hydrophobicity of the N-terminal domain is diminished reducing its ability to form α-helices (Eliezer*, et al.*, 2001). The predisposition to form β-sheet

structures is enhanced and the A30P variant decreases a-syn vesicle binding properties (Li*, et al.*, 2001, Brandis*, et al.*, 2006).

The central hydrophobic domain of a-syn is known as NAC (non-Aβ component of AD amyloid) (residues 66-95). This peptide has been implicated in AD as the second main component of amyloid plaques. It is responsible for protein-protein interaction and is believed to contribute for a-syn conformation change to an aggregation-prone β-sheet structure (Ueda*, et al.*, 1993, el-Agnaf & Irvine, 2002).

The C-terminal domain is negatively charged and is composed by a Glu-rich sequence (residues 96-140) (George, 2002). Several phosphorylation sites have been identified, and Ser-129 is phosphorylated *in vivo* (Fujiwara*, et al.*, 2002). Although it has no recognized structure, the acidic domain appears to be critical for a-syn chaperone activity. a-Syn presents 40% of homology with a chaperone named 14-3-3, suggesting a common role for both proteins, and interestingly they interact in the substantia nigra accumulating in LBs (Ostrerova*, et al.*, 1999, Kawamoto*, et al.*, 2002). Both proteins interact with tyrosine hydroxilase (TH), an enzyme involved in the synthesis of catecholamine, responsible for the formation of L-dopa. 14-3-3 induces whereas a-syn inhibits TH activity. a-Syn appears to be responsible for the inhibition of phospholipase D2 (PLD2) (Pronin*, et al.*, 2000). This enzyme hydrolyzes phosphatidylcholine to phosphatidic acid (Chen*, et al.*, 1997), when activated it is responsible for several cell responses such as Ca^{2+} mobilization, secretion, superoxide production, endocytosis, exocytosis, vesicle trafficking, recycling of membrane receptors, transport to Golgi, rearrangements of cytoskeleton, mitogenesis and cell survival (Jenco*, et al.*, 1998). When a-syn is phosphorylated, it exhibits a lower membrane affinity, and diminished binding with PLD2 resulting in the release of any membrane-bound PLD2, allowing its activation and potentially increasing membrane permeability (Bennett, 2005). A yeast two-hybrid screen identified synphilin-1 as an interacting partner (Engelender*, et al.*, 1999). Synphilin-1 may act as an adaptor protein and may be involved in vesicle transport or cytoskeleton function and both proteins co-localize in LBs in PD (Engelender*, et al.*, 1999, Wakabayashi*, et al.*, 2000). Tau has also been reported to interact with a-syn. Interestingly, a-syn induces fibrillization of tau and both proteins synergize in fiber formation (Jensen*, et al.*, 1999, Giasson*, et al.*, 2003). a-Syn may modulate tau function by stimulating protein kinase A, consequently phosphorylating tau influencing its binding to microtubules. a-Syn binds Aβ and brain vesicles via the repeat region and binds tau via its acidic C-terminus domain (Yoshimoto*, et al.*, 1995, Jensen*, et al.*, 1998).

4.1.1 Post-translational Modifications

4.1.1.1 Phosphorylation

Ser-129 is a well established phosphorylation site in a-syn (Okochi*, et al.*, 2000), Ser-87 another possible site. Casein kinases, CK1 and CK2, are able to phosphorylate a-syn and are located in the synaptosome where they also phosphorylate other synaptic vesicle proteins (Krantz*, et al.*, 1997). In addition, the G-protein-coupled receptor kinases (GRKs), GRK2 and GRK5 can phosphorylate a-syn (Pronin*, et al.*, 2000) together with members of the polo-like kinase (PLK) family (Inglis*, et al.*, 2009).

More than 90% of insoluble a-syn is phosphorylated whereas only 4% of soluble a-syn is (Fujiwara*, et al.*, 2002, Chen & Feany, 2005). Nevertheless, there is no consensus about the toxic form of a-syn. In a rat model of PD expressing the a-syn S129D mutant (mimicking a phosphorylated form of a-syn) did not display toxicity, whereas the S129A mutant (mimicking the unphosphorylated form) lead to nigostriatal degeneration (Gorbatyuk*, et al.*, 2008). In contrast, in drosophila and transgenic mouse models, the S129D variant displayed toxicity whereas S129A did not (Chen & Feany, 2005, Freichel*, et al.*, 2007).

4.1.1.2 Nitration

Nitrated a-syn has been found in LBs of PD patients in all of the putative sites of nitration (Tyr-39, -125, -133, -136) and was proposed as one of the oxidative mechanisms responsible for the formation of di-tyrosine crosslinking leading to oligomerization of a-syn (Giasson*, et al.*, 2000, Souza*, et al.*, 2000, Hodara*, et al.*, 2004). Consistently, soluble nitrated a-syn is not efficiently processed by proteases, leading to partial folding, accumulation and fibril formation (Hodara*, et al.*, 2004).

4.1.1.3 Ubiquitylation

a-Syn ubiquitylation occurs in specific residues, K12, K21 and K23 (Anderson*, et al.*, 2006), and is believed to be modified by parkin mainly in its *O*-glycosylated form at S129. It remains unclear whether monomeric a-syn requires ubiquitylation prior to its degradation, since it is natively unfolded. Instead, it may enter the 20S proteasome system directly (Beyer, 2006), then, only oligomeric forms of a-syn would require polyubiquitylation (Leroy*, et al.*, 1998). Furthermore,

both ubiquitylated a-syn and parkin colocalize in LBs and a-syn aggregation may precede ubiquitylation (Tofaris*, et al.*, 2001, Sampathu*, et al.*, 2003). Moreover, several mutations related with the ubiquitin-proteasome system are described to be associated with PD (Lim*, et al.*, 2003). These data suggest that ubiquitylation of a-syn is a pathological event associated with LBs formation.

5. Yeast as a Model

The yeast Saccharomyces cerevisiae is one of the simplest eukaryote organisms and has been employed in bread production and in beer and wine fermentation. The remarkable features of yeast lead scientists to exploit their genetic and biological processes as a model organism. Most of the cellular and biological processes such as DNA replication, recombination, cell division, protein turnover, vesicular trafficking are conserved, thus enabling the use of this simple organism for studying fundamental molecular mechanisms underlying human diseases. In 1996, *Saccharomyces cerevisiae* became the first eukaryotic organism to have its genome fully sequenced (Goffeau*, et al.*, 1996), thus increasing its genetic potential. Interestingly, approximately 31% of the yeast genes have a mammalian homologue and an additional 30% of yeast genes share domain similarities (Botstein*, et al.*, 1997). Consistently, about 30% of the genes known to be involved in human diseases may have a yeast orthologue (Bassett*, et al.*, 1996, Foury, 1997). Easy manipulation and rapid growth, DNA transformation systems, and collections of deletion mutant strains comprise some examples of the most powerful advantages of this cellular model. The generation of humanized yeast systems to study functional aspects of proteins that do not have a yeast homologue also emerged as a technique to study some cellular processes of human diseases (Mager & Winderickx, 2005). Moreover, several drugs known to target human proteins also inhibit the orthologous protein in yeast (Hughes*, et al.*, 2004). These features potentiate yeast as a genetic tool for studying complex systems as those underlying neurodegenerative diseases. Indeed, yeast has been deployed to access basic mechanisms of protein misfolding, aggregation and toxicity in Friedreich's ataxia, ALS, Huntington's disease, PD and AD.

5.1 Modeling Misfolding Diseases in Yeast

5.1.1 Friedreich's Ataxia

Friedreich's ataxia (FRDA) is an autosomal recessive disease characterized by progressive gait disturbance, limb ataxia and cerebellar dysarthria resulting in the loss of limb deep tendon reflexes and position, muscle weakness, loss of coordination, heart disorders along with other complications (Puccio & Koenig, 2000). A GAA trinucleotide repeat expansion in the gene *frataxin* is implicated in inherited cases of the disease (Campuzano*, et al.*, 1996). This expansion causes reduced expression of the protein. YFH1 (yeast frataxin homologue) displays a high homology to the human sequence (Babcock*, et al.*, 1997) and yeast strains lacking this gene are unable to grow on non-fermentable carbon sources, suggesting defective oxidative phosphorylation due to impaired mitochondrial function. Also, iron accumulates in mitochondria in these mutants, indicating an important role of iron and oxidative stress in FRDA (Babcock*, et al.*, 1997, Foury & Cazzalini, 1997, Koutnikova*, et al.*, 1997). Importantly, expression of human frataxin in the YFH1 deletion strain complements its function, in contrast, expression of frataxin including a point mutant allele from a patient with FRDA only partially complements the yeast mutant (Cavadini*, et al.*, 2000). Yeast then emerged as a good model to study some FRDA pathogenic mechanisms.

5.1.2 Amyotrophic Lateral Sclerosis

As mentioned in section 3.2, several inherited cases of ALS are related to *Sod1* copper-zinc superoxide dismutase gene (Cu, Zn-SOD) missense mutations (Kikuchi*, et al.*, 2003). Superoxide dismutase constitutes an important defense mechanism against oxidative stress in several organisms. Like frataxin, the human *Sod1* can complement *Sod1* yeast deletion mutants (Nishida*, et al.*, 1994, Rabizadeh*, et al.*, 1995). Studies revealed that ALS is not due to a reduced superoxide dismutase activity, but rather to a gain of function of mutant *Sod1* protein associated with oligomerization and inclusions formation (Bruijn*, et al.*, 1998, Cleveland, 1999, Cleveland & Rothstein, 2001, Wood*, et al.*, 2003, Kato*, et al.*, 2004). As in FRDA, yeast is being deployed to elucidate the molecular mechanisms underlying ALS.

5.1.3 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder mainly affecting the striatum and cortex of the brain, characterized by uncontrolled movements, psychiatric disturbances and cognitive impairments. An expansion of a CAG trinucleotide repeat enconding a polyglutamine domain (polyQ) within the N-terminal end of the huntingtin protein is believed to play an important role in the disease. Interestingly, several of the huntingtin interaction partners have homologous counterparts in yeast (Kalchman*, et al.*, 1997, Harjes & Wanker, 2003). This expansion is responsible for a gain of toxic function resulting in nuclear and cytoplasmic aggregation of the protein (Li & Li, 2004). Several yeast models to study the folding and behavior of proteins with an expanded polyQ were developed. Heterologous expression of huntingtin results in a polyQ length-dependent aggregation (Krobitsch & Lindquist, 2000, Muchowski*, et al.*, 2000, Meriin*, et al.*, 2002) and in one of these models it resulted in higher toxicity and reduced growth (Meriin*, et al.*, 2002). The aggregation of huntingtin is not toxic *per se* and it has been shown that aggregated expanded polyQ peptides do promote prion formation of Sup35 protein in yeast (Derkatch*, et al.*, 2004). In agreement, two anti-prion compounds were shown to inhibit polyQ aggregation (Schiffer*, et al.*, 2007). Similar to neurons, expression of expanded polyQ peptides in yeast reduced the respiratory capacity by impairing mitochondrial respiratory chain complexes II and III (Solans*, et al.*, 2006), connecting huntingtin-toxicity to apoptosis (Benchoua*, et al.*, 2006). The yeast models also elicited a connection with endocytosis. PolyQ aggregation led to a rapid cessation of endocytosis, increased in mutants affected in early endocytic events (Meriin*, et al.*, 2003). An accumulation of the expanded polyQ peptides in the nucleus for longer periods was reported and this nuclear translocation required the functional yeast metacaspase Ycal (Sokolov*, et al.*, 2006), eliciting novel mechanisms in the expanded polyQ role in HD. Hsp104 and family members of Hsp70 and Hsp40 are important in the aggregation of expanded polyQ peptides (Glover & Lindquist, 1998). Noteworthy, overexpression of Hsp104 has shown to completely suppress the growth defect associated with the expression of toxic polyQ peptides in yeast (Gokhale*, et al.*, 2005, Dehay & Bertolotti, 2006). Although mammalian cells do not present an homologous Hsp104, expression of yeast Hsp104 has shown to reduce the aggregation and toxicity of polyQ peptides either in cell lines and in mice and rat models of HD (Carmichael*, et al.*, 2000, Vacher*, et al.*, 2005, Perrin*, et al.*, 2007).

Genomic screenings were performed transforming nontoxic expanded polyQ constructs in the collection of 4850 haploid deletion strains. 52 mutants exhibited higher toxicity, associated

with protein folding, ubiquitin-proteasome system and stress responses including glutathione synthase (Gsh2) and flavohemoglobin (Yhb1) (Willingham*, et al.*, 2003). These results suggest that polyQ aggregates triggers an increased oxidative stress to a level that the cellular defense mechanisms are able to cope. Other screening was performed using toxic expanded polyQ constructs aiming to identify yeast mutants with reduced toxicity. 28 strains were identified including vesicular traffic and vacuolar protein sorting, transcription regulation and known or putative yeast prions (Giorgini*, et al.*, 2005). In addition to genetic screenings, the yeast models have been deployed as a cell-based high-throughput screening system to find chemical compounds that inhibit polyQ aggregation and toxicity. A lead compound that is structural analog of kynurenine 3-monooxygenase inhibitor was identified and validated in a fly model for HD (Zhang*, et al.*, 2005).

5.1.3 Alzheimer's Disease

One of the pathological hallmark of AD is the formation of intracellular tau aggregates with phosphoepitopes and extracellular β amyloid plaques. Yeast system robustly recapitulates this phenotype upon expression of tau-3R and tau-4R isoforms (Vandebroek*, et al.*, 2005). Interestingly, the yeast protein kinases Mds1 and Pho85 modulate the phosphorylation of tau isoforms and the human ortologues GSK-3b and cdk5 are found to represent the most important mammalian tau-kinases (Hallows*, et al.*, 2003, Vandebroek*, et al.*, 2005). Expression of tau has shown to decrease yeast life-span, which may be related to the observation that oxidative stress and mitochondrial dysfunction dramatically enhance tau aggregation in yeast (Winderickx*, et al.*, 2008).

Besides the aggregation of tau in neurofibrillary tangles, Aβ peptides also aggregate forming amyloid or senile plaques in AD. Aβ peptide expression in yeast results in reduced growth and induces heat shock response, suggesting that the peptide induces stress in the yeast cells (Caine*, et al.*, 2007). Furthermore, mutant forms of Aβ in regions known to be important for intermolecular Aβ-interaction are less prone to aggregate, moreover and Hsp104 modulates Aβ oligomerization (Bagriantsev & Liebman, 2006, von der Haar*, et al.*, 2007). These evidences suggest that folding processes play an important role in the oligomerization and aggregation of Aβ in yeast. Yeast systems are useful tools to study tau and Aβ aggregation processes, not only to

identify novel proteins or compounds affecting the process, but also by providing new insights in the molecular pathways involved in the aggregation process.

5.1.4 Parkinson's Disease

Yeast has been used to investigate and recapitulate some of the aspects reported in human brain. Consistently, WT a-syn and the mutant form A53T are delivered to the plasma membrane via the secretory pathway and once they accumulate at the plasma membrane, they start to form inclusion bodies. Inclusion development appears to be a nucleation-elongation process involving the formation of small seeds at the plasma membrane that latter detach and grow in size in the cytoplasm. Interestingly, some inclusions are thioflavin-S positive, revealing the presence of β-sheet fibrilar structures similar to the LBs of PD patients (Outeiro & Lindquist, 2003, Dixon*, et al.*, 2005, Zabrocki*, et al.*, 2005). Interistingly, A30P mutant form of a-syn appear predominantly dispersed through the cytoplasm of yeast cells, probably due to its poor membrane-binding ability and do not form inclusions (Outeiro & Lindquist, 2003, Dixon*, et al.*, 2005, Zabrocki*, et al.*, 2005). Inclusion formation is followed by an inhibition of phospholipase D, blockage of ER-to-Golgi transport and retarded endocytosis in yeast cells (Outeiro & Lindquist, 2003, Zabrocki*, et al.*, 2005, Cooper*, et al.*, 2006). Moreover, the A30P mutant does not affect phospholipase D or endocytosis. These data suggests that the plasma membrane nucleation process is critical for the inclusion formation. a-Syn toxicity appears to correlate with the membrane-binding and the ability to form α-helices and not with the fibrillization rate of the a-syn mutant proteins (Volles & Lansbury, 2007). These results were reproduced in *Schizosaccharomyces pombe* where WT and A53T variants of a-syn do not target to the plasma membrane and despite the extensive aggregation, the proteins did not display toxicity (Brandis*, et al.*, 2006).

The first genetic screening in yeast, performed in 2003, identifyed 86 yeast deletion mutants with enhanced a-syn toxicity. Several of these mutants lacked functions involved in lipid metabolism, vesicular transport, ubiquitin proteasome system, defenses against oxidative stress or mitochondrial activities (Willingham*, et al.*, 2003), providing new hints of the processes underlying PD. The search for modulators of a-syn toxicity also lead to the identification of several proteins involved in ER-to-Golgi transport including the Rab GTPase, Ypt1. Interestingly, overexpression of Rab1, the mouse homologue of Ypt1, protected dopaminergic neurons against a-syn toxicity (Cooper*, et al.*, 2006). Oxidative stress role in a-syn toxicity was also investigated. The induction of

free radical generation upon the addition of ferrous or ferric ions was shown to enhance aggregation and toxicity of a-syn (Zabrocki*, et al.*, 2005, Griffioen*, et al.*, 2006). Furthermore a higher sensitivity to peroxide hydrogen of yeast cells expressing WT and A53T variants of a-syn was reported. Interestingly, a-syn expression in yeast resulted in the production of ROS, externalization of phosphatidylserine and the release of cytochrome *c* from mitochondria, evidences of the apoptotic cell death program in yeast cells (Flower*, et al.*, 2005, Buttner*, et al.*, 2008), observations similar to fetal dopaminergic neurons (Xu*, et al.*, 2002). The yeast system was also used to identify compounds that reduce a-syn toxicity. This lead to the identification of quercetin and (-)-epigallocatechin-2-gallate, two flavonoids with metal chelating and radical scavenging properties (Griffioen*, et al.*, 2006).

These examples show the versatility of yeast in high throughput analysis allowing researchers to gain fundamental insights on mechanisms of a-syn-mediated cellular degeneration.

Chapter II

Protein Glycation in *Saccharomyces cerevisiae*: Argpyrimidine Formation and Methylglyoxal Catabolism

Gomes RA, Sousa Silva M, Vicente Miranda H, Ferreira AEN, Cordeiro C, Ponces Freire A. 2005. *FEBS J*. **272**: 4521-4531.

1. Abstract

Methylglyoxal is the most important intracellular glycation agent, formed nonenzymatically from triose phosphates during glycolysis in eukaryotic cells. Methylglyoxal-derived advanced glycation end-products are involved in neurodegenerative disorders (Alzheimer's, Parkinson's and familial amyloidotic polyneurophathy) and in the clinical complications of diabetes. Research models for investigating protein glycation and its relationship to methylglyoxal metabolism are required to understand this process, its implications in cell biochemistry and their role in human diseases. We investigated methylglyoxal metabolism and protein glycation in *Saccharomyces cerevisiae*. Using a specific antibody against argpyrimidine, a marker of protein glycation by methylglyoxal, we found that yeast cells growing on D-glucose (100-mm) present several glycated proteins at the stationary phase of growth. Intracellular methylglyoxal concentration, determined by a specific HPLC based assay, is directly related to argpyrimidine formation. Moreover, exposing nongrowing yeast cells to a higher D-glucose concentration (250 mm) increases methylglyoxal formation rate and argpyrimidine modified proteins appear within one hour. A kinetic model of methylglyoxal metabolism in yeast, comprising its nonenzymatic formation and enzymatic catabolism by the glutathione dependent glyoxalase pathway and aldose reductase, was used to probe the role of each system parameter on methylglyoxal steady-state concentration. Sensitivity analysis of methylglyoxal metabolism and studies with gene deletion mutant yeast strains showed that the glyoxalase pathway and aldose reductase are equally important for preventing protein glycation in *Saccharomyces cerevisiae*.

2. Introduction

The glycation of extracellular proteins plays a major role in diseases like diabetes *mellitus* and related clinical complications, where D-glucose is the main glycation agent (Bucala & Cerami, 1992, Brownlee, 1995). In neurodegenerative diseases of amyloid type, where protein β-fibrils accumulate with time in specific human tissues and organs, glycation may lead to a folding transition causing the formation of β-fibrils from unstructured protein deposits and activate receptor-mediated cellular responses (Du Yan*, et al.*, 1997, Bouma*, et al.*, 2003). In Alzheimer's disease (β-amyloid deposits) and FAP (transthyretin deposits) glycation is present in extracellular amyloid deposits (Vitek*, et al.*, 1994, Chen*, et al.*, 2004, Gomes*, et al.*, 2005).

Intracellular protein glycation also occurs in amyloid fibrils in Alzheimer's disease (tau deposits) and Lewy inclusion bodies of α-synuclein in Parkinson's disease (Yan*, et al.*, 1994, Castellani*, et al.*, 1996). As the concentration of D-glucose is very low inside living cells, other glycation agents must be present. Among these, methylglyoxal, a product of the non-enzymatic phosphate β-elimination of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis, is likely to be the most significant *in vivo* (Richard, 1993).

Methylglyoxal reacts irreversibly with amino groups in proteins, forming methylglyoxal advanced glycation end-products (MAGE) in a slow non-enzymatic process (Booth*, et al.*, 1997, Thornalley, 1999). N^{ε}-(carboxyethyl)lysine and methylglyoxal-lysine dimers are the main products of the reaction of methylglyoxal with lysine residues, while with arginine it forms N^{δ}-(5-methyl-imidazolone-2-yl)-ornithine and N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-l-ornithine, commonly known as argpyrimidine (Shipanova*, et al.*, 1997, Westwood & Thornalley, 1997). Argpyrimidine is a specific marker of protein glycation by methylglyoxal (Shipanova*, et al.*, 1997). It has been detected in renal tissues (Oya*, et al.*, 1999) and lens proteins from diabetic patients (Ahmed*, et al.*, 1997) and in diabetic rat kidney mesangial cells (Padival*, et al.*, 2003). It was also found in human carcinoma cells exposed to high glucose concentration (Sakamoto*, et al.*, 2002) and in neurodegenerative disorders of amyloid type such as FAP (Gomes*, et al.*, 2005).

Because AGEs formation is an irreversible non-enzymatic process, preventing or delaying its occurrence may only be accomplished by reducing the amount of glycation agents such as methylglyoxal. This α-oxoaldehyde is mainly catabolised by two enzymatic pathways, whose relative importance is largely unknown (Figure II.1). The first is the glyoxalase pathway (Racker, 1951), comprising the enzymes glyoxalase I (lactoylglutathione methylglyoxallyase, EC 4.4.1.5) and glyoxalase II (hydroxyacylglutathione hydrolase, EC 3.1.2.6). It converts methylglyoxal to D-lactate using GSH as specific cofactor. The second is aldose reductase (aldehyde reductase, EC 1.1.1.21) that reduces methylglyoxal to 1,2-propanediol in a NADPH dependent two-step reaction (Vander Jagt & Hunsaker, 2003).

Yeast cells growing on D-glucose show a high glycolytic flux and a high rate of methylglyoxal formation (Martins*, et al.*, 2001), hinting that glycation might occur in these cells. Protein glycation by methylglyoxal in yeast, monitored by argpyrimidine formation in proteins, was evaluated in a set of null mutant yeast strains for genes involved in methylglyoxal detoxification: ΔGLO1, glyoxalase I gene; ΔGLO2, glyoxalase II gene; ΔGSH1, γ-glutamyl cysteinyl syntethase gene; ΔGRE3, aldose reductase gene; ΔYAP1, the transcription factor Yap1p gene. Yap1p closely

correlates with glutathione metabolism (Moye-Rowley, 2003) and its activity is directly regulated by methylglyoxal in yeast, being therefore essential to the cell's response to the continuous and unavoidable methylglyoxal formation (Maeta*, et al.*, 2004). A kinetic model of methylglyoxal metabolism in *S. cerevisiae*, based on experimentally determined parameters, was developed to probe the relative importance of each enzyme in preventing glycation.

The mathematical model described here has been submitted to the Online Cellular Systems Modeling database and can be accessed at http://jjj.biochem.sun.ac.za/database/gomes/index.html free of charge.

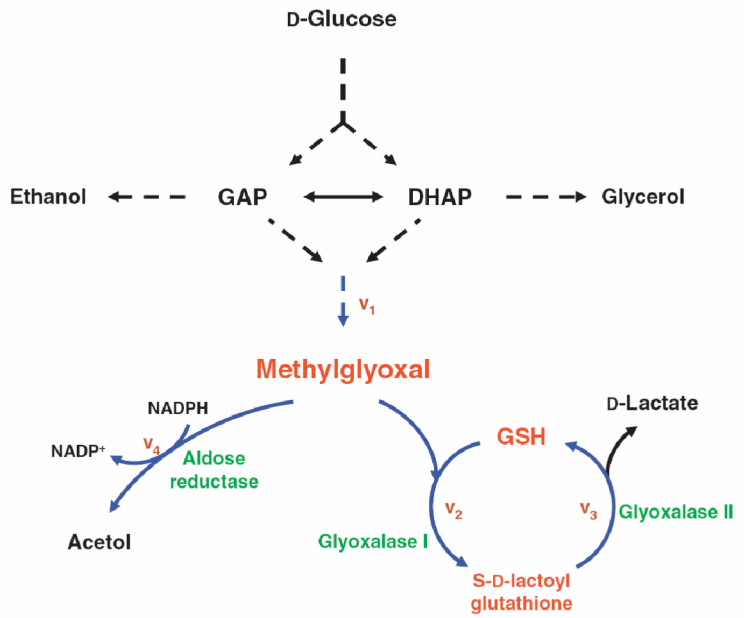


Figure II.1 Methylglyoxal metabolism in *S. cerevisiae*. Methylglyoxal is formed non-enzymatically from DHAP and GAP during glycolysis. It is converted into D-lactate by the glyoxalase system or acetol through aldose reductase. This metabolic map was used to build a mathematical model comprising the reactions represented by blue arrows, with rate equations v_i (dark red). Dynamic variables are marked red. Metabolites taken as constant or not considered in the model are marked black. Triose phosphates concentrations are constant and therefore methylglyoxal formation rate (v_1) is also constant. Detailed rate equations, parameters and reference steady-state conditions are given in Table II.1.

3. Experimental Procedures

3.1 Reagents and Materials

Peptone, yeast extract and agar were from Difco while D-glucose (microbiology grade) was from Merck (Rahway, NJ, USA). Mes, potassium dihydrogen phosphate, methylglyoxal 1,1-dimethyl acetal and monobromobimane were from Fluka (St Louis, MO, USA). Digitonin was

from Cal-Biochem (San Diego, CA, USA). Coomassie brilliant blue G, Ponceau S, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), glass beads (452–600 microns), S-D-lactoylglutathione (SDL-GSH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 1,2-diaminobenzene were from Sigma (St Louis, MO, USA). 2,3-Dimethylquinoxaline was from Aldrich (St Louis, MO, USA) while NADPH and GSH were from Roche (Indianapolis, IN, USA). Solvents were of HPLC grade while all other reagents were of analytical grade.

3.2 Yeast Strains and Culture Conditions

Saccharomyces cerevisiae strains from the Euroscarf collection (Frankfurt, Germany) were: BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0), ΔGLO1 (isogenic to BY4741 with YML004c::KanMX4), ΔGLO2 (isogenic to BY4741 with YDR272w::KanMX4), ΔGSH1 (isogenic to BY4741 with YJL101c::KanMX4), ΔGRE3 (isogenic to BY4741 with YHR104w::KanMX4) and ΔYAP1 (isogenic to BY4741 with YHR161c::KanMX4). ΔGRE3ΔGLO1 strain (*MATa*; *his3*Δ200; *leu2*Δ1; *ura3-52*; *trp1*Δ1; *lys2-801*; *ade2-101*; glo1::HIS3; gre3::KanMX4) was kindly provided by Dr. J. Prieto (Department Biotech, Instituto de Agroquimica y Tecnologia de los Alimentos, Valencia, Spain). Strains were kept in YPGlu [0.5% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) D-glucose] agar slopes (2% agar) at 4 °C and cultured in liquid YPGlu medium with 100 mM D-glucose. Experiments with non-dividing yeast cells were performed in 0.1 M Mes/NaOH pH 6.5 with 250 mM D-glucose.

3.3 Methylglyoxal Preparation

High purity methylglyoxal was prepared by acid hydrolysis of methylglyoxal 1,1-dimethyl acetal as reported (Kellum*, et al.*, 1978), followed by fractional distillation under reduced pressure in nitrogen atmosphere (McLellan*, et al.*, 1992). Once prepared, methylglyoxal solutions were standardized by enzymatic assay with glyoxalase I and II (Racker, 1951). Purity was verified by HPLC analysis and ^{13}C NMR (Bruker advance 400 MHz, USA).

3.4 Metabolite Assay

Samples were extracted with 2.5 M $HClO_4$, stirred, kept on ice for 10 min and immediately analysed (as in the case of methylglyoxal assay) or stored at 80 °C. Methylglyoxal concentration

was determined by reverse phase HPLC as 2-methylquinoxaline after derivatization with 1,2-diaminobenzene, as described (Cordeiro & Ponces Freire, 1996). For quantification, a calibration curve was obtained by plotting known methylglyoxal concentrations against ratios of analytic peak height to internal standard (1,2-dimethylquinoxaline) peak height. Glutathione was assayed by reverse phase HPLC with fluorescence detection ($\lambda_{emission,max}/\lambda_{excitation,max}$ 397/490 nm) after derivatization with monobromobimane, as described previously (Sousa Silva*, et al.*, 2005). D-Glucose was enzymatically assayed with hexokinase/D-glucose 6-phosphate dehydrogenase (D-glucose assay kit, Boehringer Mannheim), following the manufacturer's instructions. HPLC analysis were performed with a Beckman-Coulter high-pressure binary gradient pump 126, a Beckman-Coulter 168-diode-array detector (1 nm resolution, 200–600 nm) and a Jasco FP-2020 Plus fluorescence detector. For methylglyoxal assay a Merck LichroCART 250–2 (250 mm x 2 mm) column with stationary phase Purospher 100 RP-18e, 5 µm, was used at a flow rate of 0.3 ml.min^{-1}. For GSH assay, a Merck LichroCART 250-4 (250 mm x 4 mm) column with stationary phase Lichrospher 100 RP-18, 5 µm, was used at a flow rate of 1 ml.min^{-1}.

3.4 Analysis of Argpyrimidine Modified Proteins By Western Blot

Total yeast protein extraction was performed by glass bead lyses as described (Ausubel*, et al.*, 1990). Briefly, cells were harvested by centrifugation and suspended in 100 mM potassium phosphate buffer pH 7.4, containing 1 mM PMSF. An equal volume of glass beads was added and shaken in a vortex stirrer at maximum speed for five cycles of 1 min followed by 1 min of cooling on ice. The homogenate was centrifuged at 8000 g for 15 min at 4 °C and the supernatants were retained. Protein concentration was determined using the Bio-Rad Bradford assay kit.

