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Doutoramento em Bioquímica

(Especialidade: Regulação Bioquímica)

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Tese orientada pelo Professor Doutor Carlos Alberto Alves Cordeiro

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

Gomes RA, Sousa Silva M, Quintas A, Cordeiro C, Freire A, Pereira P, Martins A, Monteiro E, Barroso E, Ponces Freire A. 2005. Argpyrimidine, a methylglyoxal-derived advanced glycation end-product in familial amyloidotic polyneuropathy. *Biochem J.* **385**: 339-345.

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.

Gomes RA, Vicente Miranda H, Sousa Silva M, Graça G, Coelho AV, Ferreira AEN, Cordeiro C, Ponces Freire A. 2007. Protein glycation and methylglyoxal metabolism in yeast: Finding peptide needles in protein haystacks. *FEMS Yeast Res. In press.*

Gomes RA, Oliveira L, Silva M, Sousa Silva M, Costa C, Coelho AV, Ascenço C, Quintas A, Cordeiro C, Ponces Freire A. 2007. *In vivo* protein glycation: Structural and functional effects on yeast enolase. *