Proteins (30 µg protein per lane) were separated by SDS/PAGE in a Mini-protean 3 (Bio-Rad), using a 12% polyacrylamide separation gel and a 6% polyacrylamide stacking gel. Proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech), using the Mini Trans-Blot system (Bio-Rad). Transfer was performed with 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% (v/v) methanol. Pre-stained standard proteins (Bio-Rad) were also loaded on the gel. Total proteins were stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm the amount of protein transferred. The membrane was blocked overnight at 4 °C in 1% (v/v) blocking solution in TBS (50 mM Tris with 150 mM NaCl pH 7.5). The blots were probed with anti-argpyrimidine monoclonal antibody, a kind gift from Dr. K.

Uchida (Nagoya University, Japan), diluted 1:2000 in 0.5% (v/v) blocking solution in TBS for 2.5 h at room temperature (25 °C). Washes, secondary antibody and detection procedures were performed using the BM Chemiluminescence Western Blotting Kit (Roche) following the manufacturer's instructions. Each immunoblot was repeated three times from independent experiments.

3.5 Enzyme Activities Assay and *in Situ* Kinetics

Enzymatic activities were determined *in situ* using *S. cerevisiae* permeabilized cells. Permeabilization was achieved by incubation with 0.01% (w/v) digitonin in 0.1 mM Mes/NaOH, pH 6.5 for 15 min at 30 °C, in an orbital shaker incubator (Infors HT). Enzyme activities were determined at 30 °C in a 1.5 ml reaction volume, in 0.1 M Mes, pH 6.5 and 70 mM of KH_2PO_4. All assays were performed on a Beckman DU-7400 diode array spectrophotometer, with temperature control and magnetic stirring, essential to maintain isotropic conditions.

Aldose reductase activity was measured by following NADPH oxidation at 340 nm in the presence of methylglyoxal. Apparent kinetic parameters were determined by varying NADPH concentration at fixed methylglyoxal concentrations. NADPH concentration was varied in the range of 0.03-0.13 mM and methylglyoxal concentration was changed between 0.25 and 6 mM. Glyoxalase I activity was assayed by SDLGSH formation (followed at 240 nm) in the presence of GSH and methylglyoxal (Racker, 1951). Apparent kinetic parameters were determined by varying GSH concentration at fixed methylglyoxal concentrations. GSH concentration was varied in the range 0.4-6 mM and methylglyoxal concentration was changed between 0.6 and 4 mM. Glyoxalase II activity was determined by following GSH formation, using SDLGSH as substrate (Martins*, et al.*, 2001). Kinetic parameters were determined by varying SDLGSH initial concentration between 0.1 and 1.5 mM.

3.6 Modeling and Computer Simulation

Modeling and computer simulation were used to evaluate the relative importance of a few critical parameters of methylglyoxal catabolism on the methylglyoxal steady-state concentration in *S. cerevisiae*. The parameters considered were methylglyoxal influx, total thiol moiety concentration, NADPH concentration and enzyme activities (glyoxalase I, glyoxalase II and aldose reductase). Methylglyoxal metabolism in yeast was represented by a set of ordinary differential equations describing methylglyoxal formation from the triose phosphates, its reaction with GSH,

aldose reductase and the glyoxalase pathway (Figure II.1 and Table II.1). Two-substrate sequential enzyme rate equations were assumed for aldose reductase and glyoxalase I while a single substrate irreversible Michaelis–Menten rate equation was assumed for glyoxalase II. NADPH concentration was considered to be constant at 1.7 mM (Vaseghi*, et al.*, 1999) and the GSH concentration was initially set at 4 mM (this work). In the model, we also assumed a constant methylglyoxal formation rate, calculated from the previously reported intracellular concentrations of dihydroxyacetone phosphate (0.12 mM) and D-glyceraldehyde 3-phosphate (2.5 mM) (Hynne*, et al.*, 2001) and the first order decomposition rate constants of 6.36×10^{-3} min^{-1} and 6.60×10^{-4} min^{-1}, respectively (this study). Model parameters were determined by classic initial rate analysis or full time-course analysis (Martins*, et al.*, 2001, Sousa Silva*, et al.*, 2005). In the latter, the optimization step was performed using the differential evolution algorithm (Storn & Price, 1997) implemented in the library AGEDO (Abecasis*, et al.*, 2004). Simulations were performed with the software package PLAS (A.E.N. Ferreira, University of Lisbon, Portugal; http://www.dqb.fc.ul.pt/docentes/aferreira/plas.html).

4. Results

4.1 Protein glycation in yeast cells is a fast and non-random process

Yeast strains growing in YPGlu medium (100 mM D-glucose) reach the stationary phase of growth in 9 days. At this time, cytosolic proteins were extracted and analysed by western blotting. Argpyrimidine-modified proteins were observed in all strains except BY4741 (Figure II.2B). Compared to a total protein Coomassie blue stained gel (Figure II.2A) it is evident that only a few proteins are glycated. The high immunoreactivity observed reveals that argpyrimidine-modified proteins may appear before the stationary phase of growth. A time course of argpyrimidine formation in yeast proteins was then performed (Figure II.3A). Accumulation of the same argpyrimidine-modified proteins, starting after only 3 days of growth, was observed. ΔGLO1 and ΔGRE3 strains showed the highest and similar levels of argpyrimidine-modified proteins, hinting that both enzymes are equally important in preventing MAGE formation. This result led us to investigate argpyrimidine formation in a yeast strain lacking both aldose reductase and glyoxalase I genes (ΔGRE3ΔGLO1 strain). This strain is more prone to argpyrimidine formation than any other strain analysed (Figure II.3B). Argpyrimidine-modified proteins are observed after only 2 days of

growth and the intensity of the immunoreactive proteins is much higher after 3 days of growth than after 9 days of growth for any other strains in which glycation occurs. Surprisingly, the ΔGLO2 strain, lacking glyoxalase II, presents very low glycation levels, detectable only after 9 days of growth.

Although glycation has been described as a non-enzymatic process, where all proteins are putative targets, only three major argpyrimidine-modified proteins were observed by immunoblotting, with apparent molecular weights of 52, 40 and 35 kDa (Figure II.2B and II.3). Protein glycation in yeast cells is a fast and non-random process whereby specific protein targets for argpyrimidine formation appear to exist.

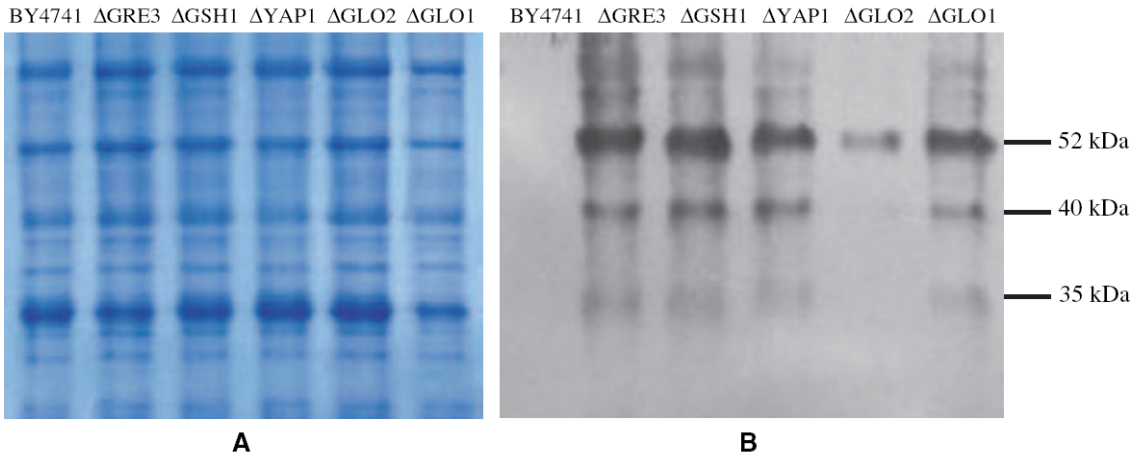


Figure II.2 Protein glycation in yeast cells. (A) Total protein Coomassie blue stained gel of the reference strain (BY4741) and mutant strains (ΔGRE3, ΔGSH1, ΔYAP1, ΔGLO2 and ΔGLO1). (B) Argpyrimidine formation in intracellular proteins from the same yeast strains as in (A), probed by western blotting with a specific anti-argpyrimidine Ig. Proteins were extracted after 9 days of growth, at the stationary phase. Equal amounts of protein were loaded (30 μg). The membrane was incubated with the primary antibody for 2.5 h and immunocomplexes were visualized by chemiluminescence western blotting. Three major argpyrimidine immunoreactive protein bands with molecular masses of 52, 40 and 35 kDa are clearly observed. Representative gels and immunoblots, from a set of more than three experiments, are shown.

Methylglyoxal concentration in yeast cells, reaching a maximum at the end of the exponential phase, is in agreement with the observed glycation phenotypes (Figure II.4). Methylglyoxal concentration is significantly increased in yeast strains where argpyrimidine modified proteins are observed (ΔGLO1, ΔGRE3, ΔGSH1, ΔYAP1 and ΔGRE3ΔGLO1). The occurrence of glycation in the form of argpyrimidine modified proteins depends on increasing the intracellular methylglyoxal steady-state concentration.

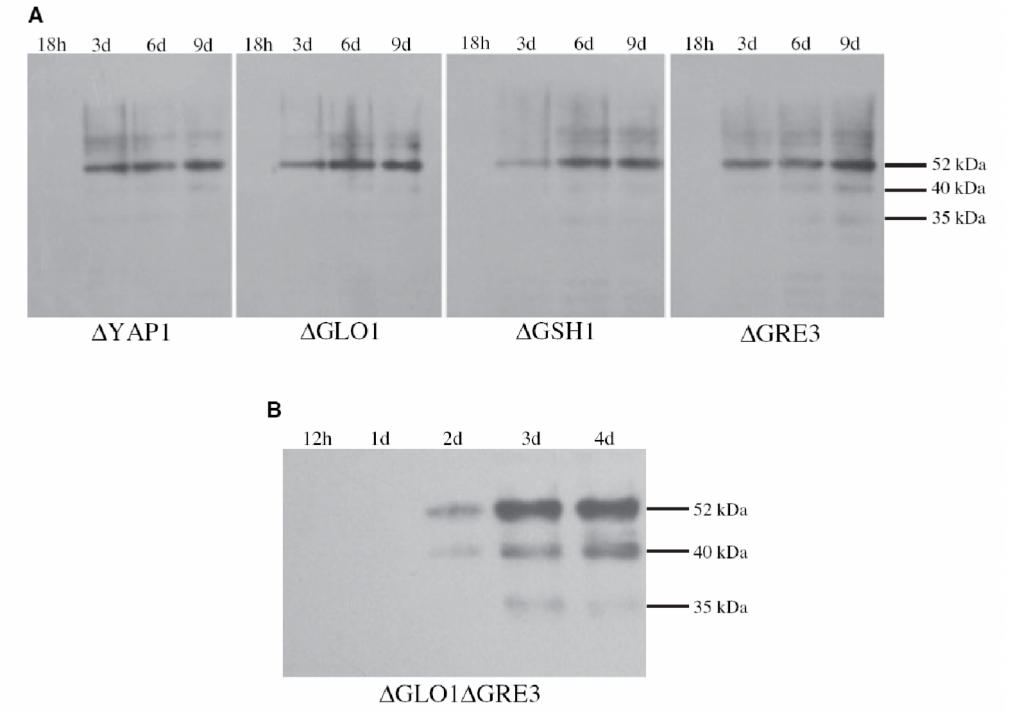


Figure II.3 Time course of argpyrimidine formation in yeast. (A) Time course of argpyrimidine formation in single gene deletion strains. Yeast strains and growth time are shown. (B) Time course of argpyrimidine formation in a double mutant ΔGRE3ΔGLO1, lacking glyoxalase I and aldose reductase. Argpyrimidine formation is a much faster process in this strain. In all immunoblots, the same three major immunoreactive protein bands are visible (52, 40 and 35 kDa). AGEs-modified proteins were detected by western blot as described. Representative immunoblots, from a set of more than three experiments, are shown.

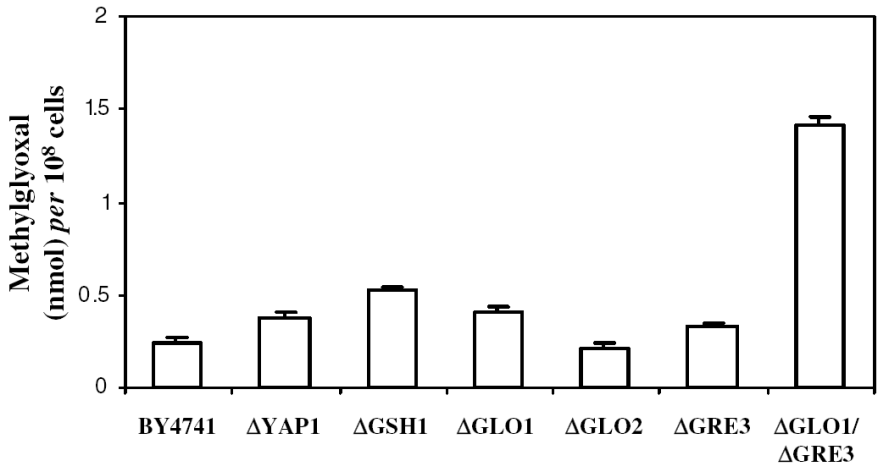


Figure II.4 Methylglyoxal concentration in yeast cells at the end of the exponential phase (18 h of growth). Methylglyoxal was quantified by HPLC as 2-methylquinoxaline after derivatization with 1,2-diaminobenzene. Yeast strains showing glycation present higher levels of methylglyoxal, compared with the reference strain. Data are the averages from three independent experiments ± SD.

4.2 Sensitivity Analysis of Methylglyoxal Metabolism in Yeast

Glyoxalase I and aldose reductase emerged as the most important glycation preventing enzymes. To investigate the relative importance of the glyoxalase pathway and aldose reductase on methylglyoxal catabolism in yeast, a kinetic model was developed (Figure II.1 and Table II.1). The roles of glyoxalase I, glyoxalase II, aldose reductase activities and initial GSH concentration on methylglyoxal steady-state concentration were first investigated (Figure II.5). Glyoxalase I, as well as aldose reductase and GSH concentration, showed marked effects on methylglyoxal concentration (Figure II.5A, C and D). Absence of glyoxalase I (describing the ΔGLO1 strain) predicts a threefold increase of methylglyoxal concentration (Figure II.5A), while absence of aldose reductase activity (ΔGRE3 strain) causes a twofold increase (Figure II.5D). Methylglyoxal concentration is also highly sensitive to GSH concentration and, as it decreases to very low levels (5% in the ΔGSH1 strain as compared to the reference strain) methylglyoxal concentration increases threefold (Figure II.5C). Glyoxalase II activity has virtually no effects on methylglyoxal concentration. Only when glyoxalase II activity decreases to 0.031% of its reference value does methylglyoxal concentration increases by 10%. Without glyoxalase II (ΔGLO2 strain) the model predicts a threefold increase of methylglyoxal concentration, identical to the one predicted in the absence of glyoxalase I activity (Figure II.5B). This is neither in agreement with methylglyoxal concentration measurements nor with the glycation phenotypes for the ΔGLO1 and ΔGLO2 strains.

Table II.1 Rate equations and kinetic parameters of the methylglyoxal metabolic model represented in Figure II.1. Note that in this model there is conservation of the *S*-glutathionyl group: with the given initial values, *S*-glutathionyl total = GSH(0) = GSH(t) + SDLGSH(t) at any time t.

Rate equations	Differential equations	Parameters and initial values	Reference steady-state
$v_2 = \frac{V_1 \times \text{MGO} \times \text{GSH}}{(Km_1 + \text{MGO})(Km_2 + \text{GSH})}$ $v_3 = \frac{V_2 \times \text{SDLGSH}}{Km_3 + \text{SDLGSH}}$ $v_4 = \frac{V_3 \times \text{NADPH} \times \text{MGO}}{(Km_4 + \text{MGO})(Km_5 + \text{NADPH})}$	$\frac{d\,\text{MGO}}{dt} = v_1 - v_2 - v_4$ $\frac{d\,\text{SDLGSH}}{dt} = v_2 - v_3$ $\frac{d\,\text{GSH}}{dt} = v_3 - v_2$	$v_1 = k_1\,\text{GAP} + k_2\text{DHAP}$ $= 2.41 \times 10^{-3}$ mM·min^{-1} $V_1 = 186.45$ mM·min^{-1} $V_2 = 8.09$ mM·min^{-1} $V_3 = 17.85$ mM·min^{-1} $k_1 = 6.36 \times 10^{-3}$ min^{-1} $k_2 = 6.60 \times 10^{-4}$·min^{-1} $Km_1 = 3.56$ mM $Km_2 = 1.64$ mM $Km_3 = 0.91$ mM $Km_4 = 0.65$ mM $Km_5 = 0.075$ mM NADPH = 0.17 mM GAP = 0.12 mM DHAP = 2.50 mM GSH(0) = 4.00 mM SDLGSH(0) = MGO(0) = 0	MGO = 4.30×10^{-3} mM GSH = 4.00 mM SDLGSH = 1.81×10^{-4} mM

To explore synergistic effects of both pathways on methylglyoxal steady-state concentration, glyoxalase I and aldose reductase activities were varied independently (Figure II.6). In the extreme case where both enzymes are absent (describing the ΔGRE3ΔGLOI strain) methylglyoxal concentration does not reach a steady state and increases with time (Figure II.6). Although methylglyoxal clearance through glyoxalase I represents 60% of its catabolism, aldose reductase may be crucial when the glyoxalase system is limited, namely by GSH depletion in oxidative stress conditions. The pattern of methylglyoxal increase, predicted by simulation, agrees with the glycation phenotypes of all strains studied (except the ΔGLO2 strain) and was confirmed by methylglyoxal assay.

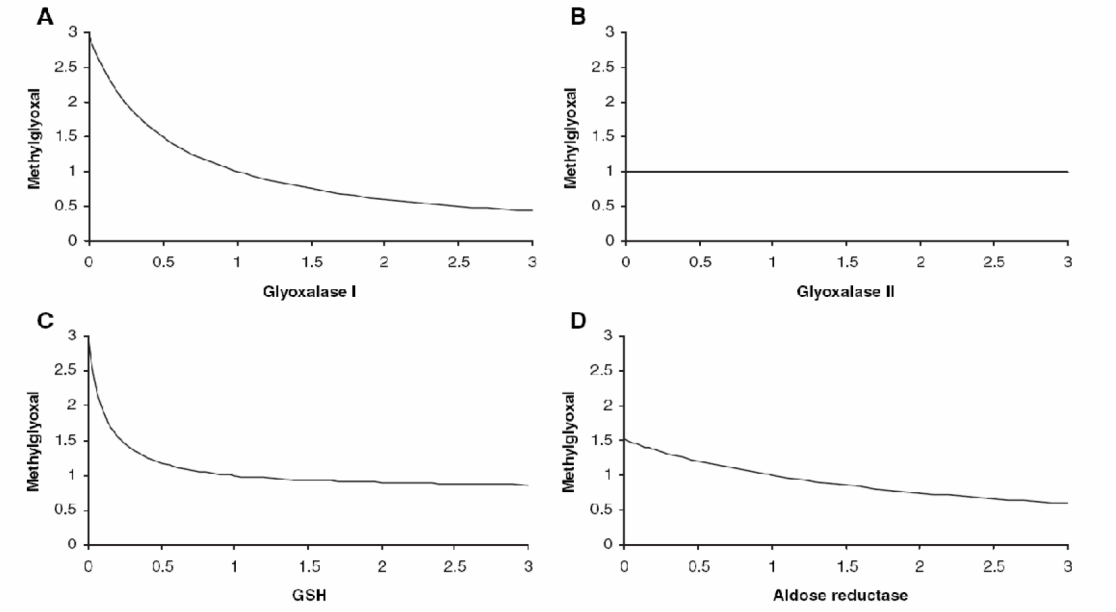


Figure II.5 Sensitivity analysis of methylglyoxal metabolism in *S. cerevisiae*. Single parameter variation. The effects of system parameters on the methylglyoxal intracellular steady-state concentration were investigated by finite parameter changes (between zero- and threefold) around the reference steady state. All values are fold variations relative to the reference state (normalized values). System parameters were: glyoxalase I activity (A), glyoxalase II activity (B), initial GSH concentration (C) and aldose reductase activity (D).

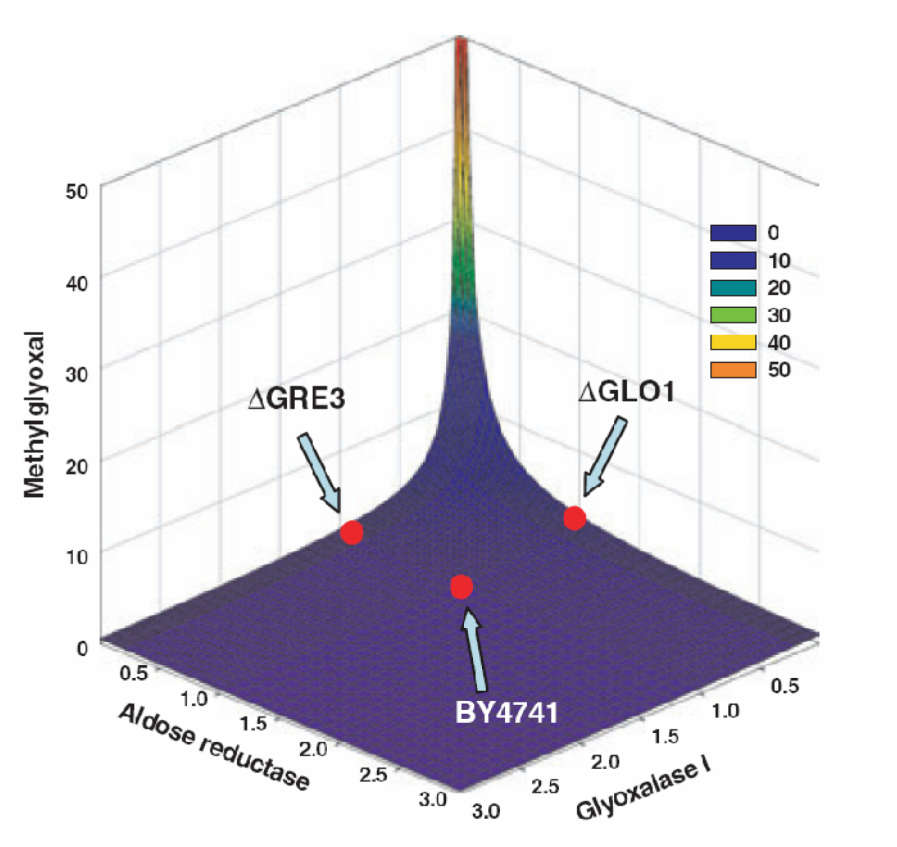


Figure II.6 Sensitivity analysis of methylglyoxal metabolism in *S. cerevisiae*. Synergistic effects of glyoxalase I and aldose reductase activities on methylglyoxal steady-state concentration. The reference strain BY4741 (glyoxalase I and aldose reductase reference activities) and the mutants ΔGRE3 (reference activity of glyoxalase I and no aldose reductase activity) and ΔGLO1 (reference activity of aldose reductase and no glyoxalase I activity) represent the conditions marked by red dots. All values are fold variations relative to the reference state (normalized values).

4.3 Predicting Glycation Phenotype

According to modeling and computer simulation, there is a linear relationship between methylglyoxal steady-state concentration and its formation rate (Figure II.7A). Therefore, a sudden increase in methylglyoxal concentration could promote argpyrimidine formation in BY4741 strain. In yeast (Inoue*, et al.*, 1998, Aguilera & Prieto, 2001, Aguilera & Prieto, 2004), mesangial cells (Padival*, et al.*, 2003) and in human carcinoma cells (Sakamoto*, et al.*, 2002) an overproduction of methylglyoxal can be caused if glucose catabolism is increased. Challenging BY4741 cells with a high D-glucose concentration (250 mM) in non-growing conditions, increases methylglyoxal concentration and argpyrimidine-modified proteins were observed after 1 h (Figure II.7B and C). Increased methylglyoxal concentration is directly related to glucose consumption (Figure II.7B).

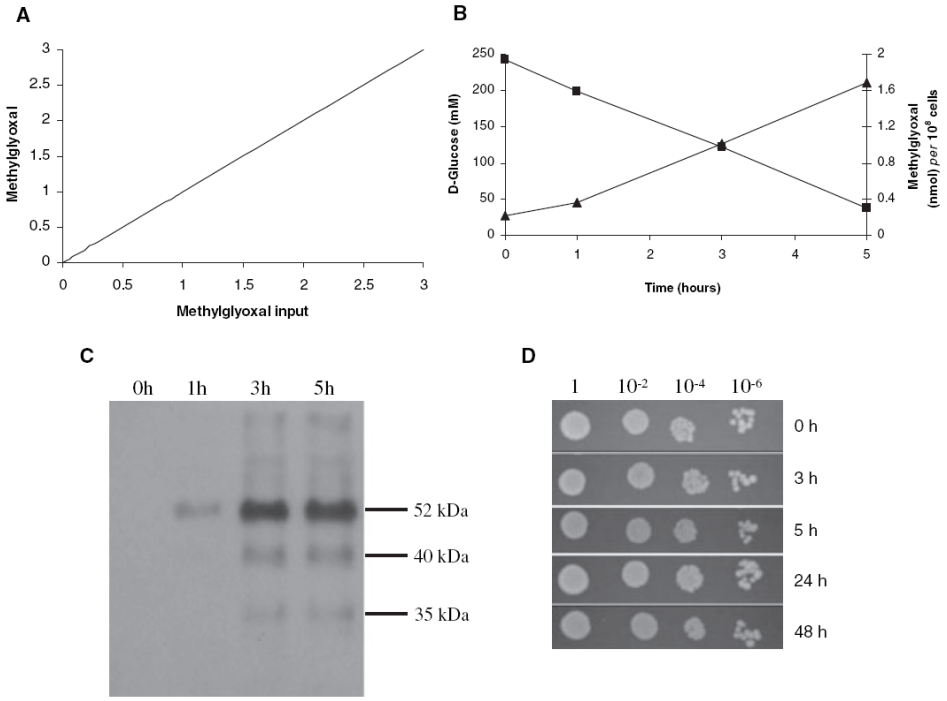


Figure II.7 Predicting glycation phenotype: increasing methylglyoxal concentration causes the formation of argpyrimidine-modified proteins within 1 h in *S. cerevisiae*. (A) Simulated effect of finite changes of methylglyoxal input in methylglyoxal steady-state concentration. All values are fold variations relative to the reference state (normalized values). (B) D-Glucose consumption (squares) and methylglyoxal formation (triangles) in non-dividing BY4741 cells challenged with 250 mM D-glucose. (C) Formation of argpyrimidine-modified proteins in the reference strain in high D-glucose (250 mM). AGEs-modified proteins were detected by western blot as described. Equal amounts of protein were loaded. (D) Viability assay of BY4741 yeast cells after exposure to high D-glucose. Incubation times are indicated, as well as dilution factors. Representative results from a set of more than three experiments are shown.

Interestingly, the same three major argpyrimidine-modified proteins are observed. However, in non-growing cells, intracellular protein glycation is a much faster process. Although the glycated proteins are the same, indicating that a similar glycation mechanism is present, cells have to deal with these modifications at an earlier stage. *De novo* protein synthesis is not occurring and the dilution effect caused by cell division is also absent. BY4741 cells, submitted to these experimental conditions remain viable (Figure II.7D) and do not undergo apoptosis, as shown by DNA fragmentation pattern analysis (data not shown). In the same experimental conditions of high D-glucose medium and non-dividing cells, all other strains show the same unchanged viability, even after 48 h (data not shown).

5. Discussion

We observed for the first time the formation of argpyrimidine modified proteins in yeast cells. Although protein glycation has been primary associated with complex organisms and long-lived proteins exposed to high levels of glycation agents, this non-enzymatic, spontaneous and irreversible post-transcriptional modification also affects short-lived organisms like yeast. When growing in YPGlu medium (100 mM D-glucose), argpyrimidine modified proteins are observed only in null mutant yeast strains for genes involved in methylglyoxal catabolism (ΔGLO1, ΔGRE3, ΔGSH1, ΔYAP1 and ΔGLO2). By contrast, non-growing BY4741 presents argpyrimidine modified proteins after only 1 h of exposure to high D-glucose medium. Formation of argpyrimidine modified proteins in these conditions indicates that cells can prevent AGEs formation only until anti glycation defenses are overcome. ΔGRE3 and ΔGLO1 strains show similar levels of argpyrimidine modified proteins, indicating that glyoxalase I and aldose reductase are equally important in preventing methylglyoxal-derived protein glycation in yeast. In fact, the double mutant ΔGRE3ΔGLO1 strain is more prone to argpyrimidine formation than a strain lacking just one of these enzymes. Glyoxalase I is a key enzymatic anti glycation enzyme (Shinohara*, et al.*, 1998). Although glyoxalase II is part of the glyoxalase system, a strain lacking glyoxalase II activity shows very low levels of argpyrimidine modified proteins. This indicates that glyoxalase II plays a minor role in maintaining a low intracellular methylglyoxal concentration in the presence of high GSH concentration (4 mM in *S. cerevisiae*). In our model of yeast methylglyoxal metabolism, glyoxalase II activity is essential for replenishing GSH and therefore, the same methylglyoxal steady-state concentration is reached in the absence of either glyoxalase I or glyoxalase II. However, this steady state is reached after 4 days in the absence of glyoxalase II, while without glyoxalase I it is attained in only a few minutes. GSH biosynthesis in living cells also diminishes the glyoxalase II recycling effect. This explains the lower level of glycated proteins in ΔGLO2 cells and the similar methylglyoxal concentration, at the end of the exponential phase, compared to BY4741 strain.

The role of aldose reductase as an anti glycation enzyme is less clear due to its broad substrate specificity. This enzyme has been implied in the protection against methylglyoxal toxicity, an endogenous substrate for aldose reductase (Vander Jagt*, et al.*, 1992). Aguilera and co-workers demonstrated that overexpression of aldose reductase increases methylglyoxal tolerance in *S. cerevisiae* and complements glyoxalase deficiency in the ΔGLO1 strain (Aguilera & Prieto, 2001). We observed a 1.5-fold increase in methylglyoxal concentration in an aldose

reductase deficient strain, in agreement with simulated data. It is noteworthy that in the ΔGLO1 strain, a twofold increase in methylglyoxal concentration is observed, again in good agreement with simulated results. By sensitivity analysis, methylglyoxal detoxification by aldose reductase is highly relevant, assuming a significant proportion of methylglyoxal catabolism (40%). When the glyoxalase system is limited, namely by GSH depletion in oxidative stress conditions, aldose reductase may be crucial to maintain a low methylglyoxal concentration. Hence, aldose reductase is an important anti glycation enzyme for methylglyoxal induced protein glycation, almost as important as the glyoxalase pathway in yeast, and it should be considered in studies where the main goal is to prevent protein glycation.

AGEs formation is described as a non-enzymatic, irreversible modification of lysine and arginine residues slowly formed through long-term exposure to high concentration of sugars and reactive compounds like methylglyoxal. Therefore, any protein is a putative target of glycation. Here we demonstrate that protein glycation affects short-lived organisms like yeast and is fast and non-random. In agreement with this idea, in glomerular mesangial cells and human carcinoma cells, Hsp 27 is the primary target for methylglyoxal-induced AGEs formation (Sakamoto*, et al.*, 2002). Van Herreweghe and co-workers reported a specific methylglyoxal-derived AGE formed during TNF-induced cell death, indicating that protein modification by methylglyoxal might be a targeted process, with yet unknown physiological roles (Van Herreweghe*, et al.*, 2002). Due to the non-enzymatic, irreversible and deleterious nature of protein glycation, the existence of specific protein targets is quite intriguing.

An interesting feature is how non-dividing yeast cells neutralize the harmful effects of protein glycation. Answering this question will provide significant clues regarding neurodegenerative disorders, where intracellular protein glycation in quiescent cells is associated with the pathology, and diabetic polyneuropathy, where quiescent cells are exposed to high D-glucose levels. It is also important in understanding how cell ageing due to glycation can be prevented. For this purpose, yeast cells are an outstanding cell model for investigating intracellular protein glycation and its implications in cell physiology.

6. Aknowledgments

We thank Dr. J. Prieto for providing the ΔGRE3ΔGLO1 strain and Dr. K. Uchida for the gift of the anti-argpyrimidine monoclonal antibody. Work supported by grants SFRH/BD/13884/2003 (R.A.G) and POCTI/ESP/48272/2002 (M.S.S) from the Fundação para a Ciência e a Tecnologia - Ministério da Ciência, Tecnologia e Ensino Superior, Portugal.

Chapter III

Yeast Protein Glycation *in Vivo* by Methylglyoxal: Molecular Modification of Glycolytic Enzymes and Heat Shock Proteins

Gomes RA, Vicente Miranda H, Sousa Silva M, Graça G, Coelho AV, Ferreira AEN, Cordeiro C, Ponces Freire A. 2006. FEBS J **273**: 5273-5287.

Gomes RA, Vicente Miranda H, Sousa Silva M, Graça G, Coelho AV, Ferreira AEN, Cordeiro C, Ponces Freire AP. 2007. *FEMS Yeast Res.* **8**(1)*: 174-181.*

1. Abstract

Protein glycation by methylglyoxal is a non-enzymatic post-translational modification where arginine and lysine side chains form a chemically heterogeneous group of advanced glycation end-products. Methylglyoxal-derived advanced glycation end-products are involved in pathologies such as diabetes *mellitus* and neurodegenerative diseases of amyloid type. Since methylglyoxal is produced non-enzymatically from dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate during glycolysis, its formation occurs in all living cells. Understanding methylglyoxal glycation in model systems will provide important clues regarding glycation prevention in higher organisms in the context of widespread human diseases. Using *Saccharomyces cerevisiae* cells with different glycation phenotypes and MALDI-TOF peptide mass fingerprint we identified enolase2 as the primary methylglyoxal glycation target in yeast. Two other glycolytic enzymes are also glycated, aldolase and phosphoglycerate mutase. Despite enolase's activity loss, in a glycation-dependent way, glycolytic flux and glycerol metabolism remained unchanged. None of these enzymes has any effect on the glycolytic flux, excepted for extreme changes, as evaluated by sensitivity analysis, showing that yeast glycolysis is a very robust metabolic pathway. Three heat shock proteins are also glycated, Hsp71/72 and Hsp26. For all glycated proteins, the nature and molecular location of some MAGE was determined by MALDI-TOF. Yeast cells experienced selective pressure towards an efficient use of D-glucose, with high methylglyoxal formation as a side effect. Glycation is a fact of life for these cells, and some glycolytic enzymes could be deployed to contain methylglyoxal that evades its enzymatic catabolism. Heat shock proteins may be involved in proteolytic processing (Hsp71/72) or protein salvaging (Hsp26).

2. Introduction

Protein glycation is a post-translational modification whereby amino groups in arginine and lysine side chains react irreversibly with carbonyl molecules forming advanced glycation end products (AGEs). Glycation is equivalent to a point mutation, exerting profound effects on protein structure, stability and function. AGEs formation in proteins is associated to the clinical complications of diabetes *mellitus* (Brownlee, 1995), cataracts (Lyons*, et al.*, 1991), uraemia (Miyata*, et al.*, 1999), atherosclerosis (Kume*, et al.*, 1995) and age related disorders (Bucala & Cerami, 1992). Glycated proteins are present in β-amyloid deposits and tau deposits in Alzheimer's

disease (Vitek*, et al.*, 1994, Yan*, et al.*, 1994, Chen*, et al.*, 2004), in Lewy inclusion bodies of a-syn in Parkinson's disease (Castellani*, et al.*, 1996) and in transthyretin amyloid deposits in FAP (Gomes*, et al.*, 2005). In all these amyloid pathologies, β-sheet fibril structure and the presence of AGEs are common features, suggesting a possible role for glycation in amyloid formation and pathogenesis.

Methylglyoxal is the most significant glycation agent *in vivo*, being one of the most reactive dicarbonyl molecules in living cells. This compound is an unavoidable by-product of glycolysis, arising from the non-enzymatic β-elimination reaction of the phosphate group of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Richard, 1993). Methylglyoxal reacts irreversibly with amino groups in lipids, nucleic acids and proteins, forming methylglyoxal advanced glycation end-products (MAGE) (Booth*, et al.*, 1997, Westwood & Thornalley, 1997) .

Argpyrimidine, hydroimidazolones (isomers and oxidation products) and tetrahydropyrimidine (THP) are specific markers of protein glycation by methylglyoxal on arginine residues (Shipanova*, et al.*, 1997, Westwood & Thornalley, 1997). Methylglyoxal specifically forms N^{ε}-(carboxyethyl)lysine (CEL) and methylglyoxal-lysine dimer (MOLD) with lysine residues (Ahmed*, et al.*, 1997, Frye*, et al.*, 1998).

Understanding methylglyoxal catabolism and the identity of MAGE protein targets are of prime importance with regard to glycation prevention. In eukaryotic cells, two pathways are responsible for methylglyoxal detoxification. The first is the formation of D-lactate by the glutathione-dependent glyoxalase system, comprising the enzymes glyoxalase I (lactoylglutathione methylglyoxal-lyase, EC 4.4.1.5) and glyoxalase II (hydroxyacylglutathione hydrolase, EC 3.1.2.6) (Racker, 1951). The second is the producing of 1,2-propanediol by NADPH-dependent aldose reductase (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21) (Vander Jagt*, et al.*, 1992, Vander Jagt & Hunsaker, 2003). In yeast, both pathways are equally important as anti-glycation defenses against protein glycation by methylglyoxal (Gomes*, et al.*, 2005). Given its high glycolytic flux and consequently high intracellular methylglyoxal concentration, yeast is highly susceptible to protein glycation, making it a suitable eukaryotic model organism to investigate this process *in vivo* (Gomes*, et al.*, 2005). Remarkably, only a few proteins appeared to be extensively glycated, and yeast cells cope remarkably well with glycation *in vivo* by methylglyoxal, remaining viable and without apparent growth changes (Gomes*, et al.*, 2005).

In the present study, we identified the MAGE protein targets by peptide mass fingerprint and determined its nature and molecular location in the modified proteins. As some of these

proteins are glycolytic enzymes, modeling and computer simulation was used to perform a sensitivity analysis of the glycation effects on glycolytic flux.

3. Experimental Procedures

3.1 Reagents and Materials

Peptone, yeast extract, agar and yeast nitrogen base (YNB) were obtained from Difco while D-glucose (microbiology grade), KCl, NaCl, $MgSO_4$, methanol and bromophenol blue were obtained from Merck. Coomassie Brilliant Blue G, Ponceau S, PMSF, glass beads (452-600 microns), adenine, uracil, L-methionine, L-histidine, L-leucine, L-tryptophan, MES, 3-phosphoglycerate, formic acid, ammonium hydrogencarbonate, dithiothreitol and iodoacetamide were obtained from Sigma. KH_2PO_4 was obtained from Fluka, digitonin from CalBiochem and EDTA from BDH Chemicals LTD. Tris, SDS 20% (w/v) and glycine were obtained from BioRad. Modified trypsin was obtained from Promega; GELoader tips were obtained from Eppendorf; TFA and HPLC-grade acetonitrile were obtained from Riedel de Häen; type I water was obtained in a Millipore Milli-Q system; POROS 10 R2 revered-phase chromatography medium was obtained from PerSeptive Biosystems; α-cyano-4-hydroxicinamic acid (α-CHCA) and PepMix1 (mixture of peptide standards) were obtained from LaserBiolabs.

3.2 Yeast Strains and Culture Conditions

Saccharomyces cerevisiae strains, Euroscarf collection (Frankfurt, Germany), were: BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), ΔGLO1 (isogenic to BY4741 with YML004c::KanMX4) and ΔGRE3 (isogenic to BY4741 with YHR104w::KanMX4). The YEpGRE3 transformant (Aguilera & Prieto, 2001) was kindly provided by Dr. J. Prieto (Dep. Biotech. Instituto de Agroquimica y Tecnologia de los Alimentos, Valencia, Spain). Strains were kept in YPGlu [0.5% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) D-glucose] agar slopes [2% (w/v) agar] at 4 °C and cultured in liquid YPGlu medium or YNB [0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose and 0.025% (w/v) of L-methionine, L-histidine, L-leucine and uracil]. The YEpGRE3 transformants was cultured in minimal YNB medium without L-leucine [0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose, 0.02% (w/v) adenine, L-histidine, L-tryptophan and uracil].

3.3 Glycation Experiments

Cells were harvested at the end of the exponential phase, washed twice in water, suspended at a concentration of 5.2 x 10^8 in 0.1 M MES/NaOH (pH 6.5) with 250 mM D-glucose and incubated at 160 r.p.m and at 30 °C in a orbital shaker (Infors HT). Samples were taken at defined times for enzyme activity assays, metabolite measurement and protein glycation analysis by western blot.

3.4 Western Blot Analysis: Detection of Protein Glycation, Yeast Enolase and Hsp26

Total yeast protein extraction was performed by glass bead lysis as described (Gomes, *et al.*, 2005). Protein concentration was determined using the Bio-Rad Bradford assay kit. Proteins (30 μg protein per lane) were separated by SDS/PAGE in a Mini-protean 3 system (Bio-Rad), using a 12% polyacrilamide separation gel and a 6% polyacrilamide stacking gel. Proteins were transferred to PVDF membranes (Hybond-P, Amersham Pharmacia Biotech), using the Mini Trans-Blot system (Bio-Rad). Transfer was performed with 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% (v/v) methanol. Pre-stained standard proteins (Bio-Rad) were also loaded onto the gel. Total proteins were stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm the amount of protein transferred. The membrane was blocked overnight at 4 °C in 1% (v/v) blocking solution in TBS (50 mM Tris with 150 mM NaCl, pH 7.5). For argpyrimidine detection, the blots were probed with anti-argpyrimidine monoclonal antibody, a kind gift from Dr. K. Uchida (Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Japan). Other methylglyoxal-derived AGEs were probed with a polyclonal anti-methylglyoxal modification, kindly provided by Dr. R. Nagaraj (Case Western University, Cleveland, U.S.A.). An antibody to enolase, a kind gift from Dr. Park (Department of Microbiology, Chungnam National University, Korea), was used to identify this protein in membranes. The small heat shock protein Hsp26 was identified by an anti-Hsp26 antibody, a kind gift from Dr. J. Buchner (Institut für Organische und Biochemie, Technische Universität München, Deutschland). Washes, secondary antibody and detection procedures were performed using the BM Chemiluminescence Western Blotting Kit (Roche) following the manufacturer's instructions. Each immunoblot was repeated at least three times in independent experiments.

3.5 Protein Identification by Peptide Mass Fingerprint

Protein bands were excised and polypeptides subjected to reduction, alkylation and digestion with sequencing-grade modified trypsin in gel according to the method of Pandey *et al.* (Pandey*, et al.*, 2000). Sample peptides were assayed for peptide mass fingerprint (PMF) in a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems). The peptide mixture was purified and concentrated by R2 pore microcolumns (Gobom*, et al.*, 1999) and eluted directly to the MALDI plate with 0.8 µl of recrystalized matrix α-cyano-4-hydroxycinnamic acid (α-CHCA) (10 $mg.ml^{-1}$), prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA. The mixture was allowed to air dry (dried droplet method). Monoisotopic peptide masses were used to search for homologies and protein identification with PEPTIDE MASS FINGERPRINT OF MASCOT (http://www.matrixscience.com). Searches were performed in the MSDB database. A mass accuracy of 50 - 100 p.p.m. was used for external calibrations, and cysteine carbamidomethylation and methionine oxidation as fixed and variable amino acid modifications, respectively. Criteria used to accept the identification were significant homology scores achieved in Mascot (53 for 95% confidence) and a minimum of four peptides matched with protein sequence coverage greater than 10%.

3.6 Metabolite Assay

All metabolites were measured in the extracellular medium after removing the cells by centrifugation (5200 g for 3 min). D-glucose, ethanol and glycerol were enzymatically assayed using specific kits from Boehringer Mannheim, following the manufacturer's instructions.

3.7 *In Situ* Assay of Enzyme Activities

Enzyme activities were determined *in situ* using *S. cerevisiae* permeabilized cells (Cordeiro & Freire, 1995). Permeabilization was achieved by incubation with 0.01% (w/v) digitonin in 0.1 M MES/NaOH (pH 6.5) for 15 min at 30 °C, 160 r.p.m. in an orbital shaker incubator. Enzyme activities were determined at 30 °C in a 1.5 ml reaction volume. All assays were performed on a Beckman DU-7400 diode array spectrophotometer, with temperature control and magnetic stirring, essential to maintain isotropic conditions during the assay.

Enolase activity was followed by measuring phosphoenolpyruvate formation at 240 nm. The reaction mixture, containing 50 mM Tris/HCl (pH 7.4), 100 mM KCl, 1 mM $MgSO_4$,

0.01 mM EDTA and 0.5 μg of protein in permeabilized cells, was pre-incubated for 10 min and the reaction was triggered by the addition of 4 mM of 3-phosphoglycerate. In all assays, endogenous phosphoglycerate mutase activity was present at a large excess compared to enolase and, therefore, the measured activity solely depends on enolase.

3.8 Sensitivity analysis

Modeling and computer simulation were used to evaluate the effects of enolase, aldolase and phosphoglycerate mutase activity changes on glycolytic flux, defined as the rate of ethanol formation. The effect of glycerol 3-phosphate dehydrogenase activity on the steady-state methylglyoxal concentration was also investigated.

The kinetic model used in this study was based on the model of Hynne *et al.* (Hynne*, et al.*, 2001), which includes most glycolytic enzymes, although the reactions of enolase and phosphoglycerate mutase are lumped together in an overall reaction. This model was extended to include these two reactions, with kinetic equations and parameters as in the model of Teusink *et al.* (Teusink*, et al.*, 2000). The connection with methylglyoxal metabolism was achieved by including the model of Gomes and co-workers, which comprises the glyoxalase pathway, aldose reductase and methylglyoxal formation from the triose phosphates (Gomes*, et al.*, 2005). Simulations were performed with the software package POWER-LAW ANALYSIS AND SIMULATION, PLAS (A.E.N. Ferreira, Universidade de Lisboa, Portugal; http://www.dqb.fc.ul.pt/docentes/aferreira/plas.html).

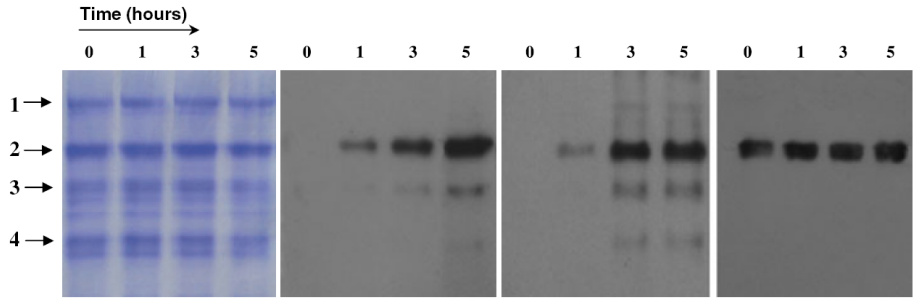
3.9 Protein Structure

Enolase dimer structure was represented by PDB entry 1ebh, containing Mg. It has 95% identity and 4% homology with enolase2. Molecular graphics images were produced using the UCSF CHIMERA package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR-01081) (Pettersen*, et al.*, 2004). Relative solvent surface accessibility was calculated according to the method of Gerstein (Gerstein, 1992).

4. Results

4.1 Identification of Glycated Proteins

When non-growing yeast cells are exposed to 250 mM D-glucose, protein glycation occurs after just 1 h in the reference strain BY4741 (Figure III.1). Against all expectations for a non-enzymatic process, glycation is primarily detectable in only one protein band of 52 kDa (Figure III.1B and III.1C). Three more protein bands, of 70, 40 and 35 kDa appear after 3 h, with much less intensity (Figure III.1B and C). To identify the proteins, protein bands were excised from the BY4741 Coomassie-stained gel (Figure III.1A) and subjected to in gel tryptic digestion.



Band Nº	Swiss Prot Code	Identified Protein	Peptides matched	Mascot Score	Molecular Weight (Da)	Sequence Coverage
1	P10591	HSP71	22	176*	69 655	42 %
	P10592	HSP72	22		69 467	41 %
2	P00925	Enolase 2	10	128	46 811	27 %
3	P14540	Aldolase	7	81	39 750	32 %
4	P00950	Phosphoglycerate mutase	12	100*	26 394	47 %
	P15992	HSP26	10		23734	52 %

* Score for the protein mixture

E Protein identification

Figure III.1 The main methylglyoxal-modified proteins in yeast are the glycolytic enzymes enolase, aldolase and phosphoglycerate mutase and the heat shock proteins Hsp71/72 and Hsp26. Non-growing BY4741 cells were incubated with 250 mM D-glucose to induce protein glycation *in vivo*. Samples, taken at the defined times, were analysed for total protein, argpyrimidine, methylglyoxal advanced glycation end-products (MAGE) and yeast enolase. Methylglyoxal modified protein bands were excised and digested in gel with trypsin for protein identification by MALDI-TOF peptide mass fingerprint. The figure shows a representative result from a set of more than three independent experiments. Equal amounts of proteins were loaded *per* lane (30 μg). (A) Total protein Coomassie-stained gel. (B) MAGE detection by western blotting. (C) Argpyrimidine detection in intracellular soluble proteins, probed by western blotting with a specific antibody towards argpyrimidine. Four major immunoreactive proteins were detected, the 52 kDa protein appearing as the main protein glycation target in yeast. (D) Enolase is the major glycation target in yeast. Total protein extract from strain BY4741, probed with antibody to enolase. The 52 kDa protein, which is highly modified by methylglyoxal, shows high immunoreactivity with anti-enolase antibody. (E) Identification of glycated proteins by MALDI-TOF peptide mass fingerprint. Criteria used for identification were significant homology scores achieved in MASCOT (53 for 95% confidence), a minimum of four peptides matched and protein sequence coverage greater than 10%.

The resulting peptide mixtures were analysed by MALDI-TOF for protein identification by peptide mass fingerprint. The 52 kDa protein was identified as enolase2 (2-phospho-D-glycerate-hydro lyase, EC 4.2.1.11), as shown in figure III.1E. To further confirm the identity of this major glycation target in yeast, a western blot analysis was performed using a specific antibody against yeast enolase, with positive results (Figure III.1D). The 40 and 35 kDa proteins were identified as two other glycolytic enzymes (Figure III.1E), aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) and phosphoglycerate mutase (D-phosphoglycerate 2,3-phosphomutase, EC 5.4.2.1), respectively. The 70 kDa protein band was identified as a mixture of Hsp71 and Hsp72 (Figure III.1E).

The same non-glycated protein bands, i.e. proteins extracted in conditions where glycation not yet occurred (Figure III.1C, lane 1), were also identified as the same proteins. The corresponding protein bands from ΔGLO1 and ΔGRE3 strains were identified as the same proteins. Greater sequence coverage was obtained in peptide mass fingerprints of non-glycated proteins. This is to be expected, because glycated proteins contain modified lysine and arginine side chains, and therefore are less amenable to trypsin hydrolysis and ionization. Moreover, due to the mass increase characteristic of AGEs, glycated peptides had no match in the databases and were therefore rejected. Nevertheless, this information can be exploited to identify the nature and molecular location of specific MAGE in glycated proteins.

4.2 Chemical Nature and Molecular Location of MAGE in Glycated Proteins

In the peptide mass spectra of all glycated proteins, several new peaks appear that do not have predicted m/z values. These could be caused by the occurrence of miscleavage associated with defined mass increases of specific MAGE. To identify some probable glycated peptides and the specific MAGE present, we performed a theoretical digestion of the identified proteins, considering up to two trypsin miscleavages (PEPTIDEMASS, Expasy, http://www.expasy.ch/tools/peptide-mass.html) and added to the resulting peptide masses the mass increment due to a specific MAGE. Using this approach with enolase, we observed that several peptides do show a mass increment of a specific MAGE. For example, the species at m/z 1723.9, present only in the peptide mass spectrum of glycated enolase, corresponds to peptide 409-422 with m/z 1669.9 plus 54 Da, a mass increase characteristic of a hydroimidazolone (Figure III.2A and B). This peptide has one miscleavage at R414, suggesting the presence of one

hydroimidazolone in this position. Interestingly, the same peptide is present only in the digestion of non-glycated enolase at an m/z of 1670.0 (Figure III.2A). Moreover, the species at m/z 1741.9 corresponds to the enolase peptide of 1669.0 Da plus 72 Da due to a CEL modification (Figure III.2B).

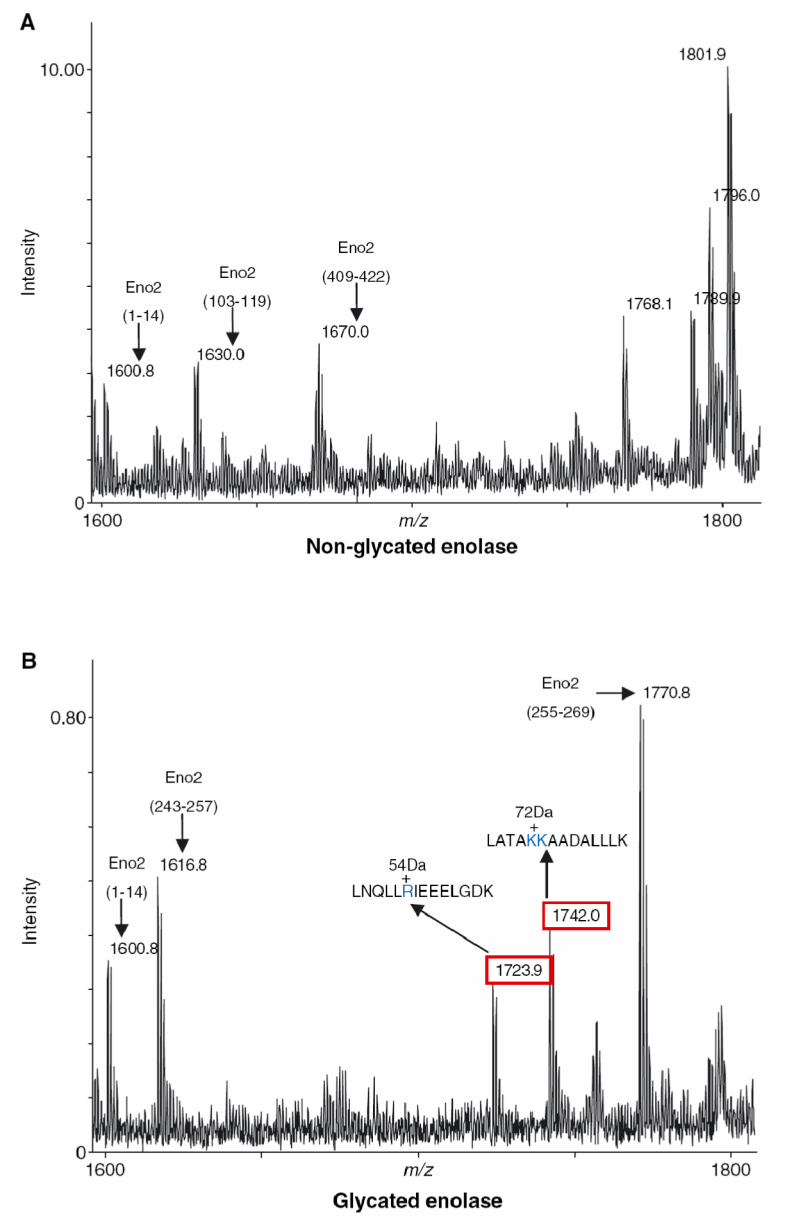


Figure III.2 Detection and molecular location of MAGE in glycated enolase *in vivo*. The figure shows a section of a MALDI-TOF spectrum of tryptic digests of glycated and non-glycated enolase. (A) Mass spectrum of non-glycated enolase. (B) Same section of MALDI-TOF spectrum from glycated enolase. New peaks are detectable, and some of them represent glycated peptides (red). In this case, a hydroimidazolone (mass increase of 54 Da) in residue R414 and a CEL (mass increase of 72 Da) in residue K336 or K337 are observed.

The peptide at m/z 1669.0 has two lysine residues at positions 336 and 337. In this case, MS/MS data would indicate which residue is modified. In the mass spectrum used to identify aldolase, the species with m/z 1082.5, which is absent in the non-glycated protein, corresponds to the aldolase peptide with a theoretical mass of 1002.5 Da plus 80 Da of argpyrimidine. Once more, the aldolase peptide of 1002.5 Da has one miscleavage in arginine residue 334. This method was applied to all mass spectra, and the results are shown in Table III.1. We assumed that arginine or lysine residue modifications make these residues resistant to proteolysis by trypsin, and therefore miscleavages associated with specific mass increases are due to glycation.

Table III.1 Identification and molecular location of MAGE in yeast glycated proteins. Glycated residues are shown in red. MG-H, hydroimidazolone; Argp., argpyrimidine; CEL, N^{ε}-(carboxyethyl)lysine.

Identified protein	Measured mass (Da)	Theoretical peptide mass (Da)	Peptide sequence	Mass increase (Da)	MAGE	Glycated residue
Enolase	1723.92	1669.91	LNQLLRIEEELGDK (409-422)	54	MG-H	R414
	1741.96	1669.03	IATAIEKKAADALLLK (330-345)	72	CEL	K336 or K337
	2178.09	2124.05	SVYDSRGNPTVEVELTTEK (9-27)	54	MG-H	R14
Aldolase	1082.66	1002.54	VWVREGEK (332-339)	80	Argp.	R335
Hsp71/72	1736.68	1664.92	IASKNQLESIAYSLK (532-546)	72	CEL	K535
	2058.94	2004.92	RLIGRNFNDPEVQGDMK (69-85)*	54	MG-H	R69 or R73

* Specific peptide from Hsp72

These data further confirm, at the molecular level, that the identified proteins are indeed glycated *in vivo*. Considering that we have sequence coverage of the identified proteins of at most 50%, a significant fraction of glycated peptides was detected. This analysis shows that the most common MAGE *in vivo* is hydroimidazolone, followed by argpyrimidine, at about half the frequency, whereas CEL and THP appear as minor modifications.

4.3 The Refolding Chaperone Pathway in Yeast Glycation

Besides the identification of Hsp71/72, another heat shock protein, Hsp26, was detected co-migrating with phosphoglycerate mutase (Figure III.1, protein band 4). In glycation conditions, more peptides from Hsp26 are discovered, while in non-glycated samples, only one or two are detected (Figure III.3). Thus, upon glycation, a larger number of Hsp26 molecules are found in the soluble protein fraction. In fact, after 5 hours incubation of BY4741 cells with 250 mM D-glucose, the amount of soluble Hsp26 increases, as evaluated by western blotting (Figure III.3C), confirming the above observation. As Hsp26 is mainly found as an insoluble 24-monomer complex that dissociates under *stress* conditions, its emergence in the soluble protein fraction is a sure sign of its activation (Stromer*, et al.*, 2003). Most peptides from phosphoglycerate mutase remain in the peptide mass spectrum from the glycated samples, as seen in figure III.3 (47% sequence coverage). Hsp26 peptides lead to a sequence coverage of 52%. We then looked for the presence of glycated peptides from both phosphoglycerate mutase and/or Hsp26. We observed that 4 peptides from phosphoglycerate mutase and one peptide from Hsp26 are glycated (Table III.2).

Table III.2 Analysis of the co-migrating proteins phosphoglycerate mutase and Hsp26 under glycation conditions; identification and molecular location of MAGE. Glycated residues are shown in red. MG-H, hydroimidazolone; Argp., argpyrimidine; THP, tetrahydropirimidine.

Identified protein(s)	Measured mass (Da)	Theoretical peptide mass (Da)	Peptide sequence	Mass increase (Da)	MAGE	Glycated residue
Phospho-glycerate mutase	1320.48	1239.69	ADRLWIPVNR (71-80)	80	Argp.	R73
	1500.55	1446.71	FGEEKFNTYRR (104-114)	54	MG-H	R113
	1574.58	1429.72	LNERHYGDLQGK (84-95)	144	THP	R87
	2360.94	2280.28	DLLSGKTVMIAAHGNSLRGLVK (169-190)	80	Argp.	R186
Hsp26	1412.63	1358.75	LLGEGGLRGYAPR (23-35)	54	MG-H	R30

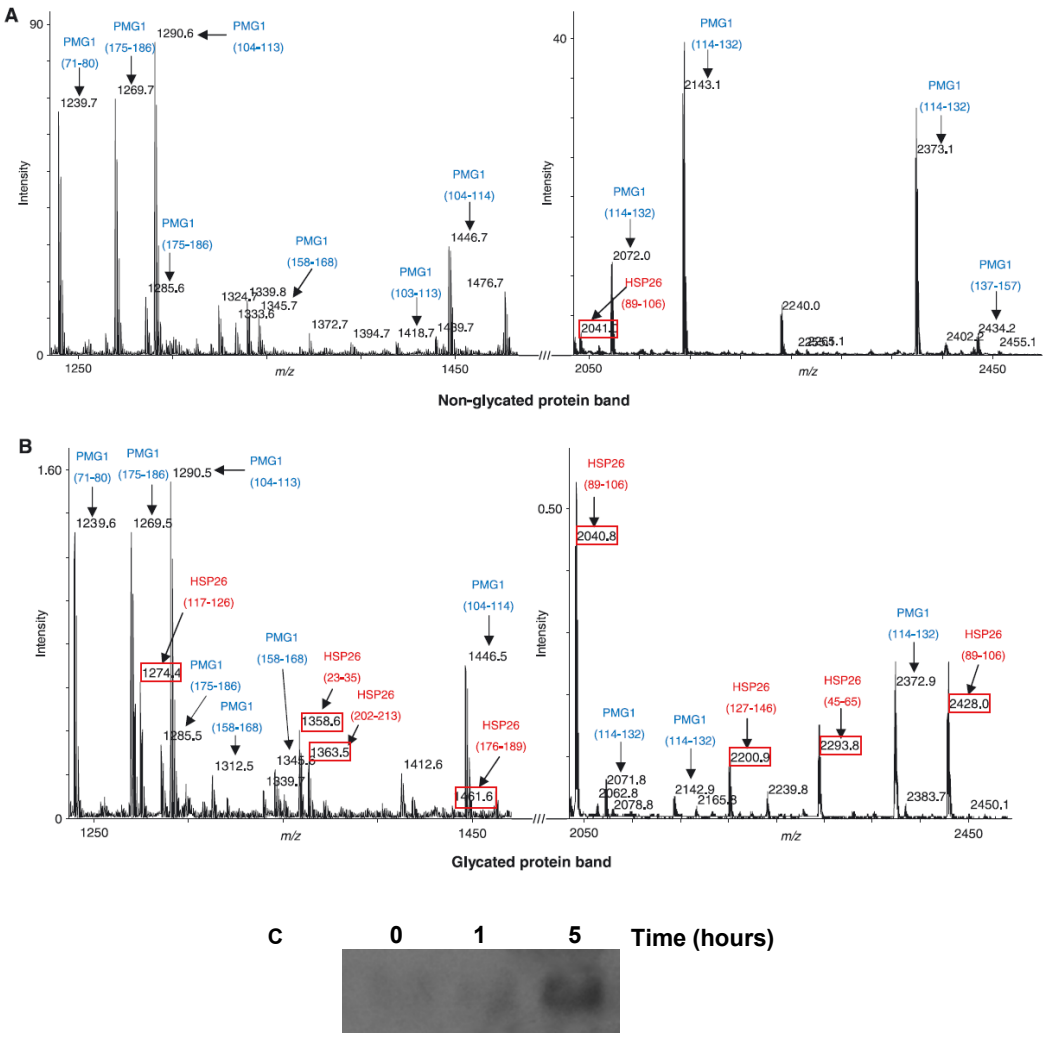


Figure III.3 Detection of Hsp26 upon glycation. (A) MALDI-TOF spectrum of tryptic digestion of the non-glycated protein band 4 (Figure III.1), mainly showing peptides from phosphoglycerate mutase and one peptide, with low intensity, from Hsp26. (B) In glycation conditions, more peptides from Hsp26, with greater intensity, appear. These data suggest that the amount of Hsp26 in the soluble protein fraction increases upon glycation. (C) Western blot detection of Hsp26 in the soluble protein fraction. After 5 hours, an increase of Hsp26 is observed, consistent with the Hsp26 activation.

4.4 Glycation Effects on Enolase Activity and Glycolysis

After identifying enolase as the primary glycation target, we investigated how its enzymatic activity was affected by glycation, in different yeast strains with distinct glycation phenotypes (Gomes*, et al.*, 2005). Strains BY4741, ΔGLO1 and ΔGRE3, with different glycation levels, were challenged with 250 mM D-glucose, and enolase activity was determined *in situ*.

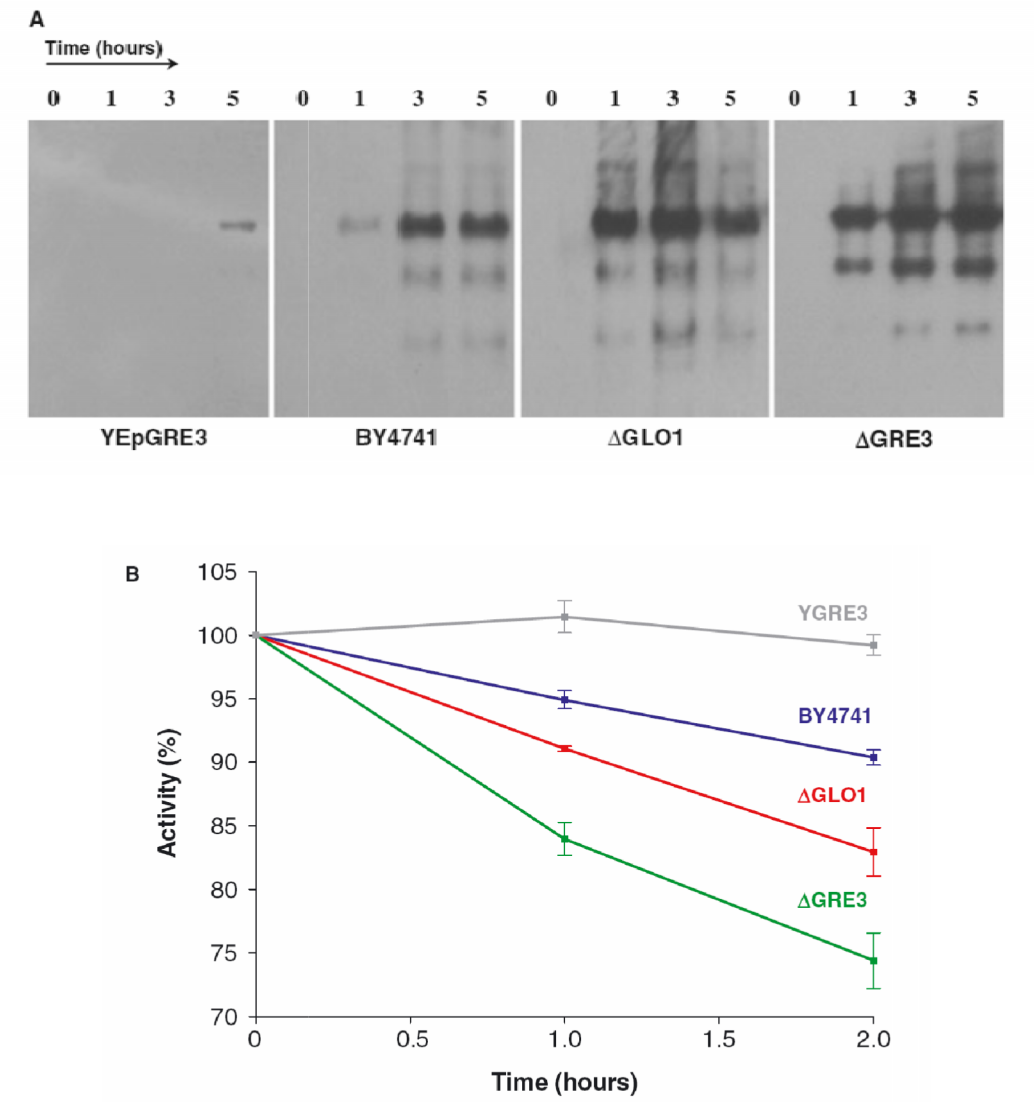


Figure III.4 *In vivo* glycation of enolase causes a decrease of its enzyme activity, directly related to glycation levels. Cells from different yeast strains, incubated with 250 mM D-glucose were sampled at indicated times. Glycated proteins were detected by western blotting using a specific anti-argpyrimidine Ig, and enzyme activity was determined *in situ*. (A) Time course of argpyrimidine formation in YEpGRE3 transformant, BY4741, ΔGLO1 and ΔGRE3 strains. As a result of aldose reductase overexpression, YEpGRE3 only shows an argpyrimidine-modified protein band after 5 hours. Strains with deficiencies in methylglyoxal catabolism (ΔGLO1 and ΔGRE3) have a higher methylglyoxal concentration (Gomes*, et al.*, 2005) and therefore higher levels of glycation. It's noteworthy that glycation increases with time. Representative immunoblots from a set of more than three experiments are shown. Equal amounts of proteins were loaded *per* lane (30 μg). (B) *In situ* enolase activity in all strains studied, at different incubation times. Percentage activity is shown relative to time zero. A decrease of enolase activity is only observed in strains with glycated enolase and this decrease is related to glycation levels. Data are average from three independent experiments ± SD.

The YEpGRE3 transformant, overexpressing aldose reductase, was used as a non-glycated control. YEpGRE3 cells are better protected against methylglyoxal-derived glycation, due to the increased *GRE3* expression and increased aldose reductase activity. In this strain, glycation was only observed after 5 h, contrasting with strains BY4741, ΔGLO1 and ΔGRE3, where it was

detected after just 1 h, with increasing respective intensities (Figure III.4A). Strains with glycated enolase (BY4741, ΔGLO1 and ΔGRE3) showed a decrease of this enzyme activity, compared to the initial value, whereas the YEpGRE3 transformant, without glycated enolase, did not show enolase activity changes (Figure III.4B). This result indicates that glycation leads to a decrease of enolase activity. Consistent with the observation that glycation increases with time (Figure III.4A), after 2 h, *in situ* enolase activity was lower than after 1 h for all strains analysed, except for the control YEpGRE3 transformant, in which enolase activity remained unchanged (Figure III.4B).

The reference strain BY4741 displayed the lowest decrease of enolase activity (5% and 10% after 1 h and 2 h, respectively), whereas strains ΔGLO1 and ΔGRE3 suffered a larger enzyme activity decrease (Figure III.4B). These results are in agreement with the corresponding glycation phenotypes. After 1 h, ΔGRE3 glycated enolase shows a 16% decrease of enzyme activity, higher than the 8% decrease observed in the ΔGLO1 strain.

Given the decrease in enolase activity caused by glycation, a study of D-glucose metabolism in these cells was performed (Figure III.5A). For this purpose, D-glucose, ethanol and glycerol were measured at different times, after incubation with 250 mM D-glucose. As three glycolytic enzymes are glycated, we expected that the glycolytic flux, measured by D-glucose consumption and ethanol formation, might be affected. Strikingly, no major differences were observed in the glycolytic flux of strains BY4741, ΔGLO1 and ΔGRE3 (Figure III.5B). Glycolytic flux remained unchanged even in strains with deficiencies in methylglyoxal catabolism, showing higher enolase glycation and consequent inactivation. As glycolysis leads unavoidably to methylglyoxal formation, which modifies three glycolytic enzymes, D-glucose metabolism could be diverted to glycerol synthesis. Increasing glycerol formation could diminish the methylglyoxal concentration, because the triose phosphate pool is reduced due to its conversion to glycerol 3-phosphate. However, no significant differences were observed in glycerol concentration between those strains (Figure III. 5B).

These results indicate that glycation *in vivo* of enolase and other glycolytic enzymes, with corresponding loss of enzyme activity, does not affect glycolytic flux. To further investigate why this is so, a sensitivity analysis using modeling and computer simulation was performed.

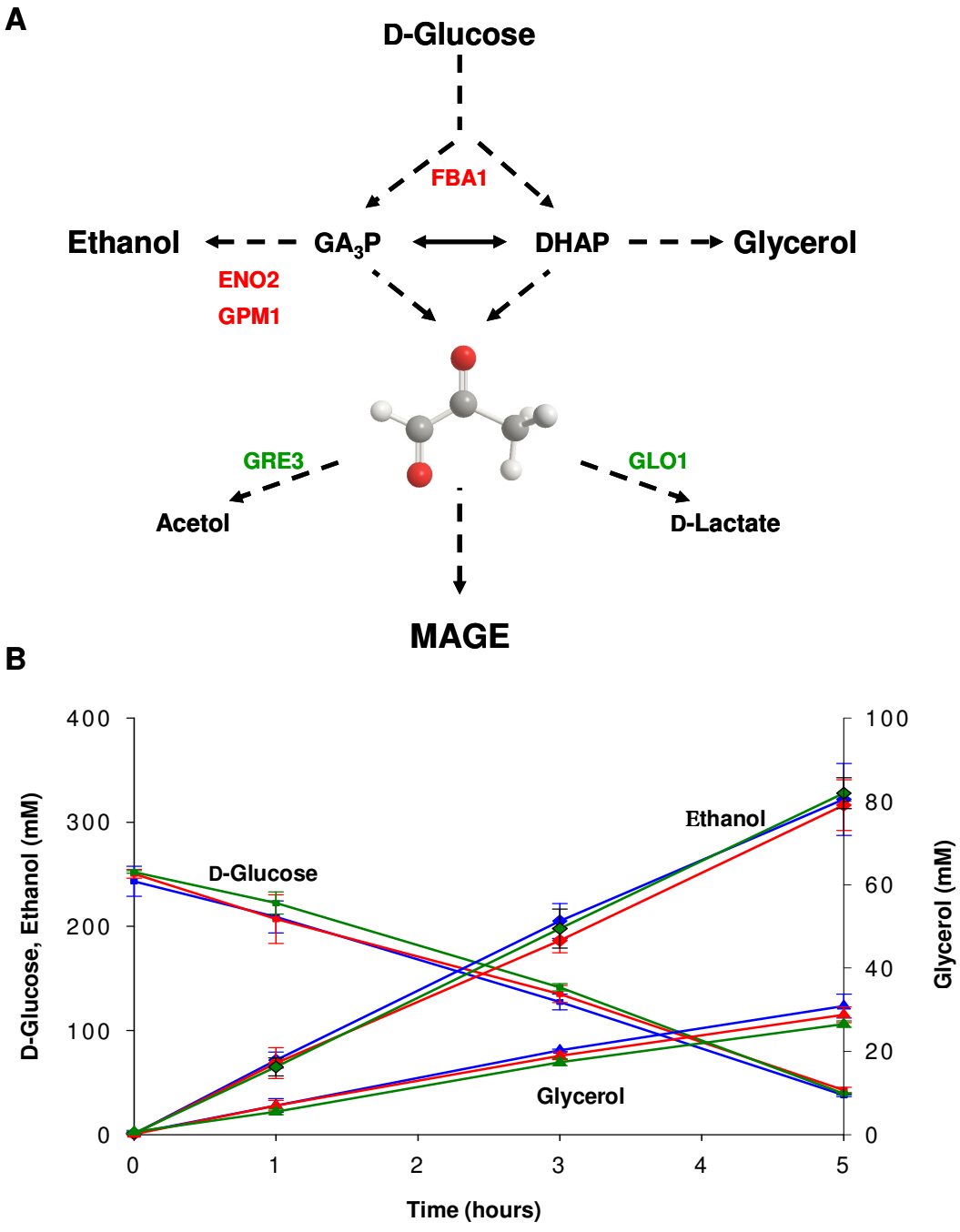


Figure III.5 Glycolysis, methylglyoxal metabolism and glycated proteins in yeast. (A) The well-known glycolytic pathway forms methylglyoxal as a non-enzymatic and unavoidable by-product. The triose phosphates are chemically unstable and suffer an irreversible β-elimination reaction of the phosphate group, forming the most powerful glycation agent *in vivo*, methylglyoxal. The main catabolic routes for methylglyoxal are the NADPH-dependent aldose reductase and the GSH-dependent glyoxalase pathway. Together, aldose reductase and glyoxalase I (green) are essential to maintain a low methylglyoxal steady-state concentration. Once formed, methylglyoxal has the potential to irreversibly modify just about any protein. However, in yeast, only one protein appears as a major target, enolase2, followed by aldolase and phosphoglycerate mutase (red). (B) Glycolysis and glycerol metabolism are unchanged by glycation. Energy metabolism appears unaffected even when three glycolytic enzymes are glycated and the major glycation target, enolase2, shows an activity loss of 20%. Strains analysed were BY4741 (blue), ΔGRE3 (green) and ΔGLO1 (red). Data shown are averages from three independent experiments ± SD.

4.5 Sensitivity Analysis of Glycation Effects on Glycolysis

The effect of changes in the activity of the main glycation targets, enolase, aldolase and phosphoglycerate mutase, on the glycolytic flux as predicted by modeling and computer simulation, is shown in Figure III.6. Aldolase and phosphoglycerate mutase have no effect on glycolytic flux, even if their activities had decreased to 1% of their reference activities (Figure III.6B and C). Glycolytic flux was more sensitive to enolase activity: a reduction of approximately 50% in ethanol formation predicted a reduction of enolase activity to 5% (Figure III.6A). A simultaneous decrease of aldolase, enolase and phosphoglycerate mutase activities to 70% of its reference activities causes a glycolytic flux decrease of less than 0.02%.

According to these results, the decrease in enolase activity caused by glycation (between 5 and 25% *in vivo*) should have no effect in glycolytic flux. This is in agreement with our experimental results, where no differences in D-glucose consumption and ethanol formation were observed among the strains BY4741, ΔGLO1 and ΔGRE3 (Figure III.5B). Even the simultaneous glycation of these three enzymes, each one losing about one-third of its reference activity, would not cause any noticeable decrease of glycolytic flux.

As glycolysis leads unavoidably to methylglyoxal formation, and glycation selectively modifies glycolytic enzymes, causing activity loss, changes in glycerol metabolism might occur. As a result of a slight decrease in the triose phosphate pool (data not shown) methylglyoxal concentration is indeed sensitive to changes in glycerol 3-phosphate dehydrogenase (EC 1.1.99.5) activity (Figure III.6D). An increasing in glycerol 3-phophate dehydrogenase activity by up to five-fold does not lead to a significant decrease in the steady-state concentration of methylglyoxal and triose phosphates. Therefore, stimulation of glycerol formation cannot lead to a decrease in methylglyoxal concentration. These predictions are consistent with the observations that glycerol metabolism is quantitatively identical in the three strains studied.

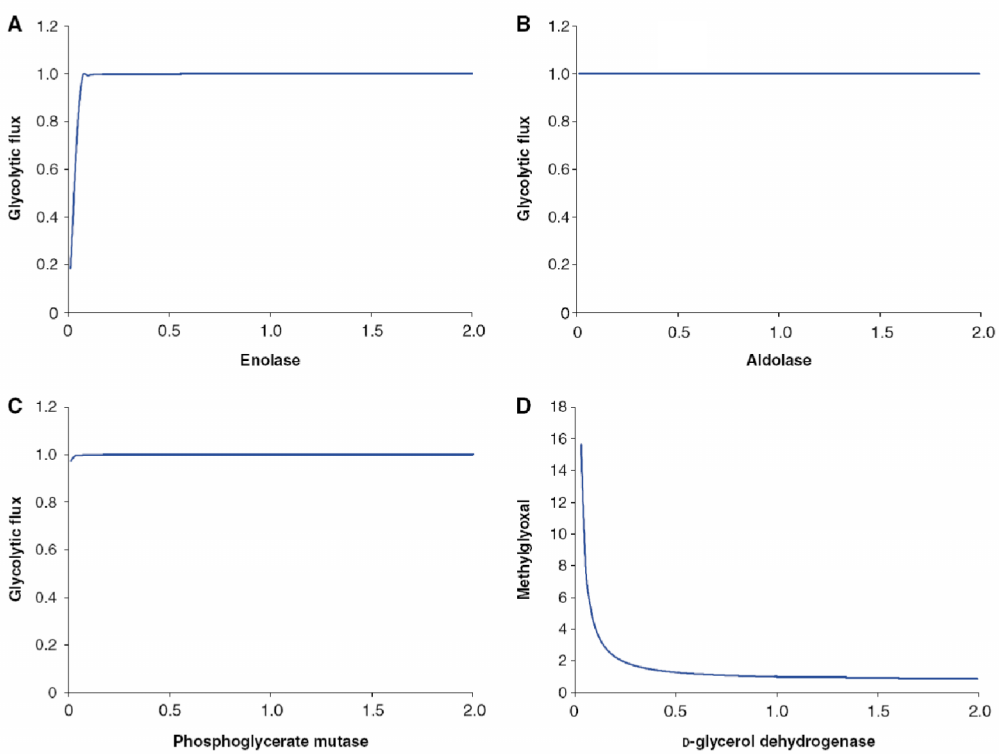


Figure III.6 Sensitivity analysis of glycation effects on glycolytic flux by modeling and computer simulation. Single, finite parameter changes (between zero-fold and two-fold) around the reference steady state were performed. All values are fold variations relative to the reference state (normalized values). System parameters were: (A) enolase activity, (B) aldolase activity and (C) phosphoglycerate mutase activity. The effect of glycerol 3-phosphate dehydrogenase activity on methylglyoxal steady-state concentration (D) was also studied. Except for extreme changes (95-99% activity loss), the glycated glycolytic enzymes enolase, aldolase and phosphoglycerate mutase have no effects on glycolytic flux. Consistent with our experimental results, enolase activity decrease due to glycation has no effects on ethanol formation and D-glucose consumption.

5. Discussion

Yeast cells evolved to use D-glucose efficiently; for this, they have a very high glycolytic flux and consequently an unavoidably high methylglyoxal production rate. Therefore, throughout evolution, defense mechanisms have developed to protect these cells against glycation. Understanding these mechanisms will provide important clues regarding glycation prevention in higher organisms. Nerve cells show a high rate of glycolysis, and several neurodegenerative diseases, like Alzheimer's and Parkinson's disorders are related to a higher AGEs formation (Yan*, et al.*, 1994, Castellani*, et al.*, 1996). Tumour cells also show a high dependence on glycolysis, the Warburg effect (Altenberg & Greulich, 2004). In these cells, expression of glyoxalase I is increased (Di Ilio*, et al.*, 1995, Davidson*, et al.*, 1999), suggesting that an increase in methylglyoxal and AGEs formation also occurs.

In yeast, protein glycation is a non-random process for which specific protein targets exist. Even though several proteins are observed in a Coomassie-stained gel, only one is highly modified by methylglyoxal. Three more protein bands appear to be slightly modified at a latter time, as judged by the western blot analysis. This is an unexpected observation, because, as glycation is a non-enzymatic process, all proteins are putative targets. We identified the four major glycation targets as the glycolytic enzymes enolase, aldolase and phosphoglycerate mutase and the heat shock proteins Hsp71/72 by MALDI-TOF peptide mass fingerprint. Under glycation conditions, Hsp26 becomes detectable in the soluble protein fraction. Of these proteins, enolase2 is clearly the primary and most relevant glycation target in yeast. Glycation introduces miscleavages and defined mass increases in the observable peptides produced by trypsin hydrolysis. Therefore, we analysed the peptide masses, looking for miscleavages associated with specific mass increases caused by the presence of MAGE in peptides containing one lysine or arginine miscleavage residue. With this approach, we confirmed at the molecular level that the identified proteins are indeed glycated *in vivo* by methylglyoxal, and in some cases, the molecular position assignment of the specific MAGE was made. In enolase2, the modified lysines (CEL) are probably the ones with the highest solvent accessibility (Figure III.7). In contrast, hydroimidazolone-modified arginines were only found in an arginine-rich crevice, located at the enolase2 dimer interface (Figure III.7). This arginine-rich cave could work as a cage for free methylglyoxal.

Glycation of enolase *in vivo* causes a decrease of its activity, directly related to methylglyoxal modification. Strains ΔGLO1 and ΔGRE3, with deficiencies in methylglyoxal catabolism and therefore higher levels of glycation (Gomes*, et al.*, 2005), cause a larger decrease in enolase activity. The YEpGRE3 transformant, overexpressing aldose reductase, does not show glycation, and no decrease of enolase activity occurs. However, in all strains analysed, D-glucose consumption and ethanol formation rates were unchanged even when glycated enolase was present. Glycerol synthesis, an alternative branching point of glycolysis, remains unchanged. These results show that glycolytic flux is not affected, despite the decreased activity of enolase in all strains in which glycation occurs.

Sensitivity analysis, by modeling and computer simulation, was used to assess the effects of each glycated glycolytic enzyme on glycolysis. For enolase, glycolytic flux is affected only when its activity decreases to 5% of its reference activity value. This is almost equivalent to an enolase null mutant yeast strain, which is not viable. The other two major glycation targets (aldolase and

phosphoglycerate mutase) have virtually no effect on glycolytic flux, in good agreement with our experimental observations.

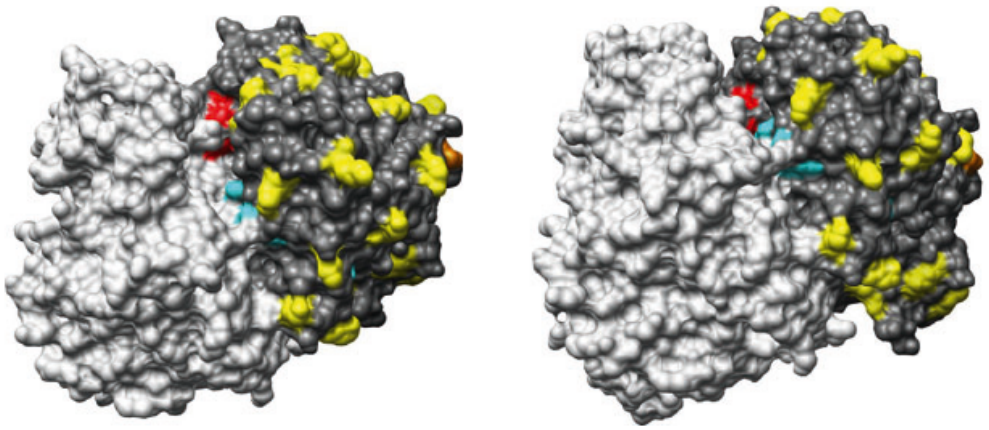


Figure III.7 Surface landscape of dimeric yeast enolase, showing solvent-exposed lysine (yellow) and arginine (cyan) residues. For greater clarity, the surface of one of the subunits is shown in light gray. Two views of the same molecule are shown, rotated clockwise by 180° along the molecule's horizontal axis. According to MALDI-TOF analysis, CEL (orange) is located at K336 or K337. K336 (47% solvent accessibility) is by far the most solvent-accessible lysine in both subunits and is likely to be glycated. Hydroimidazolones (red) were found only in R14 and R414, located in an arginine-rich cleft, deeply recessed, but solvent accessible, at the interface of the two enolase subunits. The E20-R414 ion pair is essential to dimer stability. Its disruption upon glycation will lead to dimer dissociation into inactive monomers, thus explaining at a molecular level the glycation dependent enolase inactivation. This arginine-rich cave should provide highly favoured reaction conditions for MAGE formation, therefore sequestering methylglyoxal in an arginine cage. Sequence coverage by MALDI-TOF peptide mass fingerprint is highly representative, at 27%.

As glycation is a non-enzymatic process, it is quite intriguing that it is targeted to specific proteins, being the functional aspects involved yet unknown. As methylglyoxal arises from glycolysis, perhaps these proteins are closer to the location of methylglyoxal formation than others, and methylglyoxal concentration is higher near these proteins. However, other glycolytic enzymes more closely located to methylglyoxal formation, such as triose phosphate isomerase and D-glyceraldehyde 3-phosphate dehydrogenase, are not glycated. Of the identified glycated proteins, aldolase is the only enzyme directly related to methylglyoxal formation and it only shows comparatively low glycation. Enolase, one of the most abundant proteins in yeast, could be associated with different glycolytic enzymes and therefore methylglyoxal concentration near this enzyme might be much higher than in the rest of the cell. Interestingly, in mammal cells, pure ββ-enolase binds with high affinity to the glycolytic enzymes aldolase and phosphoglycerate

mutase (the other two main glycation targets in yeast) and also to pyruvate kinase (Merkulova*, et al.*, 1997).

Protein concentration *in vivo* and arginine content might be other important parameters for protein glycation. Enolase is indeed one of the most abundant cell proteins. However, the differences between arginine content of this enzyme and most yeast glycolytic enzymes (containing between 8 and 13 arginine residues) do not explain this specific glycation. Moreover, phosphofructokinase (with 49 arginine residues) and pyruvate kinase (with 29 arginine residues) are not glycated. It is possible that arginine residues in enolase are more accessible for the reaction with methylglyoxal than they are in other proteins. This highlights the importance of the reactivity of individual proteins towards methylglyoxal, beyond a simple consideration of protein amount or number of amino groups. *In vivo*, this reactivity could depend not only of the arginine and lysine contents, or protein and glycation agent concentrations, but also of the spatial location of arginine residues in a folded protein. It is not known whether this spatial location determines glycation specificity, but it is conceivable that the 14 arginine residues in enolase are more reactive towards methylglyoxal than are the 49 arginine residues of phosphofructokinase. As demonstrated by Speer *et al.*, the reactivity of arginine peptides with methylglyoxal varies widely, due to the local chemical environment of the respective arginine residue (Speer*, et al.*, 2003). In the case of enolase2, the glycated lysines are the ones with the highest solvent accessibility (Figure III.7). Whereas glycated lysines are at the exposed surface of the protein, glycated arginines are located in an arginine-rich deep cleft, accessible to the solvent, at the interface between the two subunits (Figure III.7). Some of these arginines are involved in ion pairs that contribute to the enolase2 dimer stability. One of these ion-pairs, E20-R414 (Lebioda*, et al.*, 1989), is disrupted by R414 glycation (Figure III.8). Replacing arginine by hydroimidazolone will disrupt electrostatic interactions that stabilize the enolase2 dimer leading to its dissociation and consequent formation of inactive monomers. This molecular hypothesis for the glycation-dependent enolase 2 inactivation, albeit highly plausible, requires further research.

It has been shown that cells can prevent AGEs formation only until anti glycation defenses are overcome (Gomes*, et al.*, 2005). In these conditions, spontaneous protein glycation may be relevant to lower methylglyoxal concentrations. Enolase could indeed function as a methylglyoxal scavenger, preventing changes in the biochemical functionalities of other proteins. Being one of the most abundant proteins in cells, enolase is a good candidate for this role, as glycation of this protein would only have a limited impact on cell physiology. Indeed, our results show that,

although glycation leads to a decrease of enolase activity, no changes have been detected in glycolytic flux, even in the ΔGLO1 and ΔGRE3 mutant strains, which presents higher levels of glycation. It is noteworthy that the expression of *ENO2* (gene that code for enolase2) is induced up to 20-fold after the addition of glucose to yeast cells grown with ethanol as carbon source (Cohen*, et al.*, 1986). Again, by modeling and computer simulation, a 20-fold increase of this enzyme's activity would have no effect on glycolytic flux. Interestingly, ΔGLO1 strain appears to have larger constitutive levels of enolase than the reference strain (Figure III.9). Thus, enolase may play other roles, besides being a glycolytic enzyme.

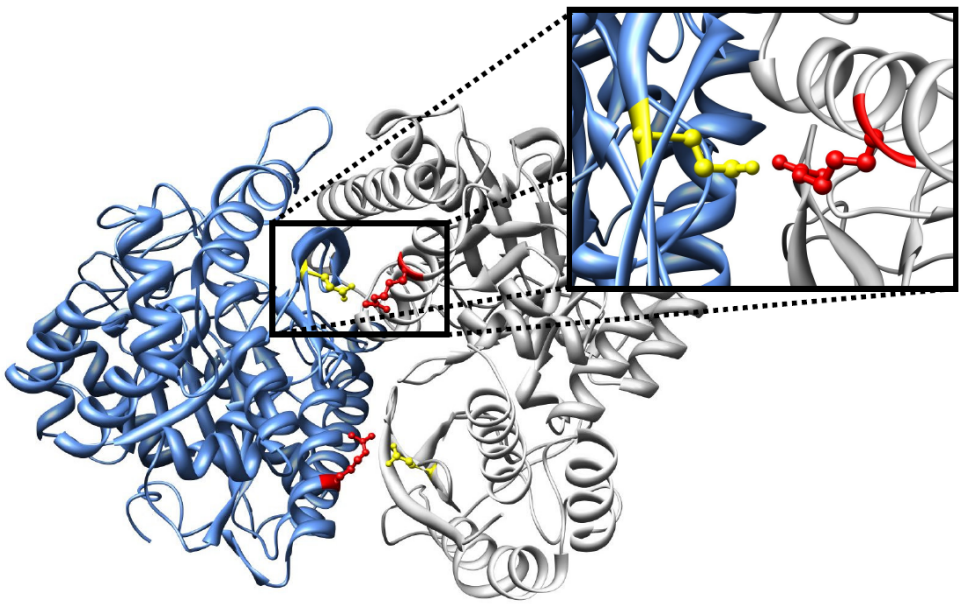


Figure III.8 Surface structure representation of enolase showing the glycation of the critical arginine residues (R414, red) located in a deep cleft at the dimer interface. Once glycated, a critical salt bridge with glutamate residue 20 is disrupted (E20-R414, magnified view) and the dimer dissociates into inactive monomers. Glutamate residues 20 are shown in yellow.

It was predicted that MAGE would be present in approximately 3-13% of cellular proteins (Ahmed*, et al.*, 2005). This is expected to have significant effects on protein structure and function, mainly by unfolding and aggregation (Ahmed*, et al.*, 2005). In the presence of denatured proteins, cells activate several pathways responsible for their recovery, preventing the detrimental effects of protein aggregation. In yeast, Hsp104 facilitates disaggregation and reactivates aggregated proteins with assistance from Hsp71 and Hsp40 (Cashikar*, et al.*, 2005). Recent data show that the small heat shock protein Hsp26 also participates in the recovery of misfolded proteins, by

rendering aggregates more accessible to Hsp104/Hsp71/Hsp40 action (Cashikar*, et al.*, 2005). The presence of Hsp26 in glycation conditions suggests that there is an activation of the refolding chaperone pathway. Moreover, glycation also affects Hsp71/72, another component of this chaperone pathway, and Hsp26 is also glycated *in vivo*. In mammal cells, the major glycation target *in vivo* is Hsp27, a protein that plays an important role in apoptosis and actin polymerization (Nagaraj*, et al.*, 2003, Padival*, et al.*, 2003). In stressed cells, increased levels of Hsp27 facilitate the repair or destruction of damaged proteins, thus promoting cell recovery. It has been shown that specific methylglyoxal modification of Hsp27 improves its chaperone activity (Nagaraj*, et al.*, 2003, Oya-Ito*, et al.*, 2006). So, glycation and/or activation of these specialized proteins (Hsp71/72 and Hsp26) could be of physiological importance in the cell response to glycation.

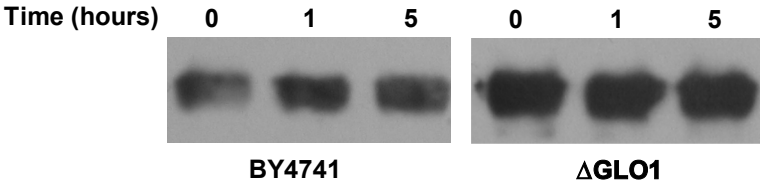


Figure III.9 Western blot analysis of enolase. Cells were incubated with 250 mM of D-glucose and samples were taken at defined times, as indicated. Enolase amount in the soluble cytosolic fraction does not vary with time. However, the glyoxalase I null mutant (ΔGLO1) shows a higher constitutive level than the reference strain BY4741. Representative immunoblots from a set of three experiments are shown.

As glycolysis is the biochemical pathway that evolved under ancient anaerobic terrestrial conditions, it is possible that specialized proteins present in higher organisms are derived from glycolytic enzymes. This could be a critical evolutionary parameter for cells with high glycolytic fluxes and high intracellular methylglyoxal concentration. Another important process is the refolding pathway, through which stress-unfolded proteins might be salvaged in a significant amounts, instead of simply processed by proteolytic pathways.

6. Acknowledgements

We thank Dr. J. Prieto for providing the YEpGRE3 transformant, Dr. H.M. Park for the gift of the anti-yeast-enolase polyclonal antibody, Dr. Johannes Buchner for the gift of the anti-Hsp26p antibody and Dr. K. Uchida for the gift of the anti-argpyrimidine monoclonal antibody. We also acknowledge Dr. Ram Nagaraj for the gift of the anti-methylglyoxal antibody and for fruitful discussions. We wish to acknowledge Ana Varela Coelho and Gonçalo Graça for providing data

from the laboratório de espectrometria de massa at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras, Portugal. Work supported by grants SFRH/BD/13884/2003 (R.A.G.), SFRH/BD/23035/2005 (H.V.M) and POCTI/ESP/48272/2002 (M.S.S.) from the Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Tecnologia, Portugal.

Chapter IV

Methylglyoxal Effects in a Yeast Model of Parkinson's Disease

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1. Abstract

α-Synuclein (a-syn) accumulation in Lewy bodies (LBs) is one of the pathognomonic features of Parkinson's disease (PD) and other synucleinopathies. Three mutations in the gene encoding a-syn are associated with familial PD and render the protein more prone to aggregation. a-Syn is a known target for post-translational modifications such as phosphorylation and ubiquitylation. Glycation was also reported in LBs, but its effects are still poorly understood.

Methylglyoxal is the most important glycation agent *in vivo*, mainly derived from glycolysis in an unavoidable process which has been extensively studied in yeast. Importantly several aspects of a-syn biology are also recapitulated in yeast, including the formation of inclusion-like structures. In this study, we investigated the effect of protein glycation in the biology of a-syn using yeast as a model organism. We observed that glycation increases both a-syn toxicity and inclusion formation. This phenotype is more evident in a glyoxalase I deficient yeast strain that is more prone to methylglyoxal glycation. Analysis of purified a-syn methylglyoxal glycation showed that K6, K10, K12, K21, K23 and K32 residues are consistently modified in all variants. Nevertheless, differences were identified with WT, A53T and E46K a-syn variants displaying MAGE modifications at K43 and K58; K45 and K58; and K60, K96 and K97, respectively. The majority of a-syn glycation sites are located in the protein N-terminal domain. In contrast to A30P a-syn variant, glycated in the first 32 residues, WT and A53T a-syn variants display modifications in the final residues of the protein N-terminal, whereas E46K is modified in a-syn C-terminal domain. Importantly, K6, K10, K12, K21 and K23 were reported as ubiquitylation sites in a-syn. In this study, we found all these residues to be glycation sites, suggesting a putative deleterious role of glycation in the clearance of a-syn via the ubiquitin proteasome pathway, wich may lead to its accumulation.

2. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disease affecting 2% of the world population over the age of 65 (Van Den Eeden*, et al.*, 2003). Muscle rigidity, resting tremor, bradykinesia and postural instability are the characteristic clinical symptoms of the disease (Forno, 1996, Burke, 1999). The loss of dopaminergic neurons from the *substantia nigra* and the intracellular accumulation of protein inclusion, named Lewy bodies (LBs), are the pathological hallmarks of PD (Gai*, et al.*, 2000, Braak*, et al.*, 2003). Although the majority of PD

cases are sporadic, the discovery of mutations in the *PARK1* gene, encoding for α-synuclein (a-syn), and the identification of this protein as the main component of LBs, implicate a-syn as a central player in the pathogenesis of PD (Polymeropoulos*, et al.*, 1997, Kruger*, et al.*, 1998, Spillantini & Goedert, 2000, Zarranz*, et al.*, 2004). a-Syn is a 14.5 kDa protein (140 amino acids) (Lavedan, 1998) with an intrinsically unfolded structure that may shift to a partially folded α-helical conformation upon interaction with membranes (Li*, et al.*, 2001). a-Syn may be subdivided in three different domains: (i) the N-terminal (residues 1-65) including seven imperfect repetitions of an unusual amino acid sequence that may acquire an α-helical conformation (Davidson*, et al.*, 1998, George, 2002, Kahle*, et al.*, 2002, Lee*, et al.*, 2002, Bussell & Eliezer, 2003); (ii) the central hydrophobic domain (residues 66-95), composed by a fragment of NAC peptide (non-Aβ component of AD amyloid), contributing to an aggregation-prone β-sheet structure (Ueda*, et al.*, 1993, el-Agnaf & Irvine, 2002); (iii) and the negatively charged C-terminal domain (residues 96-140). The functions of a-syn are still poorly understood, nevertheless is thought to be associated with synaptic function and plasticity modulation, dopamine regulation, vesicular trafficking and is found to assists vesicle fusion (Chandra*, et al.*, 2005, Klein & Lohmann-Hedrich, 2007). In LBs, a-syn is found to be phosphorylated, oxidized and ubiquitylated (Hirsch, 1993, Fujiwara*, et al.*, 2002, Hasegawa*, et al.*, 2002, Anderson*, et al.*, 2006). Although a-syn glycation was not directly identified, it colocalizes with a-syn in LBs (Castellani*, et al.*, 1996) and may be involved in the crosslinking and proteolytic resistence of a-syn deposits (Munch*, et al.*, 2000). Protein glycation involves non enzymatic reactions between carbonyl containing compounds and amino groups leading to the formation of irreversible modifications named advanced glycation end products (AGEs) (Brownlee*, et al.*, 1984). The main glycation targets are biomolecules with free amine groups such as proteins, nucleotides and also some phospholipids. Methylglyoxal is considered to be the most reactive glycation agent *in vivo* [27-29]. This compound is mainly formed by the non enzymatic β elimination of the phosphate group from the triose phosphates derived from glycolysis [30] and the glyoxalase system together with aldose reductase compose its main catabolic routes (Racker, 1951) (Vander Jagt*, et al.*, 1992, Vander Jagt & Hunsaker, 2003). In yeast, both pathways are equally important to prevent methylglyoxal glycation (Gomes*, et al.*, 2005).

To address the involvement of glycation in a-syn aggregation, we used yeast cells as a eukaryotic cell model to host human recombinant a-syn variants. Yeast cells have been shown to recapitulate a-syn dose-dependent cellular toxicity (Outeiro & Lindquist, 2003, Gitler*, et al.*, 2008).

Wild-type and A53T variants of a-syn fused to green fluorescent protein (GFP) expressed in yeast accumulate in intracellular inclusions (Outeiro & Lindquist, 2003) that colocalize with vesicle clusters (Gitler*, et al.*, 2008, Soper*, et al.*, 2008). In yeast, glycation phenotypes can be controlled through growth conditions and by deletion of genes coding for methylglyoxal catabolic enzymes (Gomes*, et al.*, 2005, Gomes*, et al.*, 2006). Thus, different a-syn variants, WT, A30P, A53T and E46K, were expressed in yeast and the effects of protein glycation on a-syn aggregation *in vivo* were investigated. Furthermore, *in vitro* glycation patterns of a-syn variants were addressed.

3. Experimental Procedures

3.1 Reagents and Materials

Peptone, yeast extract, agar and yeast nitrogen base (YNB) were obtained from Difco while D-glucose (microbiology grade) was from Merck. NaCl, ampicilin, L-methionine, L-histidine, L-leucine, dithiothreitol, iodoacetamide and trifluoroacetic acid (TFA), sequence grade modified trypsin, K_2HPO_4, Coomassie Brilliant Blue G, Ponceau S, glass beads (452-600 microns), 2-mercaptoethanol, PMSF, agarose, lithium acetate, zymolyase, tween-20, glycerol, cloranphenicol and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from Sigma. Tris, SDS 20% (w/v), acrylamide/BIS [40% (w/v)] and glycine were purchased from BioRad. EDTA was obtained from BDH chemicals LTD while MES, bromophenol blue, triton X-100, 2,5 dihydroxybenzoic acid (DHB) were obtained from Fluka. PerfectPure C-18 tips were obtained from Eppendorf; HPLC gradient grade acetonitrile was from Merck; ultrapure water was produced in a Millipore Milli-Q system. Electrophoretic protein standards were from Fermentas. Protein inhibitors were from Roche.

3.2 Bacteria, Yeast Strains and Culture Conditions

Escherichia coli strains used were (DH5α - F-; *rec*A1; *end*A1; *thi*-1; *gyr*A96; hsdR17; *sup*E44; *rel*A1;ϕ89d; *lac*Z; DM15 λ-); and BL21+ - (F-; *omp*T; $hsdS_B$ (r_B-, m_B-); dcm; gal, λ(DE3), pLysS, Cm^r). Cells were cultured in LB medium [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract] at 37 °C. Solid LB medium contained 2% (w/v) agar. Transformed DH5α strains, carrying the plasmids, grew in LB medium supplemented with 0.1 $mg.ml^{-1}$ ampicillin. BL21+ strain was grown on the same media supplemented with 34 $\mu g.ml^{-1}$ cloranphenicol.

Saccharomyces cerevisiae strains used were the BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and ΔGLO1 (isogenic to BY4741 with YML004c::KanMX4) from Euroscarf collection (Frankfurt, Germany). Strain was kept in YPGlu [0.5% (w/v) yeast extract, 1% (w/v) peptone and 2% (w/v) D-glucose agar plates 2% (w/v) agar] at 4 °C and cultured in liquid YPGlu medium at 30 °C. *S. cerevisiae* strains expressing the plasmids containing a-syn variants were cultured in synthetic medium without uracil (YNB-u) [0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose and 0.025% (w/v) L-methionine, L-histidine, L-leucine].

3.3 Plasmids and Yeast Transformation

Plasmids carrying the different a-syn genes (a-syn WT, a-syn A30P, a-syn A46K and a-syn A53T) were constructed by Dr. Tiago Outeiro (Cell and Molecular Neuroscience Unit, Instituto de Medicina Molecular, Lisboa, Portugal) who kindly provided the plasmids for yeast transformation. Human a-syn genes fused to green fluorescent protein (GFP) were cloned into the expression vector p426GPD, which contains a 2-micron origin of replication, a glyceraldehyde 3-phosphate dehydrogenase promoter, and a uracil (Ura) selection marker (Mumberg*, et al.*, 1995).

Plasmids carrying different a-syn genes were used to transform BY4741 and ΔGLO1 yeast strains using the lithium acetate method and transformants were selected on selective YNB-u agar plates, following the procedure described in the Yeast Protocols Handbook ("Small-scale LiAc Yeast Transformation Procedure", from Clontech). The same yeast strains were also transformed with the p426GPD vector alone and p426GPD vector with GFP gene, as controls. For protein expression in yeast, transformants were grown in YNB-u selective medium.

For bacterial expression, pT7-7 plasmid constructs containing a-syn genes under the T7 promoter were kindly provided by Dr. Lashuel (Laboratory of Molecular Neurobiology and Neuroproteomics, Ecole Polytechnique Fédérale de Lausanne, Switzerland).

3.4 Yeast Glycation Experiments

Cells were harvested in the exponential phase of growth (0.8 Abs_{600nm}), washed twice in type II water, suspended in 0.1 M MES/NaOH pH 6.5 (MES buffer) with 250 mM D-glucose and incubated at 160 r.p.m, 30 °C, in a orbital shaker (Infors HT) for 24h. For control, cells were incubated in MES buffer. Samples were collected for protein glycation, a-syn intracellular localization and expression studies.

3.5 Spotting Toxicity Experiments

Cells expressing different variants of a-syn-GFP fusions were grown overnight at 30 °C in liquid media containing glucose until they reached log or mid-log phase. Cultures were then normalized for OD_{600nm}, and incubated in MES buffer or 250 mM D-glucose in MES buffer for 24h. Cells were normalized for OD_{600nm} and serially 10 fold diluted and spotted onto rich (YPD) or synthetic selective solid media (YNB-u), after which they were grown at 30 °C for 2-3 days.

3.6 Cell Viability Assay

Viability was defined as the ability of a single organism to reproduce and form a colony within 48 hours (colony forming units or CFU). Both the control and 250 mM D-glucose exposed cells were serially diluted and seeded to rich solid media. Yeast cells viability was assessed by counting the number of colony forming units and the results were expressed as $CFU.mL^{-1}$ of cells.

3.7 Immunoblot Analysis of a-Syn Expression and Protein Glycation

Total yeast protein extraction was performed by glass bead lysis as described (Ausubel*, et al.*, 1990, Gomes*, et al.*, 2005). Proteins (30 μg per lane) were separated by SDS-PAGE using a Tetra cell (Bio-Rad, Hercules, California, U.S.) using a 15% polyacrilamide separation gel and a 6% polyacrilamide stacking gel and applying a constant voltage of 100V. Proteins were transferred to nitrocelulose membranes (Hybond-C extra nitrocellulose, Amersham Pharmacia Biotech) using the Mini Trans-Blot system (Bio-Rad) with transfer buffer 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% (v/v) methanol. Pre-stained standard proteins were also loaded on the gel. The membrane was stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm protein transfer and blocked overnight at 4 °C in 1% (v/v) blocking solution in TBS (50 mM Tris and 150 mM NaCl, pH 7.5). N^{ε}-(carboxyethyl)lysine (CEL) was detected by using anti-CEL KNH-30 antibody from Abcam diluted 1:5000 in blocking solution. An anti-human-a-syn antibody, diluted 1:1000 in blocking solution, was used to detect this protein in membranes (anti-synuclein 1, BD Transduction Laboratories). Washes, secondary antibody and detection procedures were performed using the ECL Western Blotting system (Amersham) following the manufacturer's instructions. Each immunoblot was repeated at least three times from independent experiments.

3.8 Microscopy Analysis

Yeast cells were harvested and washed in ultrapure water. A drop of the cell suspension was placed on a microscope slide. GFP transformants were directly examined and photographed in an inverted fluorescence microscope (Axiovert 200M, Zeiss, Germany) equipped with oil immersion objectives (Zeiss, 100 × objective, N.A. = 1.40 and Zeiss, 63 x, N.A. = 1.40 objective) and with filter sets for GFP standard (excitation 450-490 nm, emission 515-565 nm).

The number of cells in the field containing a-syn *foci*, intracellular structure localization or plasma membrane localization, were counted. At least 1000 cells were counted for each sample in triplicate from independent experiments.

3.9 Assessment of Aggregation by Semi-Denaturant Detergent-Agarose Gel Electrophoresis

Semi-denaturant detergent-agarose gel electrophoresis (SDD-AGE) was adapted from (Halfmann & Lindquist, 2008). A 1.5% agarose gel supplemented with 0.1% SDS was prepared and stacked in a casting tray. Glycated yeast cells were harvested at 2000 g for 5 minutes at room temperature. Cells were washed and suspended in spheroblasting solution (1.2M D-sorbitol, 0.5 mM $MgCl_2$, 20 mM Tris-HCl pH 7.5, 50 mM 2-mercaptoethanol and 0.5 $mg.ml^{-1}$ zymolyase 100-T) for 30 minutes at 30 °C. Cells were centrifuged at 800 g for 5 minutes at room temperature and spheroblasts resuspended in lysis buffer (100 mM Tris pH 7.5, 50 mM NaCl, 10 mM 2-mercaptoethanol, protease inhibitors, bromophenol blue, 2X TAE, 20% (v/v) glycerol and 8% (w/v) SDS). Cells were high speed vortexed for 2 minutes. Cellular debris were pelleted by centrifugation at 4000 g for 2 minutes and soluble fraction was loaded in the gel. A constant low voltage (4 $V.cm^{-1}$) was applied for 4 hours at 4 °C. Proteins were transferred to nitrocellulose membranes by capillarity overnight and a-syn was detected by western-blot as previously described. As positive control, yeast cells expressing a-syn S129A, kindly provided by Dr. Sandra Tenreiro from Cell and Molecular Neuroscience Unit, Instituto de Medicina Molecular, Lisboa, Portugal, were used.

3.10 Expression and Purification of Recombinant a-Syn

3.10.1 Expression of a-Syn Variants

E. coli BL21+ bacteria were transformed by heat shock with a-syn PT7-7 constructs. A positive colony was transferred into 5 mL of LB supplemented with antibiotics and incubated overnight at 37 °C with continuous shaking. 2.5 mL of the bacterial culture was added to a 2 L flask containing 500 ml of LB supplemented with antibiotics. The culture was grown at 37 °C under continuous shaking and the expression of the protein was induced with 1 mM IPTG when the culture reached an $OD_{600\ nm}$ of 0.4. After a 6 hour growth period, cells were harvested by a 15 minute centrifugation at 5000 g at 4°C. The a-syn containing pellet is frozen at -20 °C until required.

3.10.2 Bacterial a-Syn Containing Pellet Handling

E. coli pellet was resuspended in 20 mL lysis buffer [50 mM Tris-HCl pH 8.5, 50 mM KCl, 5 mM MgAc, 0.1% (w/v) NaN_3] supplemented with 300 μM of PMSF inhibitor and lysed in a French Press. The resulting lysate was centrifuged for 30 minutes at 18000 g at 4 °C and the supernatant boiled for 20 minutes. Centrifugation was repeated and a-syn containing supernatant filtered through a PVDF 0.22 μm pore filter and stored for further purification.

3.10.3 Chromatography/Purification

a-Syn has a negative charge in lysate solution at pH 8.5 and therefore interacts with positively charged moieties of an anion exchange column (HiPrep Q FF column, GE Healthcare Life Sciences). Column was equilibrated in solution A (20 mM Tris-HCl pH 8) with 12% of solution B (20 mM Tris-HCl pH 8, 1M NaCl). A gradient with solution B from 12-50% was applied over 7 column volumes followed by a 50-100% gradient over 3 column volumes and 1 final column volume with 100% solution B. A-Syn elutes at approximately 40% of B solution. To further purify a-syn, size exclusion chromatography was performed using a preparative Superdex 75 column (GE Healthcare Life Sciences). Column was equilibrated with solution C (50 mM Tris-HCl, 150 mM NaCl pH 7.5) and separation was carried out for 1.2 column volume in an isocratic system running in solution C.

Purified protein was concentrated in 5KDa cutoff Amicon filter (Millipore), dialysed overnight against water and lyophilised for further studies.

3.11 Purified a-Syn *in Vitro* Glycation

WT and variant forms of a-syn were glycated *in vitro* by methylglyoxal. Methylglyoxal was prepared by acid hydrolysis of 1,1-dimethoxypropanone (Fluka) followed by fractional distillation at reduced pressure in a nitrogen atmosphere. Proteins at a concentration of 1 $mg.ml^{-1}$ were incubated with 0.1 and 1 mM of methylglyoxal for 24h.

3.12 Purified a-Syn Analysis by Mass Spectrometry

Proteins (5 µg per lane) were separated by SDS-PAGE, as previously referred, and the gel was stained with Coomassie Brilliant Blue. Protein bands (corresponding to the 14.5 kDa a-syn) were excised and subjected to reduction, alkylation and digestion with sequencing-grade modified trypsin in gel, according to Pandey and co-workers (Pandey*, et al.*, 2000). The peptide mixture was purified and concentrated in PerfectPure C-18 tips microcolumns, following the manufacturer's instructions and eluted directly to the MALDI plate (Anchorchip MALDI target from Brucker Daltonics, Bremen, Germany) with 0.8 µl of DHB matrix (10 $mg.ml^{-1}$) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA. Samples were air dried. Peptide mixture was analysed by MALDI-FTICR-MS in a Bruker Apex Ultra, Apollo II combi-source (Bruker Daltonics, Breman, Germany) with a 7 Tesla magnet (Magnex corporation, Oxford UK). *In vitro* glycated samples were also analysed by ESI-FTICR-MS with the same equipment after tryptic digestion, reduction, alkylation and digestion in the same conditions already mentioned. Monoisotopic peptide masses, determined by the Snap 2 algorithm in Data analysis 3.4 software (Bruker Daltonics, Breman, Germany), were used to search for homologies with theoretical a-syn tryptic digestion using Brucker Daltonics BioTools 3.1 software. A mass accuracy of 20 ppm was considered; Cysteine carbamidomethylation and Methionine oxidation were taken into account as fixed and variable amino acid modifications, respectively.

4. Results

4.1 a-Syn Toxicity in Yeast Cells

To study the molecular mechanisms underlying a-syn aggregation and its relationship with protein glycation, yeast cells were used as cellular model system for which different glycation phenotypes exist (Gomes*, et al.*, 2005, Gomes*, et al.*, 2006). Wild type, A30P, A53T and E46K

mutant a-syn were expressed in the reference strain BY4741 and in ΔGLO1, a glycation prone strain, lacking the gene coding for glyoxalase I, the first enzyme of the glyoxalase pathway (Racker, 1951). Yeast cells expressing GFP as control and the different a-syn variants were grown to log or mid-log phase and harvested. After challenging non-growing cells to MES buffer and D-glucose for 24h, a-syn toxicity was investigated through spotting analysis (figure IV.1).

MES buffer exposure resulted in slight sensitivity to WT and E46K a-syn expression in the reference strain, whereas in ΔGLO1 showed higher sensitivity to WT, A53T, and E46K expression and some toxicity to A30P variant. In glycation conditions, BY4741 is much more sensitive to WT and E46K toxicity and in these conditions A53T also exhibits toxicity. In ΔGLO1, all variants are toxic where WT and E46K expression clearly inhibit yeast growth, followed by A53T and with less sensitivity for A30P. Interestingly, the toxicity of a-syn variants expressed in ΔGLO1 cells prior to D-glucose glycation was similar to the observed in BY4741 expressing cells after exposure to D-glucose, consistent with an increase of a-syn toxicity due to glycation.

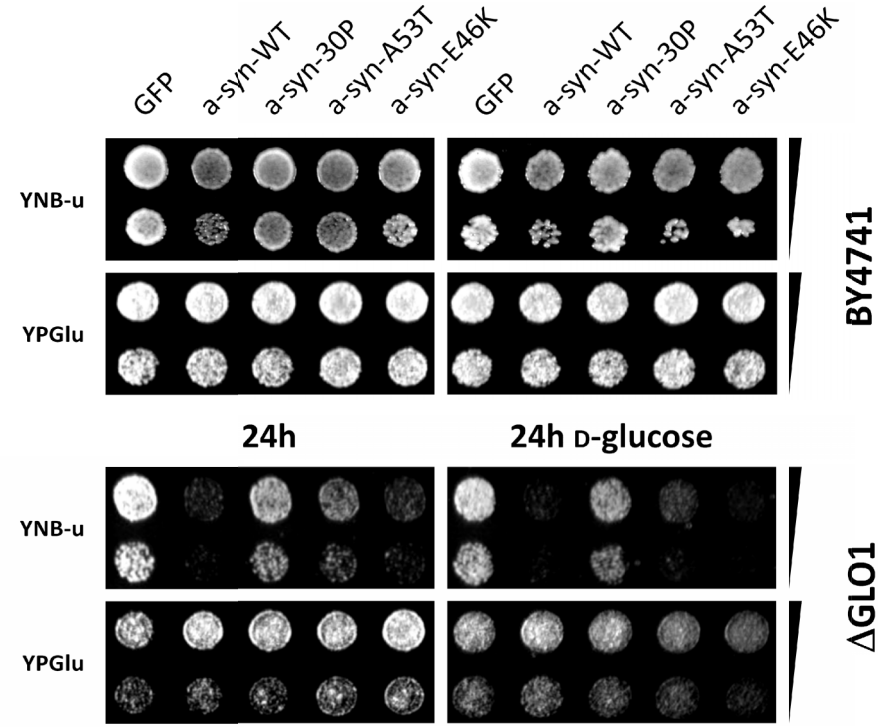

Figure IV.1 a-Syn toxicity in *Saccharomyces cerevisiae*. Yeast BY4741 and ΔGLO1 were grown in YNB-u solid medium prior and after challenging cells to 250 mM D-glucose. Control GFP, A30P and A53T has no detectable effect on growth of BY4741 strain in control conditions (24h) while WT and E46K show slight inhibition effects. In contrast, in the same growth conditions ΔGLO1 has shown to be more sensitive to WT, A53T and E46K. After D-glucose exposure, WT, A53T and E46K inhibitory effects were increased in both strains, however in ΔGLO1, a higher toxicity is displayed by WT and E46K variants of a-syn.

4.2 Viability of a-Syn Expressing Yeast Cells Under Glycation Conditions

Yeast cells viability was evaluated by the number of colony forming units (CFU). BY4741 and ΔGLO1 a-syn expressing cells were analyzed prior and after 24h MES buffer and D-glucose exposure. The control viability (GFP) is affected by incubation for 24h in MES buffer. Nevertheless, initial viability of a-syn expressing cells is widely affected. In glycation conditions, yeast cells expressing WT and A30P variants have shown the higher significant decrease of viability followed by E46K. A53T cells also display a low viability, however the difference between 24h MES buffer and 24h D-glucose is similar to GFP control cells (figure IV.2).

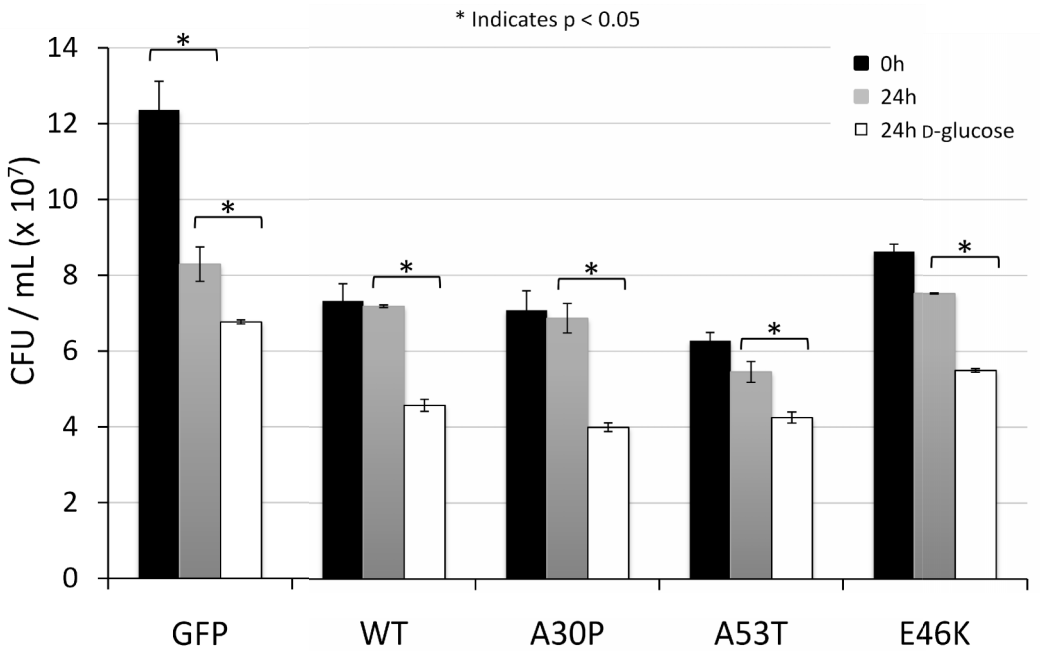

Figure IV.2 Viability of yeast BY4741 cells expressing a-syn and GFP as control. Colony forming units (CFU) of initial (0h), 24h MES buffer and 24h D-glucose exposures are shown. The bars show the average ± SD. * $p < 0.05$ between 0 and 24h MES buffer of GFP expressing cells and between 24h and 24h D-glucose of GFP, WT, A30P, A53T and E46K expressing cells.

Cell viability of ΔGLO1 yeast strain expressing a-syn variants was also investigated. In initial conditions, cells viability is lower compared to BY4741 strain either in control transformants and a-syn expressing cells. Similar to BY4741, GFP expressing cells show a high viability decrease after 24h MES buffer and in contrast, in ΔGLO1, the viability increase after D-glucose exposure. a-Syn expressing cells exhibit similar phenomenon. A53T expressing cells display the lowest viability (figure IV.3).

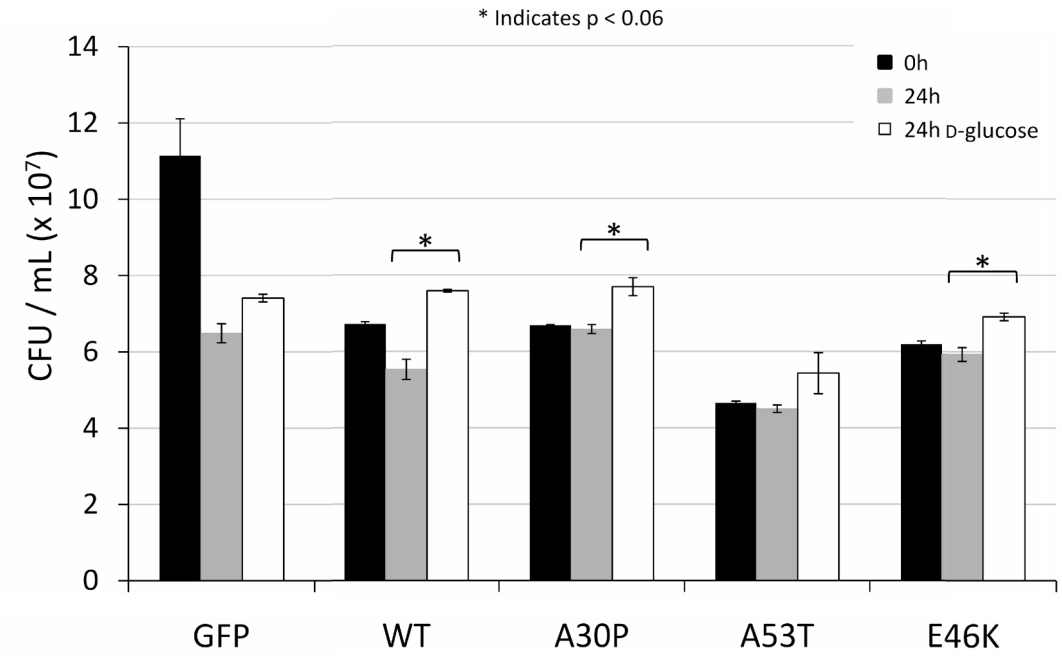

Figure IV.3 Viability of yeast ΔGLO1 cells expressing a-syn and GFP as control. Colony forming units (CFU) of initial (0h), 24h MES buffer and 24h D-glucose exposures are shown. The bars show the average ± SD. * $p < 0.06$ between 24h and 24h D-glucose of WT, A30P and E46K expressing cells.

4.3 Glycation Patterns of a-Syn Expressing Yeast Cells

BY4741 cells at the mid or late-log phase of growth show similar expression levels of the WT, A53T and E46K in contrast to A30P, expressing higher amounts as shown by western blot analysis with anti-human a-syn antibody (figure IV.4). Similar observations were previously reported (Outeiro & Lindquist, 2003).

After D-glucose exposure, a-syn content is higher, followed by increased protein glycation patterns either in BY4741 as ΔGLO1 yeast strains. Methylglyoxal glycation was probed using anti-CEL antibody, shown to modify several proteins in yeast, in contrast to argpyrimidine where glycation is more targeted (Gomes*, et al.*, 2006). In agreement with previous results, ΔGLO1 strain is more susceptible to methylglyoxal glycation (Gomes*, et al.*, 2006). In BY4741, higher glycation levels are shown in A30P, A53T and E46K in glycation prone conditions, whereas in ΔGLO1 only small variations were observed when comparing the control with a-syn expressing cells. Glycating conditions as D-glucose exposure of non-growing cells confirm the increased protein glycation levels in BY4741 and in ΔGLO1.

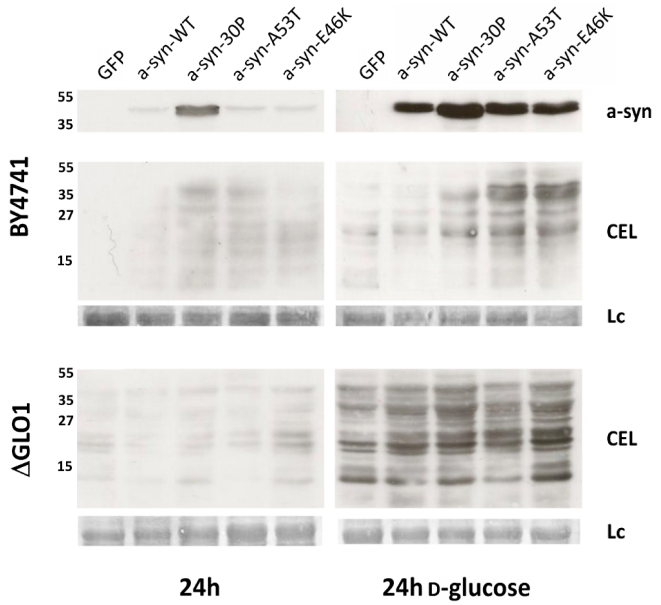

Figure IV.4 Expression of a-syn variants and *in vivo* yeast glycation levels prior and after D-glucose exposure. Western blot analysis with anti-human a-syn antibody of total protein extracts from BY4741 and ΔGLO1 yeast strains expressing WT, A30P, A53T and E46K variants of a-syn and GFP as control. Expression levels of WT, A53T and E46K are similar (~42 kDa, corresponding to the molecular weight of a-syn fused to GFP). After D-glucose exposure, expression levels are higher. Western blot analysis of methylglyoxal-modified intracellular proteins in initial and glycating conditions was probed with an anti-CEL antibody. The intensity of glycated bands in glycation conditions (D-glucose exposure) is higher and ΔGLO1 is more susceptible to glycation. Lc stands for comassie staining loading control.

4.4 Glycation Conditions Promote the Formation of a-Syn *Foci* in Yeast

Yeast cells have been shown to recapitulate some features of PD. WT and A53T variants, in contrast to A30P, were shown to accumulate a-syn in intracellular structures (Outeiro & Lindquist, 2003). When non-growing yeast cells are exposed to D-glucose, the increased intracellular methylglyoxal concentration leads to the formation of MAGE-modified intracellular proteins (figure IV.4), consistent with previous reports (Gomes*, et al.*, 2005, Gomes*, et al.*, 2006).

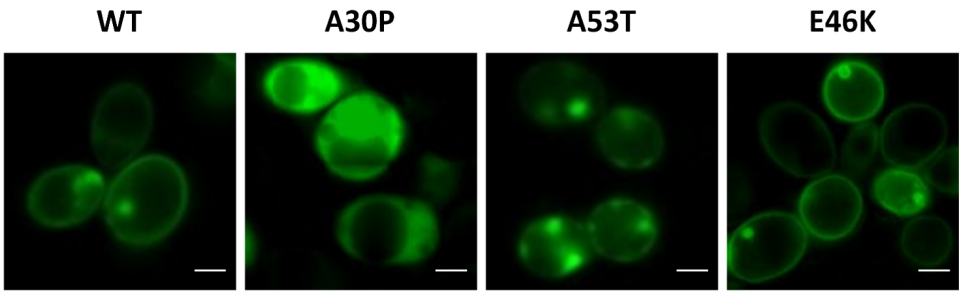

Figure IV.5 Intracelular localization of a-syn variants. Yeast BY4741 expressing a-syn WT, A53 and E46K variants of a-syn are mainly associated to the plasma membrane and display intracellular or plasma membrane aggregate-like structures. A30P a-syn is dispersed in the cytosol and do not form inclusions. Scale bar, 2 μ.

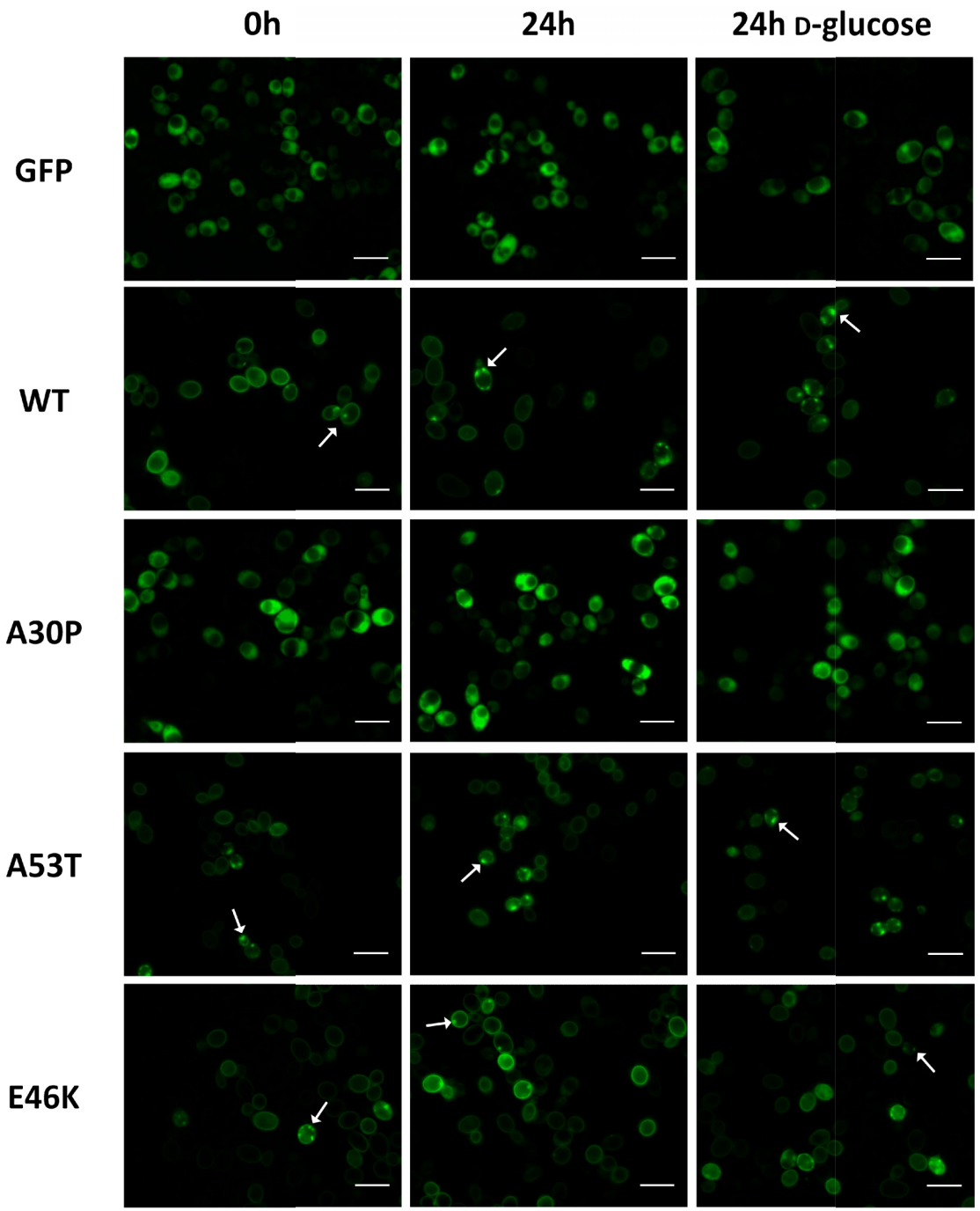

Figure IV.6 Formation of a-syn *foci in vivo*. Yeast BY4741 expressing GFP as control and a-syn variants WT, A30P, A53T and E46K fused to GFP were monitored and the number of cells in different fields containing a-syn *foci* (intracellular or plasma membrane aggregate-like structures, white arrow) were counted (detailed information in figure IV.7). GFP expressing cells together with A30P variant do not display formation of a-syn *foci*. Scale bar, 5 μ.

We analyzed the intracellular localization of a-syn variants and their inclusion formation under glycating conditions. Consistent with earlier findings (Outeiro & Lindquist, 2003), WT and A53T variants of a-syn localize mainly in association with yeast plasma membranes, while A30P is

spread throughout the cytoplasm. As for E46K, its localization is similar to WT and A53T variants, present in the plasma membrane and forming discrete a-syn *foci* (figures IV.5 and IV.6). Yeast cells challenged to D-glucose did not display differences on a-syn intracellular localization.

After 5h exposure of non-growing yeast cells to 250 mM D-glucose, a known glycation promoting condition (Gomes*, et al.*, 2005), no significant differences were established (data not shown). However, increasing D-glucose exposure up to 24h resulted in significant changes in the number of a-syn intracellular *foci*. Control cells expressing GFP alone and A30P a-syn variant did not form these inclusion-like structures. 19, 26 and 21% of yeast cells exhibit formation of inclusion expressing WT, A53T and E46K a-syn variants respectively. After 24h exposure to MES buffer, no significant changes were observed. However under glycation conditions, WT and A53T expressing cells show an increase in the percentage of inclusion containing cells to 28 and 32% respectively (figure IV.7). This results in a total 8% increase in the number of cells exhibiting a-syn WT *foci*.

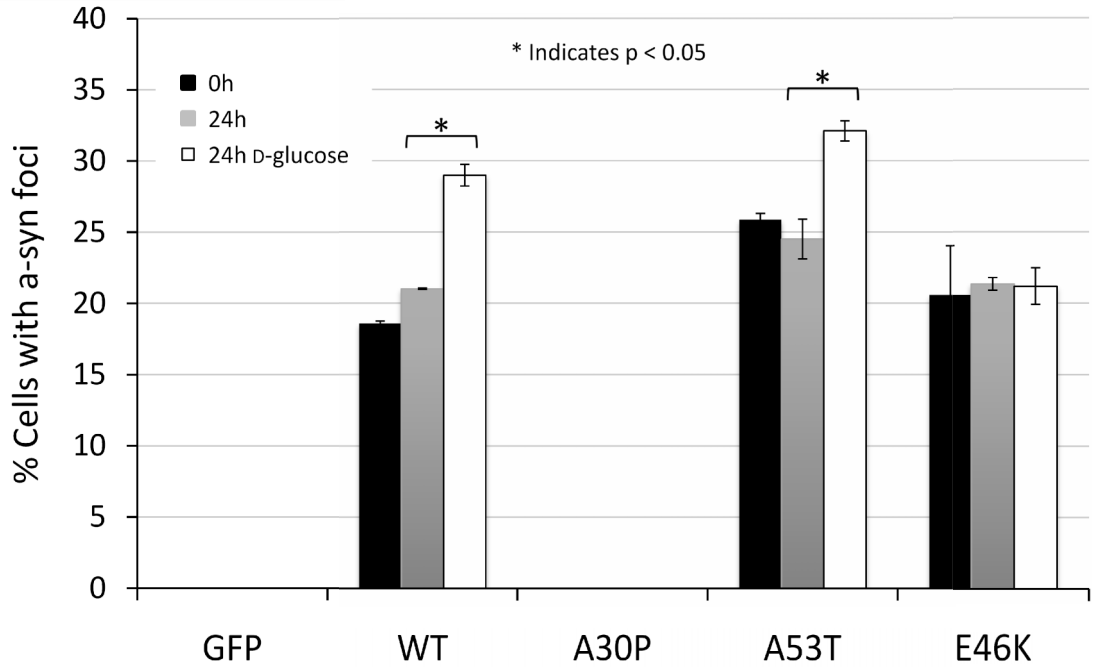

Figure IV.7 *In vivo* a-syn *foci* formation in BY4741 yeast cells. Cells expressing GFP, and a-syn variants WT, A30P, A53T and E46K containing intracellular *foci* (aggregate-like structures) were counted prior and after MES buffer and D-glucose exposure. At least 1000 cells from independent experiments were considered and the results are expressed by the percentage of cells presenting *foci*, represented in bars ± SD. * $p < 0.05$ between 24h and 24h D-glucose of WT and A30P expressing cells. Control (GFP) and A30P variant do not form intracellular inclusions.

In ΔGLO1, the number of *foci* was also evaluated. The initial number of cells with *foci* is higher than in the reference strain for WT with 24%, and E46K with 27%. A53T showed 25% of cells positive for a-syn *foci*. After 24h MES buffer, the number of cells including a-syn *foci* increased. A53T variant displayed a 6% significant increase whereas WT and E46K shown a 6 and 2% non significant increases. In contrast with BY4741, ΔGLO1 cells challenged to D-glucose did not increase the number of *foci* for A53T. As for E46K, similarly to BY4741, no significant variations were observed. WT variant have shown an increased number of a-syn *foci* positive cells, significant between initial and 24h of D-glucose exposure, however in non significant numbers compared to 24h of MES buffer (figure IV.8).

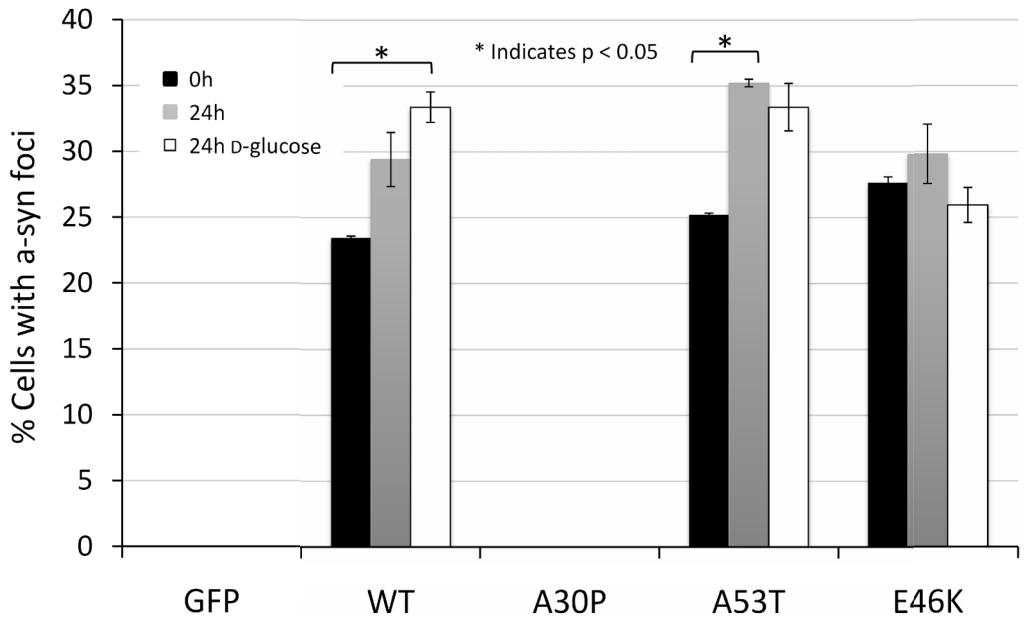

Figure IV.8 *In vivo* a-syn *foci* formation in ΔGLO1 yeast cells. Cells expressing GFP, and a-syn variants WT, A30P, A53T and E46K containing intracellular *foci* (aggregate-like structures) were counted prior and after MES buffer and D-glucose exposure. At least 1000 cells from independent experiments were considered and the results are expressed by the percentage of cells presenting *foci*, represented in bars ± SD. Control (GFP) and A30P variant do not form intracellular inclusions.

4.5 a-Syn Aggregates Formation

SSD-AGE is a well described technique to evaluate the presence of amyloid aggregates (Halfmann & Lindquist, 2008). The presence of a-syn aggregates was investigated in BY4741 yeast cells in glycation conditions (figure IV.9). Total protein extracts did not displayed any formation of

high molecular protein aggregates, in contrast to a positive a-syn S129A variant mimicking an unphosphorylated isoform of a-syn that displays large percentage of a-syn *foci* cells positive for high molecular weight protein aggregates (data not published, Sandra Tenreiro personal communication).

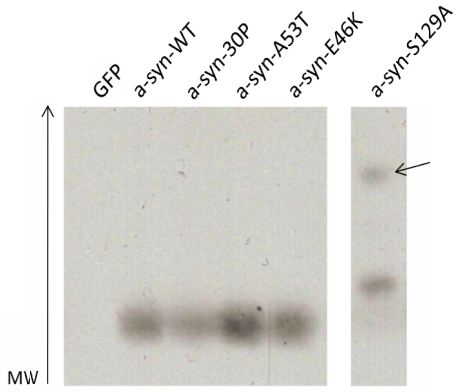

Figure IV.9 a-Syn *in vivo* amyloid aggregation. Semi-denaturant agarose-gel electrophoresis of glycated yeast BY4741 cells expressing GFP as control and a-syn WT, A30P, A53T and E46K variants. As positive control, yeast cells expressing a-syn S129A fused to GFP were used (S129A mimicks the unphosphorylated form of a-syn, is known to form a-syn aggregates in yeast - Sandra Tenreiro, unpublished data). None of the yeast expressing a-syn variants formed amyloid aggregates, while the positive control exhibit a high molecular weight aggregate (arrow indicated).

4.6 *In Vitro* a-Syn Glycation Sites

a-Syn glycation is a poorly understood process. Methylglyoxal glycation is known to increase a-syn oligomerization and decrease its lipid binding ability (Lee*, et al.*, 2009) however the extent of glycation and the modified residues are still unkown. We purified recombinant human a-syn variants in *E. coli* and performed methylglyoxal glycation *in vitro*. Unmodified a-syn was analyzed by ESI-FTICR providing a sequence coverage of 73% for all a-syn variants (data not shown) without any identified glycated peptides within 20 ppm mass accuracy. After 0.1 mM methylglyoxal 24h glycation, no glycated peptides were identified (data not shown), whereas incubation of a-syn with 1 mM of methylglyoxal for 24h led to the modification of several peptides for all a-syn variants. This chosen concentration is 10 fold lower than in previous works (Lee*, et al.*, 2009), and is often used in *in vitro* methylglyoxal glycation experiments. The exact location of the probable modifications was not fully accessed since different tryptic peptides of a-syn may display the same exact molecular weight. This fact is due to the peculiar sequence of a-syn with seven imperfect repetitions of an 11 amino acid residues sequence, thus, for instance, accurate mass alone cannot distinguish between TKEGVVHGVATVAEK and EGVVHGVATVAEKTK sequences. Also,

the majority of peptides identified contained two miscleavages, raising questions on which lysine is modified. For instance, if the peptide MDVFMKGLSKAK presents a molecular weight increase of 72 Da (the molecular weight of CEL), it is not possible to distinguish between K6 or K10 modification based only on MS data. Sequencing this peptides performing tandem-MS analysis was not achieved. The modified peptides have shown to be resistant to fragmentation by low energy collision-induced dissociation (CID) or electron capture dissociation (ECD). More energetic methods will be required, most likely combining infrared multiphoton dissociation (IRMPD) and ECD. Nevertheless, using the accurate massa data, it was possible to ascertain that WT a-syn was modified in K6, K10, K12 and probable K21, K23, K32, K43 and K58 residues also modified; A30P in K21, K23, K32 and probably in K6, K10 and K12; A53T in K45, K58 and probably in K6, K10, K21, K23 and K32; and E46K in K6, K10, K12, K60, K96, K97 and probably in K21, K23 and K32. The sequence coverage of the proteins was 73% for ET, A30P and E46K and 66% for A53T, where almost all the C-terminal domain of all variants was not ionized (residues 103-140). Detailed information is included in tables IV.1-4. The high resolution of the mass spectrum is illustrated in figure IV.10.

Table IV.1 Identification and molecular location of post-translational modifications of purified methylglyoxal-glycated a-syn WT. Detailed informations of glycated peptides are included and modified residues are shown in red. Z indicates peptide charge.

Measured mass (Da)	z	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	Glycated residue
375.45269	+4	1498.76440	12	MDVFMKGLSKAK (1 – 12)	K6 and/or K10
1213.12154	+2	2425.23531	1	MDVFMKGLSKAKEGVVAAAEK (1 – 21)	K6, K10 and K12
993.53857	+2	1986.05021	9	EGVVAAAEKTKQGVAEAAGK (13 – 32)	K21 and/or K23
1108.10869	+2	2215.19285	7	EGVVAAAEKTKQGVAEAAGKTK (13 – 34)	K21 and/or K23 and/or K32
798.92765	+2	1596.85916	7	TKEGVVHGVATVAEK (44 – 58) EGVVHGVATVAEKTK (46 – 60)	K45 and/or K58

Table IV.2 Identification and molecular location of post-translational modifications of purified methylglyoxal-glycated a-syn A30P. Detailed informations of glycated peptides are included and modified residues are shown in red. Z indicates peptide charge.

Measured mass (Da)	z	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	Glycated residue
1221.14868	+2	2385.25076	20	MDVFMKGLSKAKEGVVAAAEK (1 – 21)	K6 and/or K10 and/or K12[1]
801.44214	+2	1601.87448	1	GLSKAKEGVVAAAEK (7 – 21)	K6 and/or K10
1193.14187	+2	2385.25076	10	EGVVAAAEKTKQGVAEAAGKTK (13 – 34)	K21, K23 and K32

[1] M1 or M5 is oxidated

Table IV.3 Identification and molecular location of post-translational modifications of purified methylglyoxal-glycated a-syn A53T. Detailed informations of glycated peptides are included and modified residues are shown in red. Z indicates peptide charge.

Measured mass (Da)	z	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	Glycated residue
750.88688	+2	1500.74366	14	MDVFMKGLSKAK (1 – 12)	K6 and/or K10[1]
1108.10124	+2	2215.19285	1	EGVVAAAEKTKQGVAEAAGKTK (13 – 34)	K21 and/or K23 and/or K32
964.52825	+2	1928.03350	7	TKEGVVHGVTTVAEKTK (44 – 60)	K45 and K58

[1] K6 or K10 are acetylated

Table IV.4 Identification and molecular location of post-translational modifications of purified methylglyoxal-glycated a-syn E46K. Detailed informations of glycated peptides are included and modified residues are shown in red. Z indicates peptide charge.

Measured mass (Da)	z	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	Glycated residue
335.44230	+4	1498.76440	12	MDVFMKGLSKAK (1 – 12)	K6 and/or K10
750.88459	+2	1500.74366	11	MDVFMKGLSKAK (1 – 12)	K6 and/or K10[1]
1213.12002	+2	2425.23531	2	MDVFMKGLSKAKEGVVAAAEK (1 – 21)	K6, K10 and K12
687.39815	+2	1373.76347	17	AKEGVVAAAEKTK (11 – 23)	K12 and/or K21
1108.10994	+2	2215.19285	8	EGVVAAAEKTKQGVAEAAGKTK (13 – 34)	K21 and/or K23 and/or K32
1115.10461	+2	2229.20850	3	TKEQVTNVGGAVVTGVTAVAQK (59 – 80)	K60
1146.61018	+2	2292.20817	2	TVEGAGSIAAATGFVKKDQLGK (81 – 102)	K96 and K97

Table IV.5 Summary of a-syn *in vitro* glycated residues. Identification and molecular location of post-translational modifications.

a-Syn variant	Modification	Glycated residue
WT	Defined location	K6, K10, K12
	Probable locations	K21, K23 K32, K43, K58
A30P	Defined location	K21, K23, K32
	Probable locations	K6, K10, K12
A53T	Defined location	K45, K58
	Probable locations	K6, K10, K21, K23, K32
E46K	Defined location	K6, K10, K12, K60, K96, K97
	Probable locations	K21, K23, K32

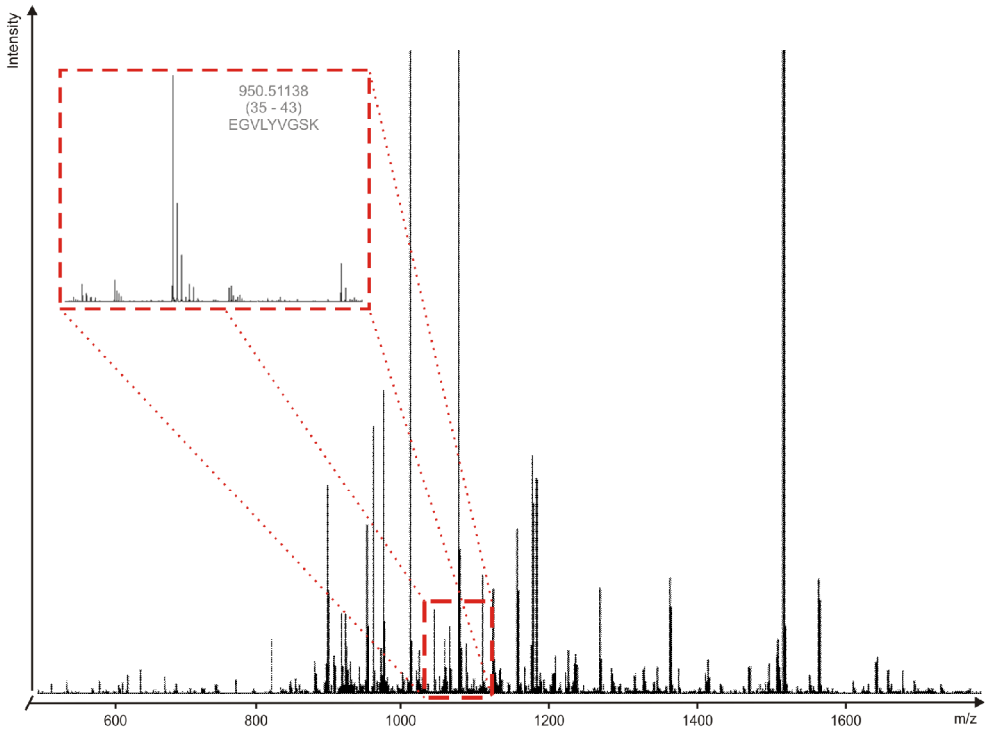

Figure IV.10 ESI-FTICR mass spectrum of *in vitro* glycated a-syn WT. The high resolution of the mass spectrum is illustrated by showing the isotopic pattern of the ion corresponding to the unmodified a-syn peptide 35-43.

5. Discussion

Despite recent efforts in understanding the processes underlying the aggregation of a-syn, these are still far from being understood. The occurrence of sporadic cases of PD suggest that non-genetic factors, such as post-translational modifications may contribute to the misfolding and further oligomerization of a-syn generating highly toxic species. Protein glycation has been reported in several neurodegenerative diseases and is undoubtly involved in amyloidoses. For example, glycated proteins are found in Alzheimer's disease (neurofibrillary tangles and amyloid β plaques), prion diseases (prion protein), familial amyloidotic polyneuropathy (transthyretin) and in Parkinson's disease (LBs). Some questions remains to be elicited whether glycation of susceptible proteins are the triggering events in the disease or just a result of its reaction towards low turnover aggregated species that are highly insoluble and protease resistance.

Our study suggests that protein glycation affect a-syn *foci* formation *in vivo*. Glycation conditions promote the formation of WT and A53T a-syn variants intracellular *foci*. By contrast, E46K was not affected by glycation and A30P a-syn variant did not form any inclusion-like

structures. In a yeast strain deficient for glyoxalase I, the main methylgyoxal catabolic enzyme, the percentage of cells expressing WT a-syn containing positive *foci* is higher than in the reference strain. Both results suggest that in glycating conditions, WT a-syn is more prone to accumulate and form intracellular *foci*. This phenomenon is independent of a-syn expression levels, since the amount of a-syn present in BY4741 and ΔGLO1 are similar (data not shown).

The formation of a-syn *foci* in yeast cells expressing a-syn WT, A53T and E46K seem to correlate with its toxicity. Previously, WT and A53 variants have shown to display more toxicity in yeast cells in a concentration dependent manner (Outeiro & Lindquist, 2003). In this study, ΔGLO1 cells, showing higher percentage of cells containing a-syn *foci*, are much more sensitive to a-syn toxicity than the reference strain prior to D-glucose challenge. Thus, methylglyoxal increased formation in this strain may be responsible for enhancing a-syn toxicity. Interestingly after D-glucose exposure, ΔGLO1 yeast cells are more viable than BY4741. These results suggest that despite a-syn is more toxic to the cells, their viability is higher.

We have further investigated if the differences in a-syn variants *foci* formation depend on a-syn glycation patterns. To that purpose, recombinant a-syn was purified from E. coli and methylglyoxal glycation patterns were accessed by peptide mass fingerprinting. In all variants only 72.9% of the protein was identified. This sequence coverage is due to the ineffective ionization of the fragment of the acidic C-terminal domain (residues 103-140), with a theoretical molecular weight of 4285.726 Da. K6, K10, K12, K21, K23 and K32 are consistently CEL modified in all a-syn variants (with the exception of K12 in A53T). All these modifications are located in the N-terminal of a-syn (residues 1-65), the region associated with a-syn binding ability to membranes. K6, K10 and K12 were reported to be ubiquitylated in 293T cells cotransfected with human a-syn and ubiquitin (Nonaka*, et al.*, 2005) and a-syn present in LBs is ubiquitylated in K12, K21 and K23 (Anderson*, et al.*, 2006). Since all these targets were modified by CEL, we hypothesize that in cells a-syn glycation might occur in these targets inhibiting protein ubiquitylation and disturbing proteasome degradation. Interestingly WT, A30P and A53T a-syn variants fused to an unstable green fluorescent protein were shown to accumulate in yeast, indicating proteasome impairment (Outeiro & Lindquist, 2003). Moreover, several other studies reported a-syn to impair proteasome mediated protein degradation (Tanaka*, et al.*, 2001, Snyder*, et al.*, 2003, Chen*, et al.*, 2005, Nonaka & Hasegawa, 2009).

Together with the highlighted consistent modifications between all a-syn variants, some different glycation targets were identified. WT is shown to be modified at K43 and K58. These

modifications are also located in the protein N-terminal and similarly, A53T variant displays K45 and K58 glycation. Both variants were shown similar toxicity levels and are shown to form higher percentage of cells containing a-syn *foci*. In contrast, E46K, exhibit the highest toxicity but the percentage of cells containing a-syn *foci* is not affected by 24h exposure to MES buffer and D-glucose. It is the only variant displaying modifications in the C-terminal region with K60, K96 and K97. These results let us hypothesize that although a-syn is an intrinsically unfolded protein, lysine exposure to the solvent is different between the variants. Furthermore, A30P variant was the less glycated variant with modified residues only in the first 32 amino acids of the N-terminal region, in contrast to the other variants displaying modifications in the first 58 (WT and A53T) and 97 (E46K) residues.

In agreement with *in vitro* results, a-syn K12, K23, K32, K45 and K58 residues were found to be glycated in a healthy adult rat (*Rattus norgevicus*) midbrain (see chapter V). Only K6, K10 and K43 were not confirmed to be modified in this mammalian model.

In summary, we showed that protein glycation increases a-syn *foci* formation in yeast cells. The process underlying glycation effects is unknown, but nevertheless, glycation may interfere with a-syn aggregation process and may diminished the effectiveness of the cell quality control system by modifying the a-syn ubiquitylation sites impairing protein ubiquitylation and further proteasome degradation. This observation suggests that glycation defenses may contribute to an effective therapeutic strategy for PD.

6. Acknowledgements

H.V.M was supported by fellowship SFRH/BD/23035/2005, S.T. by fellowship SFRH / BPD / 35767 / 2007 and L.M.A.O. by fellowship SFRH/BD/23604/2005. TFO is supported by an EMBO installation grant and by Marie Curie IRG. Work was also supported by PTDC/QUI/73430/2006 and by pluriannual funding of Centro de Química e Bioquímica, FCUL (Chem-LVT-Lisboa-612) from Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Tecnologia, Portugal.

Chapter V

Alpha-Synuclein Extraction and Characterization in Different Brain Regions

Vicente Miranda, H., de Oliveira, R. M., Graça, G., Cordeiro, C., Outeiro, T.F., Manuscript in preparation.

1. Abstract

Alpha-synuclein (a-syn) is the major component of proteinaceous inclusions known as Lewy bodies, the pathological hallmark of Parkinson's Disease and other synucleinopathies. Phosphorylation, oxidation, ubiquitylation, and glycation are post-translational modifications associated with the aggregation process of a-syn. Nevertheless, the role of these modifications is still poorly understood. In this study we developed a method for the extraction and partial purification of a-syn from different brain regions. The method results in a 5-fold enrichment in a-syn content upon exposure to heat. Two-dimensional gel electrophoresis and mass spectrometry analysis were performed and about 60% of a-syn sequence coverage was achieved by peptide mass fingerprinting. Several lysine and arginine residues were found to be glycated, acetylated or phosphorylated. Interestingly K10, K12 and K21 are known ubiquitylation sites and in this study, all these sites were shown to be post-translationally modified with N^{ε}-(carboxyethyl)lysine (CEL) and/or an acetyl-group. This method enables the systematic study of a-syn post-translational modifications, providing important clues about the role of these modifications on a-syn aggregation and toxicity. Moreover, out results suggest a role for glycation and acetylation on a-syn biology by modulating both the structure and processing of this protein via the ubiquitin proteasome system.

2. Introduction

Parkinson's disease (PD) is the second most common progressive neurodegenerative disorder with a prevalence of around 2% in people over the age of 65 years old (Van Den Eeden*, et al.*, 2003). Muscle rigidity, resting tremor, bradykinesia, rigidity and postural instability are the most common symptoms of the disease (Forno, 1996, Burke, 1999). The majority of PD cases are sporadic, although several genes have been associated with familial cases of the disease. Mutations and multiplications in the gene PARK1, encoding for alpha-synuclein (a-syn), are associated with familial cases (Polymeropoulos*, et al.*, 1997, Kruger*, et al.*, 1998, Zarranz*, et al.*, 2004). Pathologically, the disease is characterized by the loss of nigrostriatal dopaminergic neurons and the accumulation of proteinaceous intraneuronal cytoplasmic inclusions named Lewy bodies (LBs), primarily composed of a-syn protein (Gai*, et al.*, 2000, Braak*, et al.*, 2003).

Although the function of a-syn is still poorly understood, it is thought to be associated with vesicular trafficking, synaptic function and plasticity modulation, and dopamine neurotransmission regulation (Klein & Lohmann-Hedrich, 2007). a-Syn is a target for several post-translational modifications such as oxidation, phosphorylation, glycation and ubiquitylation (Hirsch, 1993, Castellani*, et al.*, 1996, Fujiwara*, et al.*, 2002, Hasegawa*, et al.*, 2002, Anderson*, et al.*, 2006). However, the role of the post-translational modifications on a-syn biology remains unclear. Phosphorylation may play an important role in the aggregation process of the protein since about 90% of a-syn found in LBs is phosphorylated (Fujiwara*, et al.*, 2002). Moreover, glycation may be involved in the chemical crosslinking and proteolytic resistance of a-syn protein deposits (Castellani*, et al.*, 1996, Munch*, et al.*, 2000). Although it remains unclear whether monomeric a-syn requires ubiquitylation prior to its degradation, a-syn is found to be ubiquitylated in K11, K21 and K23 residues (Anderson*, et al.*, 2006).

The elucidation of the molecular mechanisms underlying a-syn misfolding and its associated proteotoxicity is essential for a better understanding of the role of a-syn in the neuropathology associated with PD. In this study we developed a specific method to analyse and characterize the post-translational modifications of a-syn extracted from different brain regions of different mammalian models.

3. Experimental Procedures

3.1 Reagents and Materials

Dithiothreitol, iodoacetamide and trifluoroacetic acid (TFA), sequence grade modified trypsin, K_2HPO_4, Coomassie Brilliant Blue G, Ponceau S, 2-mercaptoethanol, PMSF, agarose, tween-20, glycerol, thiourea, CHAPS (3-(3-cholamidopropyl dimethylammonio)-1 propanesulphonate) and NP-40 were purchased from Sigma. Tris, SDS 20% (w/v), acrylamide/BIS [40% (w/v)] and glycine were purchased from BioRad. EDTA was obtained from BDH chemicals LTD while, bromophenol blue, triton X-100, were obtained from Fluka. PerfectPure C-18 tips were obtained from Eppendorf; HPLC gradient grade acetonitrile was from Merck; ultrapure water was produced in a Millipore Milli-Q system. Electrophoretic protein standards were from Fermentas. Protein inhibitors were from Roche. Urea, IPG buffer, IPG strips and PVDF membranes Hybond-P were from Amersham Biosciences. CHCA (alpha-Cyano-4-hydroxycinnamic acid) was from Fluka.

3.2 Protein Extraction

Brains from Wistar rats (female, aged 32 weeks) obtained from Harlan, Barcelona, Spain, were disected and stored at -80 °C prior to use. The different brain regions (cerebellum, cortex, hippocampus, and midbrain) were homogenized in RIPA Buffer [50mM TrisHCl pH7.4, 150mM NaCl, 2 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) SDS] in the presence of proteases inhibitors. The tissue extract was centrifuged at 18 000 g 30 min. 4 °C and the soluble fraction isolated. After protein quantification (Bio-Rad Bradford assay kit), lysates were heated at 90 °C for 30 min and centrifuged at 18 000 g 15 min. 4 °C. Protein concentration was determined in the soluble fraction.

3.3 SDS-PAGE and Western Blot

Proteins, 30 μg of soluble tissue protein and 3 μg of heat-purified protein, were separated by SDS/PAGE using a Tetra cell (Bio-Rad, Hercules, California, U.S.) using a 15% polyacrilamide separation gel and a 6% polyacrilamide stacking gel and applying a constant voltage of 100V. Proteins were transferred to PVDF membranes, using the Mini Trans-Blot system (Bio-Rad). Transfer was performed with 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% (v/v) methanol. Pre-stained standard proteins were also loaded onto the gel. Total proteins were stained with Ponceau S solution [0.5% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm the amount of protein transferred. The membrane was blocked overnight at 4 °C in 1% (v/v) blocking solution in TBS (50 mM Tris with 150 mM NaCl, pH 7.5). The blots were probed with anti-synuclein-1 (S63320 BD Transduction Laboratories). Washes, secondary antibody and detection procedures were performed using the ECL Western Blotting system (Amersham) following the manufacturer's instructions. Each immunoblot was repeated at least three times in independent experiments. Protein intensities were assayed using ImageJ - Image Processing and Analysis in Java (Abramoff*, et al.*, 2004).

3.4 Bi-Dimensional Electrophoresis

Protein samples, containing 50 μg of total protein, were precipitated with acetone overnight at -20°C. Proteins were pelleted by centrifugation at 18 000 g 10 min. 4°C. Pellet was ressuspended in rehydration buffer [7 M urea, 2 M Thiourea, 4% (w/v) CHAPS, a 2% (v/v) IPG buffer, 60 mM dithiothreitol (DTT), and bromophenol blue 0.002% (w/v)] to a final volume of 125 μL and loaded onto 7 cm pH 3-10 NL IPG strips by in-gel rehydration 12 hours at 30V in the IPGphor II (Amersham Biosciences Europe GmbH, Freiburg, Germany). Proteins were focused as

described (Lamy*, et al.*, 2009). After focusing, proteins in the IPG strips were reduced by soaking with 1% (w/v) DTT; 50 mM tris-HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol; 2% (w/v) SDS at room temperature for 15 min, then alkylated with 65 mM iodoacetamide, 50 mM tris-HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol, 2% (w/v) SDS for 15 min at room temperature. Equilibrated strips were then horizontally applied on top of a 15% SDS-PAGE mini gel and proteins were separated vertically at 18 °C, using a Tetra cell (Bio-Rad, Hercules, California, U.S.) and applying a constant voltage of 100V. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue (CBB) R-250, dissolved in 40% (v/v) methanol, 10% (v/v) acetic acid overnight and destained with 10% (v/v) acetic acid for 48 h. Gels, prepared in triplicate of independent experiments were also analysed by western blot following the previously described methodology (section V 3.2).

3.5 In Gel Protein Digestion

Protein bands were manually excised from the gels using disposable scalpels, washed in milliQ water, and distained in 50% acetonitrile (ACN) and subsequently with 100% ACN. Cys residues were reduced with 10 mM DTT and alkylated with 50 mM iodoacetamide as described (Pandey*, et al.*, 2000). Gel pieces were dried by centrifugation under vacuum and rehydrated in digestion buffer containing 50 mM NH_4HCO_3 and 6.7 ng/µL of trypsin at 4 °C. After 30 min the supernatant was removed and discarded and 20 µL of 50 mM NH_4HCO_3 were added. Digestions were allowed to proceed at 37 °C overnight (16-18 hours). After digestion, the remaining supernatant was removed and stored at -20 °C.

3.6 Mass Spectrometry

Samples were desalted and concentrated using PerfectPure C-18 microcolumns and eluted directly to the MALDI target AnchorChip (Bruker Daltonics, Bremen) with the appropriated matrix, according to the manufacturer procedure. Matrix solution of CHCA was prepared at a concentration of 10 mg/mL in 50% ACN with 0.1% TFA. Peptide mixtures were analyzed by MALDI-FTICR-MS in a Bruker Apex Ultra, Apollo II combi-source (Bruker Daltonics, Breman, Germany), with a 7 Tesla magnet (Magnex corporation, Oxford UK). Monoisotopic peptide masses were determined using the SNAP 2 algorithm in Data Analysis software version 3.4 (Bruker Daltonics). External calibration was performed by using BSA tryptic digest, processed and analyzed with Biotools 3.1 (Bruker Daltonics, Bremen).

4. Results

4.1 a-Syn Enrichment

Initially, we confirmed the ubiquitous expression of a-syn in the brain, and found it is more abundant in the cortex and hippocampus than the midbrain or in the cerebellum (figure V.1 A). One of the major limitations for the characterizing of a-syn from tissues extracts is the difficulty in the extraction and analysis by mass-spectrometry, followed by the presence of several other proteins with similar molecular weight, hindering specific epitopes required for imunoblotting experiments. The method we developed is based on the thermostability of a-syn. Heating protein extracts precipitates all thermally unstable proteins whereas more stable proteins remain in the soluble fraction. This step was responsible for an average 5-fold increase of a-syn content in the soluble fraction of tissue extracts (figure V.1 B). In addiction, the number of total proteins clearly decreased, enabling a considerable purification of a-syn (figure V.2).

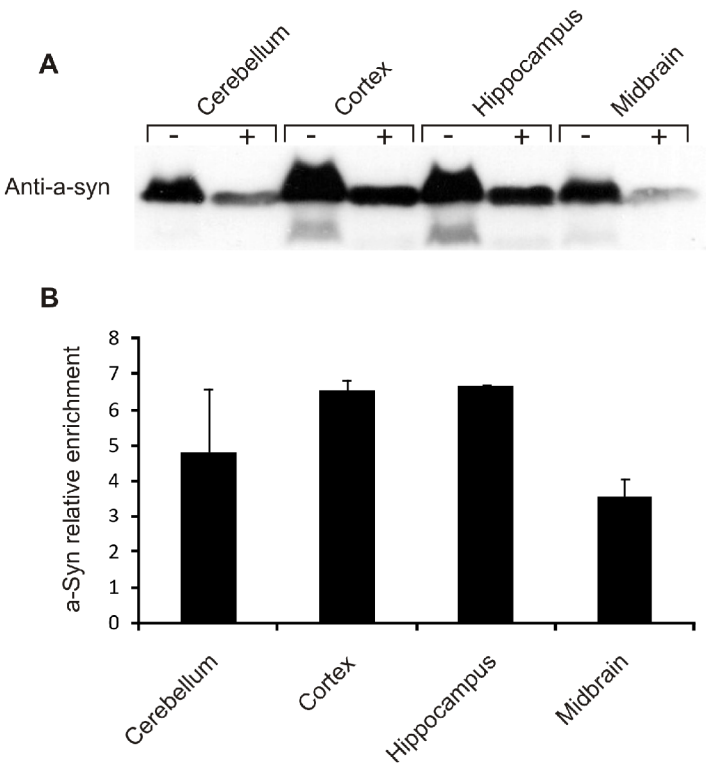

Figure V.1 Representative Western blot of brain protein extracts stained for a-syn. **A**. 30 μg of protein extract (-) and 3 μg of heated protein extracts (+) from cerebellum, cortex, hippocampus and midbrain are a-syn positive and expressed higher amounts in the cortex and hippocampus. **B**. After heating, the amount of a-syn present in the soluble fraction increased ~5-fold.

4.2 a-Syn Identification

Direct analysis by mass spectrometry of the protein band separated in 15% bis-acrilamide SDS-PAGE showed that a-syn co-migrated with several other proteins including myelin (data not shown). To circumvent this problem, the proteins were separated by bi-dimensional electrophoresis (2D-PAGE). This analysis showed that after heat purification, the number of proteins in the soluble fraction of protein extracts decreased. The western blot analysis revealed the location of the a-syn spot on the gel (red arrowhead in figure V.2).

Spot excision, tryptic digestion and peptide mass fingerprinting confirmed the presence of a-syn with a sequence coverage of 41% and 50% for CRA_a and CRA_b protein sequences of a-syn, respectively (figure V.3). Detailed information of the peptides identified is included in the tables VI.1 and VI.2. Two different isoforms of a-syn, one with 140 amino acids (CRA_a) and the second with 149 amino acids (CRA_b) are expressed in the rat. The CRA_a isoform displays higher homology to the human a-syn sequence with 95% identity compared to 76% of CRA_b.

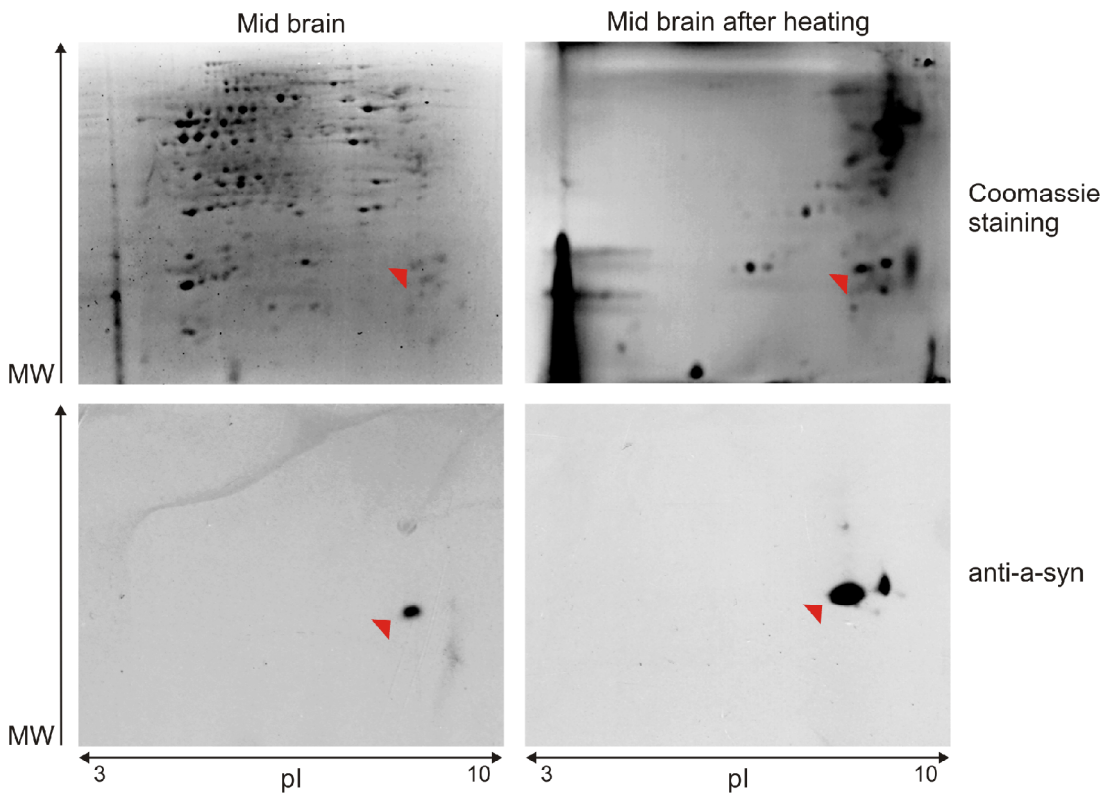

Figure V.2 Representative bi-dimensional separation of midbrain protein extracts Coomassie staining of soluble extracts (upper left) and heated extracts (upper right) show a clear decrease in the number of proteins after heat. Western blot probed for a-syn shows an enrichment in of a-syn after heating(lower right). (▲) Indicates the a-syn positive spots.

Table V.1 Peptide mass fingerprinting of a-syn protein (isoform CRA_a) in the midbrain.

Measured mass (Da)	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence
951.51506	951.51457	0	EGVLYVGSK (35 – 43)
1072.57788	1072.59970	20	AKEGVVAAAEK (11 – 21)
1180.65461	1180.65721	3	TKEGVLYVGSK (33 – 43) EGVLYVGSKTK (35 – 45)
1325.70776	1325.70596	1	EGVVHGVTTVAEK (46 – 58)
1928.04797	1928.04473	1	EQVTNVGGAVVTGVTAVAQK (61 – 80)

Table V.2 Peptide mass fingerprinting of a-syn protein (isoform CRA_b) from midbrain.

Measured mass (Da)	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence
951.51506	951.51457	0	EGVLYVGSK (35 – 43)
1055.68188	1055.70477	20	LIALRVKSR (116 – 124)
1072.57788	1072.59970	20	AKEGVVAAAEK (11 – 21)
1180.65461	1180.65721	3	TKEGVLYVGSK (33 – 43) EGVLYVGSKTK (35 – 45)
1325.70776	1325.70596	1	EGVVHGVTTVAEK (46 – 58)
1529.76278	1529.78325	20	SRYREHSWRPR (123 – 133)
1928.04797	1928.04473	1	EQVTNVGGAVVTGVTAVAQK (61 – 80)

4.3 a-Syn Post-translational Modifications

Protein spots containing both a-syn isoforms were analysed for the presence of post-translational modifications. The modified peptides increased the sequence coverage to 53% and 71%. This is the first report of the presence of glycation in a-syn in the form of CEL in 5 possible lysine residues for the CRA_a isoform (K12, K23, K32, K45 and K58) and in 9 possible lysine and arginine residues in the form of CEL and *N*d-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl) ornithine (tetra-hydropyrimidine) (K12, K23, K32, K45, K58, R124, R126, R131, R133). Acetylation also occurs possibly in K10, K12 and or K21, while phosphorylation occurred in either T33, S42 and or T44. Detailed information is included in figure V.4 and tables V.3 and V.4.

Table V.3 Identification and molecular location of post-translational modifications of a-syn protein (isoform CRA_a) from *Rattus norvegicus* midbrain. Modified residues are shown in red. PTM, acetyl and phosph are the abbreviatures for post-translational modifications, acetylation and phosphorylation, respectively.

Measured mass (Da)	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	PTM	Glycated residue
1131.58974	1131.60043	20	TKQGVAEAAGK (22 – 32) QGVAEAAGKTK (24 – 34)	CEL	K23 and/or K32
1144.61649	1144.62083	4	AKEGVVAAAEK (11 – 21) EGVVAAAEKTK (13 – 23)	CEL Acetyl	K12 K21
1260.64435	1260.62354	20	TKEGVLYVGSK (33 – 43) EGVLYVGSKTK (35 – 45)	Phosph	T 33 and/or S42 and/or T44
1728.95151	1728.98542	20	GLSKAKEGVVAAAEKTK (7 – 23)	Acetyl	K10 and/or K12 and/or K21
1928.04797	1928.03349	7	TKEGVVHGVTTVAEKTK (44 – 60)	CEL	K45 and K58

The exact location of the modifications was not fully accessed since different a-syn tryptic peptides may display the exact same molecular weight. This fact is due to the peculiar sequence of a-syn with seven imperfect repetitions of an unusual 11 amino acid residues sequence of a KTKEGV consensus, thus, for instance, only with the accurate peptide mass, we cannot distinguish between TKQGVAEAAGK or QGVAEAAGKTK sequences.

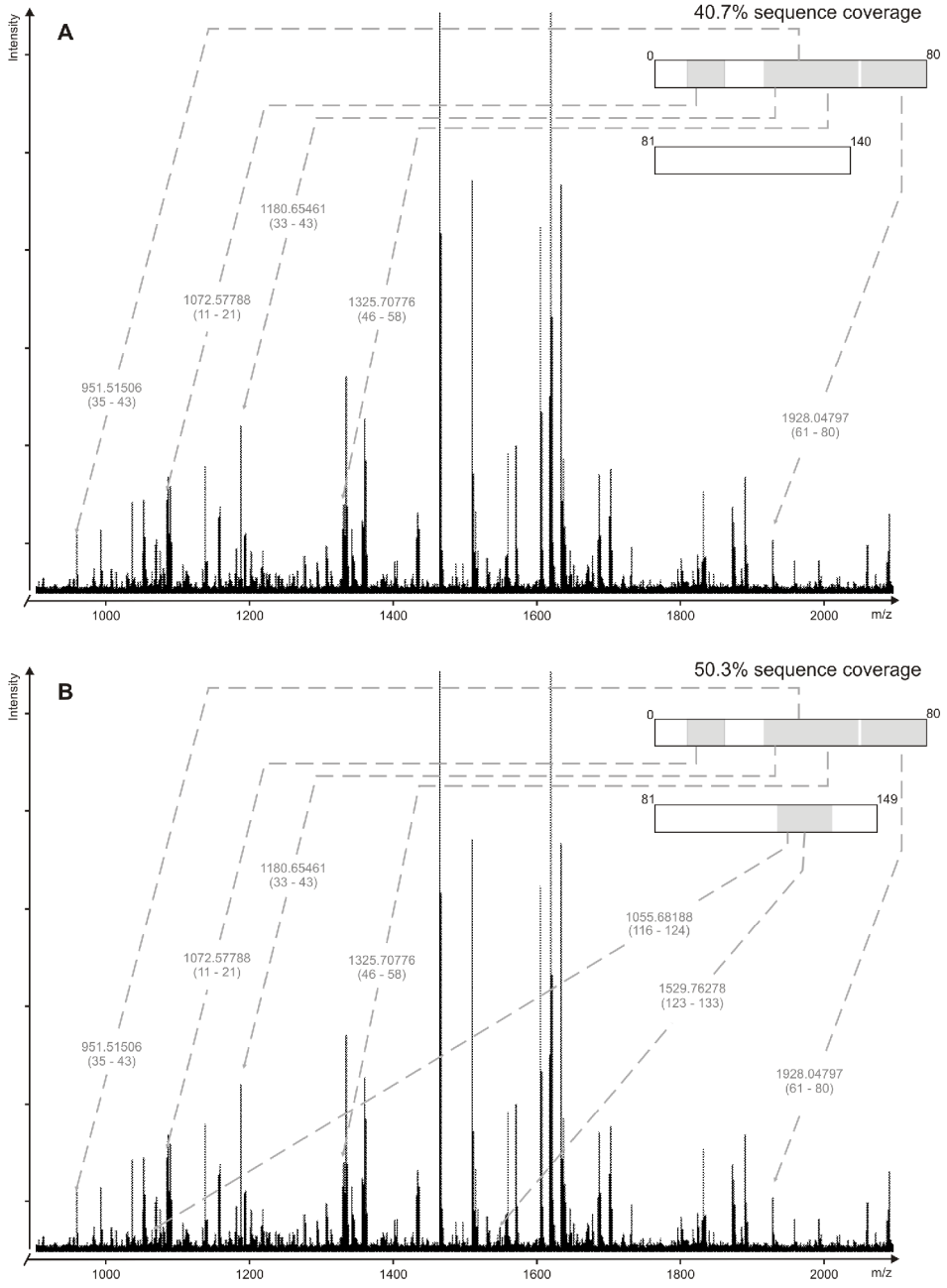

Figure V.3 Peptide mass fingerprinting analysis of a-syn positive protein spot from *R. norvegicus* midbrain bi-dimensional electrophoresis. **A.** About 41% of a-syn isoform CRA_a sequence was identified. **B.** About 50% of isoform CRA_b sequence was identified. Peptides are shown in the spectrum and the corresponding protein regions are in dark grey.

Table V.4 Identification and molecular location of post-translational modifications of a-syn protein (isoform CRA_b) from *Rattus norvegicus* midbrain. Modified residues are shown in red. PTM, acetyl and phosph are the abbreviatures for post-translational modifications, acetylation and phosphorylation, respectively.

Measured mass (Da)	Theoretical peptide mass (Da)	Deviation (ppm)	Peptide sequence	PTM	Glycated residue
951.51506	951.51322	1	VKSRYR (121 – 126)	THP	R124
1131.58974	1131.60043	20	TKQGVAEAAGK (22 – 32) QGVAEAAGKTK (24 – 34)	CEL	K23 and/or K32
1144.61649	1144.62083	4	AKEGVVAAAEK (11 – 21) EGVVAAAEKTK (13 – 23)	CEL Acetyl	K12 K21
1238.62828	1238.61507	10	EHSWRPRK (127 – 134)	THP	R131 and/or R133
1260.64435	1260.62354	20	TKEGVLYVGSK (33 – 43) EGVLYVGSKTK (35 – 45)	Phosph	T 33 and/or S42 and/or T44
1429.70873	1429.68455	17	YREHSWRPR (125 – 133)	THP	R126 and/or R131
1728.96151	1728.98542	20	GLSKAKEGVVAAAEKTK (7 – 23)	Acetyl	K10 and/or K12 and/or K21
1928.04797	1928.03349	7	TKEGVVHGVTTVAEKTK (44 – 60)	CEL	K45 and K58

5. Discussion

Post-translational modifications in a-syn have been studied in protein extracted from LBs of PD patients and from mammalian models. The process requires several laborious techniques involving ion-exchange and size-exclusion chromatography, Percoll density gradient centrifugation, and immunoprecipitation, among others. With this novel method, based on the thermostability of a-syn, enabled us to partially purify and enrich the a-syn content up to 5-fold. It is well described that the abundance of a-syn is different between different brain regions, and our results are in

agreement with previous reports (Wersinger*, et al.*, 2004). The relative amount of a-syn, which is critical for adequate characterization by mass spectrometry techniques, was successfully increased in heated tissue lysates.

After enrichment, we analysed the a-syn positive protein spots in 15% SDS PAGE electrophoresis. Only two a-syn peptides were identified, together with several myelin peptides. These results indicate that a-syn co-migrate with other thermostable proteins with similar molecular weights. Bi-dimensional electrophoresis was performed and a-syn was identified by peptide mass fingerprint with 41 and 50% sequence coverage for both isoforms CRA_a and CRA_b. The acidic C-terminal domain of the CRA_a isoform (residues 96-140) (George, 2002) was not identified since the resulting tryptic peptide has a theoretical molecular weight of 4265 Da that could not be detected by FTICR-MALDI-MS, the mass spectrometry technique used in this study. This peptide represents 31% of a-syn CRA_a sequence, thus, other mass spectrometry techniques are recommended to acess the full sequence coverage.

With the positive identification of a-syn, we searched the presence of post-translational modifications. To identify modified peptides, we performed a theoretical digestion of the identified proteins, considering up to three trypsin miscleavages and added to the resulting peptide masses the mass increment due to specific modifications, namely, methylglyoxal-derived advanced glycation end product N^{ε}-(carboxyethyl)lysine (CEL), acetyl and phospho groups. Using this approach we observed that several peptides do show a mass increment compatible with these modifications. The most interesting results came from the finding of glycated peptides in the normal *rat* midbrain. Thus far, no direct evidence of glycation in a-syn had been reported, only the co-localization of AGEs with a-syn in LBs (Castellani*, et al.*, 1996). Altough full peptide sequencing is necessary to locate and identify all modifications, some locations were determined. We verified that K12, K21 and K23 were targets for glycation and/or acetylation. Interestingly, these residues were previously described to be targets for ubiquitylation *in* vivo. Our data enable us to hypothesize that if these residues are modified prior to ubiquitylation, abnormal ubiquitylation may occur, leading to a-syn accumulation and, possibly, proteasome impairment.

Altough our data did not confirm S129 to be phosphorylated, since the C-terminal peptide was not detected, T33, S42 and/or T44 phosphorylation sites were discovered. Furthermore, as mentioned above, acetylation was observed in agreement with previous reports (Anderson*, et al.*, 2006).

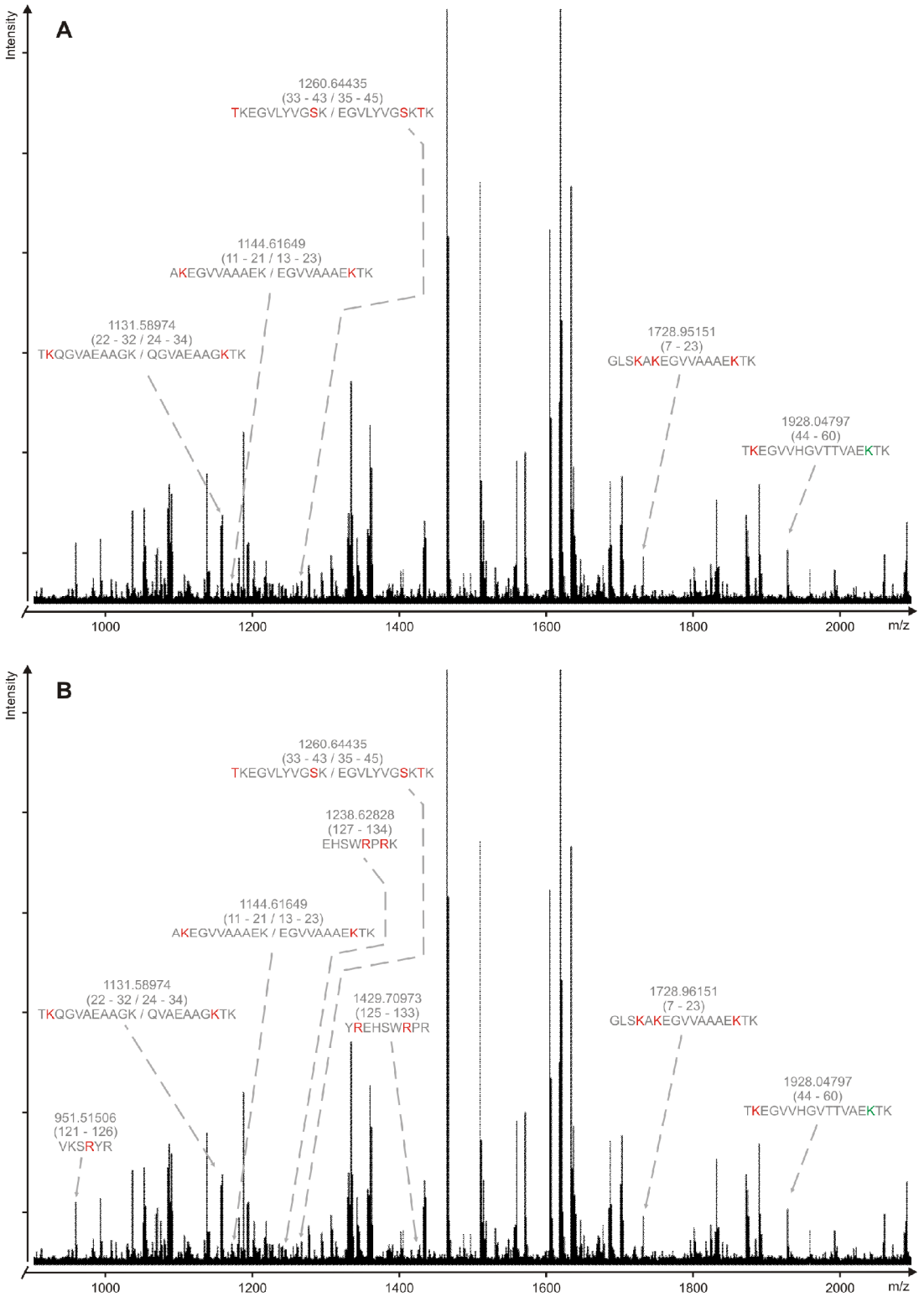

Figure V 4. Post-translational modification of a-syn from *R. norvegicus*. **A.** Peptides with post-translational modification of a-syn isoform CRA_a. **B**. Post-translational modifications of a-syn isoform CRA_b. Modified peptides are depicted in the spectrum and the modified residues are highlighted in red.

In conclusion, this novel method, comprising the heat of tissue lysates, followed by mass spectrometry analysis of bi-dimensional electrophoresis separated proteins, enables a fast characterization of a-syn and provides insights into the role of these modifications in a-syn aggregation and toxicity.

6. Acknowledgements

Work supported by grants SFRH/BD/23035/2005 (H.V.M) and SFRH/BPD/41416/2007 (R.O.) from the Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Tecnologia, Portugal. TFO is supported by an EMBO installation grant and by Marie Curie IRG. Work was also supported by pluriannual funding of Centro de Química e Bioquímica, FCUL (Chem-LVT-Lisboa-612) from Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Tecnologia, Portugal.

Chapter VI

Concluding Remarks

Concluding Remarks

The occurrence of protein glycation has been reported in several neurodegenerative diseases and is clearly involved in amyloidosis. Glycated proteins may misfold, become insoluble and protease resistant, and form amyloid structures. Nevertheless, some important questions remain to be answered, namely whether glycation of susceptible proteins is one of the triggering events in the disease process or just an outcome from glycation reactions with low turnover aggregated protein species. Several results point that glycation may be an early event promoting or accelerating the abnormal protein deposition into β fibrils structures, followed by increased protease resistance and insolubility. For example, in Alzheimer's disease, glycation of diffused neurofibrillary tangles (Takeda*, et al.*, 2000), along with AGEs in diffuse amyloid β senile plaques (Sasaki*, et al.*, 1998) are observed; in Parkinson's disease, AGEs are present in very early Lewy bodies; and in prions disease AGEs occurs in astrocytes prior to the formation of PrP^{res} (Choi*, et al.*, 2004). Regardless of the chronology of AGEs formation, it is known that its accumulation is related to sustained inflammatory responses and oxidative stress, common features in neurodegenerative diseases. Glycation is also known to modify protein structure with consequent loss of function and toxicity enhancement. Glycation may then be understood as a dynamic contributor to neurodegenerative diseases, promoting, accelerating or stabilizing pathological features and inducing several cellular responses leading to cell dysfunction, damage and death.

In this work, protein glycation by methylglyoxal was investigated *in vitro* and *in vivo*, using the yeast *Saccharomyces cerevisiae*. This simple eukaryote model, easy to manipulate genetically, is a remarkable "living test tube"in which to perform metabolic studies. Methylglyoxal is the main protein glycation agent *in vivo* and it is commonly accepted that the glyoxalase system is its major catabolic pathway. We showed, for the first time in microorganisms, that glycation is a process that modifies yeast proteins in a methylglyoxal concentration dependent reaction. Different glycation phenotypes were achieved in yeast strains deficient in genes encoding for methylglyoxal catabolic enzymes. Moreover, contrary to the belief that the glyoxalase system is the main methylglyoxal catabolic system, a deletion mutant of aldose reductase was found to display similar protein glycation levels as well as a similar methylglyoxal intracellular concentration. A kinetic mathematical model of methylglyoxal metabolism was developed, enabling to predict different phenotypes in yeast cells. Sensitivity analysis showed that both glyoxalase system and aldose

reductase catabolic pathways are equally important in preventing an intracellular methylglyoxal concentration increase. NADPH and glutathione concentrations are also key variables in maintaining a low steady-state methylglyoxal concentration. Importantly, glutathione is essential to the glyoxalase system, as is NADPH to aldose reductase. Furthermore, glutathione is kept in the reduced form due to the action of glutathione reductase, a NADPH dependent enzyme. Both these variables are also essential to prevent oxidative stress. In stress conditions, the decrease of glutathione and NADPH concentrations diminishes the normal activity of the glyoxalase system and aldose reductase, leading to an increase in methylglyoxal concentration and, consequently, in protein glycation. *YAP1* is responsible for the up-regulation of genes involved in anti oxidant responses, including glutathione synthesis and glutathione-dependent antioxidant systems (Inoue*, et al.*, 1999). Interestingly, its activity is modulated by methylglyoxal (Maeta*, et al.*, 2004), raising the glutathione concentration upon activation, suggesting that this may represent a cellular defense mechanism to cope with methylglyoxal toxicity. In agreement, we showed that *YAP1* deletion yeast strain was glycated.

Argpyrimidine is a specific type of methylglyoxal modification occurring in arginine residues, and was recently associated with neurodegenerative diseases (Gomes*, et al.*, 2005). In contrast to the common belief that glycation is a random process glycation in yeast was found to occur at specific protein targets. Three glycolytic enzymes were shown to display MAGE: enolase 2, phosphoglycerate mutase, and aldolase. Despite the loss of enolase 2 activity upon glycation, the major glycation target, yeast glycolytic metabolism was not affected. Importantly, arginine glycation depends on the surrounding chemical environment (Speer*, et al.*, 2003, Ahmed*, et al.*, 2005), suggesting the arginine-rich cave located at the dimer interface of enolase 2 may provide a favorable environment for glycation to occur. This could indicate that this protein may function as a methylglyoxal scavenger.

In addition to the glycolytic enzyme targets, heat shock proteins, such as Hsp70/71 and Hsp26 were also MAGE modified. The majority of neurodegenerative diseases are often associated with misfolding proteins such as a-syn in PD, TTR in FAP and tau in AD.

Proteostasis is ensured by a complex cellular quality control system composed by molecular chaperones and the protein degradation systems, which include the ubiquitin proteasome pathway. Protein glycation may interfere with the folding processes, promoting the misfolding of glycated proteins. Hsp26 is known to bind to misfolded proteins, and to be associated for the refolding processes (Ehrnsperger*, et al.*, 1997, Haslbeck, 2002, Cashikar*, et al.*,

2005). Interestingly, yeast cell activated Hsp26 upon methylglyoxal glycation, prossibly to cope with the consequent misfolding events caused by glycation. The processes underlying Hsp26 activation are still poorly understood. However, glycation of Hsp27 and α-crystallins improves their chaperone activity (Nagaraj*, et al.*, 2003, Oya-Ito*, et al.*, 2006). Thus, we hypothesize that Hsp26 and Hsp71/71 MAGE modifications may promote their chaperone activity. This is an important question which remains to be addressed in the future.

As mentioned above, glycation may play an important role in the misfolding and aggregation processes of proteins. The behavior of huntingtin and a-syn, proteins associatedwith neurodegenerative disorders, was previously investigated in yeast (Krobitsch & Lindquist, 2000, Muchowski*, et al.*, 2000, Outeiro & Lindquist, 2003). Protein quality control systems are highly conserved, from yeast to man, affording the opportunity to use yeast as a simple cell system in which to study complex fundamental biological problems. Furthermore, a-syn expression in yeast was shown to recapitulate central aspects of PD, including the formation of inclusion-like structures and cytotoxicity (Outeiro & Lindquist, 2003). Thus, we sought to investigate how post-translational modifications interfere with misfolding and aggregation to understand their contribution to cytotoxicity. Using yeast cells, we established a relationship between glycation and the formation of a-syn inclusion-like structures. In a glycating environment, achieved by growing cells in the presence of a high D-glucose concentration and by deleting glyoxalase I. We verified that, under these conditions, the number of yeast cells displaying a-syn fluorescent *foci* increased and the different mutant forms of a-syn displayed different phenotypes. While a-syn WT, A53T and E46K variants were mainly associated with the plasma membrane of yeast cells, the A30P mutant was distributed throughout the cytosol, and did not form inclusion-like structures. While the percentage of yeast cells expressing WT and A53T a-syn variants increased after exposure to D-glucose, the formation of inclusions did not change in E46K expressing cells. Since methylglyoxal glycation is known to diminish a-syn membrane binding ability and to induce its oligomerization (Lee*, et al.*, 2009), we suggest that glycation may induce a-syn membrane release resulting in the formation of intracellular inclusion-like structures. It will be important to address this hypothesis in the future and further investigate the biochemical nature of the observed a-syn fluorescent *foci*.

To better understand how glycation of a-syn may contribute to the different behavior of the various mutant forms, we glycated purified a-syn *in vitro*. Methylglyoxal was shown to react with specific lysine residues of a-syn. All a-syn variants displayed consensus modifications at residues K6, K10, K12, K21, K23 and K32, which are located in the N-terminal region of the protein

and represent the first 6 lysine residues in the protein sequence. The N-terminal region of a-syn is important for membrane binding, where seven imperfect repetitions of 11 amino acid motifs are believed to be responsible for the formation of α-helices upon interaction with lipid structures (Davidson*, et al.*, 1998, George, 2002, Bussell & Eliezer, 2003). This phenomenom, together with the extensive glycation detected in a-syn led us to hypothesize that methylglyoxal glycation may directly affect a-syn binding to membranes. Additional glycation sites were identified for WT, E46K and A53T a-syn, but E46K was the only a-syn variant displaying modifications near the C-terminal region. Altough the secondary structure of all a-syn variants appears to be similar, the solvent exposure of the lysine residues is likely to be different. Interestingly, the glycation patterns of WT and A53T variants were similar, suggesting that the similar behavior of WT and A53T in yeast cells may also be related to their glycation phenotypes. One interesting question that we will address in the future is the exact location of the modifications. Also, we will investigate the glycation sites of the different a-syn variants expressed in yeast cells upon glycation, and determine how it affects a-synmembrane binding to further confirm our hypothesis.

The characterization of a-syn post-translational modifications from different sources requires laborious purification techniques. Here, we developed a novel method based on the thermostability of a-syn to partially purify, enrich and analyze these modifications. Heating rat brain protein lysates allowed at least a 5-fold enrichment of a-syn content in the soluble fraction. This was followed by a clear decrease in the number of proteins as judged by 2D-PAGE. The analysis of the a-syn protein spot by peptide mass fingerprinting provided around 60% sequence coverage, which is sufficient to identify post-translational modifications. This method showed, for the first time, that a-syn is a target of methylglyoxal modification *in vivo*. In agreement with the a-syn glycation pattern observed *in vitro*, we found that a-syn from an adult rat was modified in the residues K12, K23, K32 and K58. Moreover, other lysine residues were found to be glycated and acetylated, and also T33 and/or S42 and/or T44 were found to be phosphorylated.

Interestingly, a-syn is ubiquitylated in residues K12, K21 and K23 in Lewy bodies (Anderson*, et al.*, 2006). Furthermore, mammalian cell lines also showed to ubiquitylate a-syn in residues K6, K10 and K12 (Nonaka*, et al.*, 2005). Taken togheter, our *in vitro* and *in vivo* results showed that all mentioned ubiquitylation sites are also probable targets for glycation. Thus, we hypothesize that a-syn glycation may influence the degradation of a-syn by the ubiquitin-proteasome pathway, leading to the accumulation and oligomerization of the protein. Furthermore, the accumulation of mis-ubiquitylated protein may impair the normal activity of the

proteasome. In agreement, a-syn was found to impair proteasome activity in different systems (Tanaka*, et al.*, 2001, Outeiro & Lindquist, 2003, Snyder*, et al.*, 2003, Chen*, et al.*, 2005, Nonaka & Hasegawa, 2009). Thus, it would be of great interest to evaluate the effects of a-syn glycation in the activity of the proteasome system. Also, it would be important to investigate a-syn post-translational modifications in transgenic mouse models of PD, at different stages of the disease. A comparative analysis of human *post-mortem* tissue from healthy and PD patients would be essencial to further validate these data.

The studies hereby reported provide important insight into the process of protein glycation, misfolding and the development of neurodegenerative diseases such as PD. It is now possible to raise several other important questions. The yeast model has proven to be extremely versatile and may be employed for addressing some of those questions. Thus, together with the method we developed for extracting and purifying a-syn we expect to contribute not only to the understanding of the effects of glycation in the aggregation process of a-syn, but also to the understanding of how post-translational modifications, in general, affect the biologyof a-syn. This knowledge will be essential for the development of improved therapeutic strategies to neutralize a-syn toxicity and also to establish new diagnostic methods for PD.

Appendix

Technique Development:
Kinetic Assay for Measurement of Intracellular Enzyme Concentrations in Situ

Vicente Miranda, H., Ferreira, A.E.N., Cordeiro, C., Ponces Freire, A. 2006, Analytical Biochemistry, Volume 354, Issue 1, 1 July 2006: 148-150.

Vicente Miranda, H., Ferreira, A.E.N., Quintas, A., Cordeiro, C., Ponces Freire,A., 2008, Biochemistry and Molecular Biology Education, Volume 36, Issue 2, 135-138.

1. Introduction

Metabolic pathway analysis requires accurate data regarding enzyme kinetic parameters and metabolite concentrations inside living cells. Intracellular enzyme concentration is a critical parameter that is notoriously difficult to obtain. We propose a kinetic method, based on enzyme addition, to quantify the amount of specific enzymes in metabolic pathways. The kinetic parameters known for the vast majority of enzymes were obtained *in vitro* with purified enzymes. More recently, with the emergence of sequenced genomes, the use of recombinant proteins as enzyme sources are now common practice, replacing the laborious purification from the original organism by the production of large amounts of protein from a suitable host such as *Escherichia coli*. However, this approach poses major problems regarding metabolic pathway analysis. Post-translational modifications are not likely to occur, and specific protein–protein interactions may be absent. Better strategies are clearly needed for investigating enzymes in a way more closely resembling the *in vivo* state. Recently, noninvasive techniques such as fluorescence spectroscopy and nuclear magnetic resonance (NMR) are assuming greater importance but still endure serious limitations (Neves*, et al.*, 1999, Martins*, et al.*, 2001). Fluorescence spectroscopy is restricted to a few metabolites, namely NAD(P)H, or to the use of specific probes that may be introduced in a nondestructive way in living cells. *In vivo* NMR shows great promise but is hampered by its limited time resolution and sensitivity. Moreover, intact systems do not allow good control of individual enzymes regarding substrate concentration and reaction conditions such as pH. An alternative approach is the study of enzymes *in situ* (Cordeiro & Freire, 1995). This invasive technique is based on the selective cell membrane permeabilization to low-molecular weight molecules, whereas all macromolecules, namely enzymes, remain within the cell boundaries at unchanged concentrations (Serrano*, et al.*, 1973). This method preserves most cellular structures, protein-protein interactions, and intracellular enzyme concentration. Membrane permeabilization to low-molecular weight molecules can be achieved in destructive conditions by using physical (freeze-thawing) or chemical (organic solvents or detergents) methods. Selective membrane permeabilization, made possible by the use of digitonin, relies on the distinct cholesterol (or other sterols) content of different cellular membranes. Permeabilization may be achieved in a sequential way in which all cellular membranes are successively permeabilized by digitonin titration, a method used for identifying intracellular enzyme location (Heise & Opperdoes, 1999). Cell permeabilization is still a destructive process,

and the extent of cellular damage may be difficult to evaluate. It is always necessary to use suitable marker enzymes for different cellular compartments to evaluate the extent of permeabilization, and studies in cell-free extracts should be used as controls. In most cases, except for membrane proteins or multi-subunit enzymes, the results obtained are similar to those obtained *in situ* (Fredrich, 1984). If differences do arise, they should be carefully investigated because they might reveal important regulatory aspects. *In situ*, all enzymes in a permeabilized cell compartment are active; therefore, interferences might occur. Interfering reactions may be eliminated by the use of suitable mutants, readily available for *Saccharomyces cerevisiae* (Euroscarf collection) or inhibitors. However, *in situ* enzymology works at its best when combined with time course analysis of metabolic pathways, exploiting ‘‘interfering reactions’’ to gain kinetic information. Once a detailed kinetic model is defined and different metabolite concentration time courses are obtained, it is possible to fit the model parameters using genetic algorithms. Time course analysis was performed in a cell-free extract of *Leishmania infantum* to obtain all kinetic parameters of glyoxalase I and glyoxalase II (Sousa Silva*, et al.*, 2005). Ultimately, these methods are used to obtain detailed information on the metabolic network topology, an emerging field in systems biology (Voit, 2002).

To measure intracellular protein concentration *in situ*, we propose a kinetic method based on enzyme addition.

2. Method Basis

This method can be used for enzymes with a rate equation in which there is a direct proportionality between initial rate and total enzyme concentration. This property can be expressed by Eq. (1):

$$v = k_{cat} \cdot E_0 \cdot f(\mathrm{A}) \qquad (1)$$

In this equation, f is a known function of the substrate concentration A and does not depend on the enzyme concentration. For example, for Michaelis–Menten enzymes, $f = \mathrm{A}/(K_m + \mathrm{A})$, which will be known if the Michaelis constant is determined.

To measure an enzyme concentration *in situ*, the only requirement is the addition of known amounts of the same enzyme in a purified form and to study the variation of the initial rate

at a constant substrate concentration. It should be noted that the catalytic constant (k_{cat}) of the added enzyme must be the same as the catalytic constant of the intracellular enzyme. Under these assumptions, at a constant substrate concentration, the dependence of the initial rate v on the amount of added enzyme E_{add} follows the straight-line Eq. (2):

$$v = k_{cat} \cdot E_{in} \cdot f_{in}(\mathrm{A}) + k_{cat} \cdot E_{add} \cdot f_{add}(\mathrm{A}) \qquad (2)$$

In this equation, k_{cat} is the catalytic constant, E_{in} is the assayed enzyme concentration, and f_{in} and f_{add} are functions of substrate concentration only. The concentration of the assayed enzyme is related to the x intercept of the straight-line according to Eq. (3):

$$E_{in} = -\text{x intercept} \cdot \frac{f_{add}(\mathrm{A})}{f_{in}(\mathrm{A})} \qquad (3)$$

In general, the f functions of the assayed and the added enzymes do not need to be the same. They only need to be known *a priori* from the kinetic characterization of the two enzymes. In particular, if these functions are equal (i.e., if the added enzyme has the same kinetic properties as the assayed enzyme), then Eq. (3) reduces to Eq. (4) and the concentration of the assayed enzyme is simply the absolute value of the x intercept:

$$E_{in} = -\text{x intercept} \qquad (4)$$

This method is illustrated by measuring glyoxalase I (*S*-D-lactoylglutathione:methylglyoxal lyase, EC 4.4.1.5) and glucose-6-phosphate 1-dehydrogenase (D-glucose-6-phosphate:NADP+ 1-oxidoreductase, EC 1.1.1.49) concentration *in situ*.

3. Experimental Procedures

S. cerevisiae cells (BY4741, MATα, *his3*Δ1, *leu2*Δ0, *met*15Δ0, and *ura*3Δ0) were grown in yeast–peptone–dextrose (YPD) rich medium (D-glucose [2%, w/v], yeast extract [0.5%, w/v], and peptone [1%, w/v]) and harvested by centrifugation (5200g at 4 °C in an Eppendorf 5804R) at the end of the exponential phase. Cells were permeabilized with digitonin (0.01%, w/v) for 15 min at 30 °C in 0.1 M MES-NaOH (pH 6.5) buffer (Cordeiro & Freire, 1995). Total protein concentration

was determined with the modified Bradford assay (Cordeiro & Freire, 1994). Total yeast protein extraction was performed by glass bead lysis as described previously (Ausubel*, et al.*, 1990). Enzyme activities were measured in a Beckman DU7400 diode-array spectrophotometer, with stirring and temperature control in the cuvette, in a reaction volume of 1.5 ml. Glyoxalase I activity was measured by following *S*-D-lactoylglutathione formation at 240 nm in 0.1 M MES-NaOH (pH 6.5) buffer in the presence of 6 mM glutathione (BoehringerMannheim) and 6.5 mM methylglyoxal. Methylglyoxal was prepared by acid hydrolysis of 1,1-dimethoxypropanone (Fluka) followed by fractional distillation at reduced pressure in a nitrogen atmosphere. D-Glucose-6-phosphate 1-dehydrogenase activity was measured by following NADPH formation at 340 nm in 0.05 M Hepes–HCl (pH 7.5) in the presence of D-glucose-6-phosphate and $NADP^+$. Apparent kinetic parameters were determined by varying $NADP^+$ concentration at fixed D-glucose-6-phosphate concentrations. $NADP^+$ concentration was varied in the range of 0.1 to 1 mM, and D-glucose-6-phosphate concentration was changed between 0.1 and 6 mM. For the enzyme concentration assay, activity was measured in the presence of 0.5 mM NADP+ and 2 mM D-glucose-6-phosphate. Kinetic parameters were determined by time course analysis (Martins*, et al.*, 2001, Sousa Silva*, et al.*, 2005). The optimization was performed using the differential evolution algorithm (Storn & Price, 1997) implemented in the software library AGEDO (Abecasis*, et al.*, 2004). Taking a suitable amount of permeabilized yeast cell suspension and identical protein amount in homogenate, glyoxalase I, and D-glucose-6-phosphate 1-dehydrogenase activities were measured at a fixed substrate concentration. Then purified *S. cerevisiae* glyoxalase I (Sigma) or purified *S. cerevisiae* D-glucose-6-phosphate 1-dehydrogenase (Sigma) was added in a known amount to each assay.

4. Results and Discussion

As expected, according to Eq. (2), a linear relationship was obtained when plotting initial rate versus amount of added enzyme (figure A.1). These relationships are observed for both enzymes, and the lines are parallel in all cases regarding purified enzyme, *in situ* assay, and homogenate. The amount of glyoxalase I or glucose-6-phosphate 1-dehydrogenase *in situ* is given by the x intercept according to Eq. (4), that is, 0.04 ± 0.006 and 0.012 ± 0.0016 μg, respectively, in the given amount of cells (figure A.1 and table A.1). Glyoxalase I represents only 0.53% of the total protein amount, whereas D-glucose-6-phosphate 1-dehydrogenase represents 0.12%.

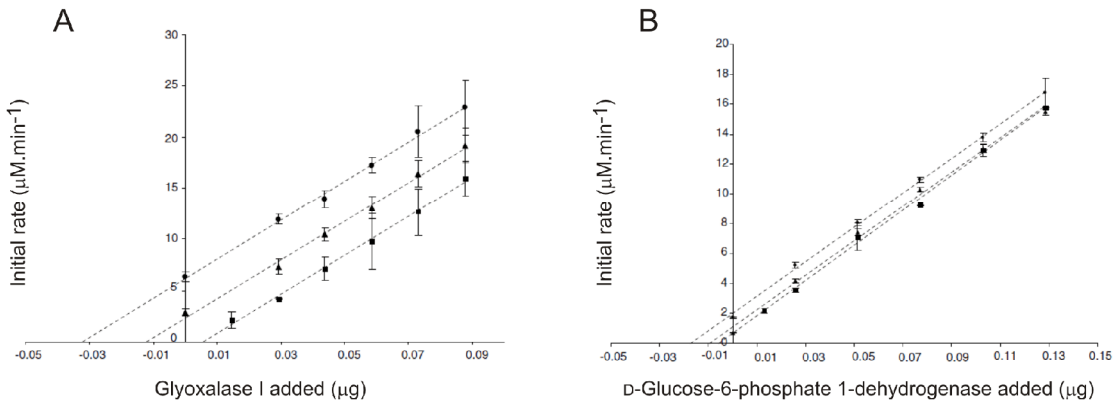

Figure A.1 Measurement of enzyme amount *in situ* in *Saccharomyces cerevisiae*. (A) Glyoxalase I activity was measured in permeabilized cells (6.46 x 10^6 cells containing 7.56 μg total protein, (●), homogenate 7.56 μg total protein, (▲), and commercial enzyme (■). The assay contained 6 mM glutathione and 6.5 mM methylglyoxal in 0.1M MES–NaOH (pH 6.5) in a 1.5-ml volume. (B) D-Glucose-6-phosphate 1-dehydrogenase activity was measured in permeabilized cells (9.82 x 10^6 cells containing 9.95 μg total protein, (●), homogenate 9.95 μg total protein, (▲), and commercial enzyme (■). The assay contained 2 mM D-glucose-6-phosphate and 0.5 mM of $NADP^+$ in 0.05 M Hepes–HCl (pH 7.5). Formation of NADPH was monitored at 340 nm. In all cases, initial rate varies linearly with added enzyme amount for both enzymes. Moreover, the lines are parallel for each enzyme regardless of whether the enzyme is in situ, homogenate, or purified. Data are from three experiments with standard deviations shown.

Table A.1 Determination of enzyme concentration, catalytic constant, and specificity constant for Saccharomyces cerevisiae glyoxalase I and D-glucose-6-phosphate 1-dehydrogenase *in situ*

Enzyme	Glyoxalase I	D-Glucose-6-phosphate 1-dehydrogenase
Number of Cells (10^6)	6.46	9.82
Total protein (μg)	7.56	9.95
Enzyme assay (μg)	0.04 ± 0.006	0.012 ± 0.0016
Molecular weight (Da)	37 209	57 521
Enzyme concentration (10^{-20} mol $cell^{-1}$)	16.7	2.08
Molecules per cell	100 500	12 500
Michaelis constants (mM)	MGO: 3.56[a] GSH: 1.64[a]	G6P: 0.22 $NADP^+$: 0.34
Limiting rate (μM min^{-1})	23.3	2.68
k_{cat} (10^4 min^{-1})	3.24	1.97
k_s (10^7 M^{-1} min^{-1})	MGO: 0.91 GSH: 1.98	G6P: 8.99 $NADP^+$: 5.80

Note. MGO, methylglyoxal; GSH, glutathione; G6P, D-glucose-6-phosphate; NADP+, Nicotinamide adenine dinucleotide phosphate (oxidized)

[a] Value from Gomes and colleagues (Gomes, *et al.*, 2005).

The accurate measurement of enzyme concentration is usually achieved with pure enzymes only and is essential in enzymology if reliable k_{cat} or k_s measurements are to be achieved. There are, however, many other situations where an estimate of enzyme amount, as provided by the proposed method, is useful. Protein expression changes can easily be monitored in this way. Alternative methods are based on immunoblotting techniques (for which specific antibodies must be produced) or quantitative proteomics, relying on the use of high-resolution mass spectrometers and requiring synthetic peptides.

5. Acknowledgements

Work supported by grant SFRH/BD/23035/2005 (H.V.M) from the Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Tecnologia, Portugal.

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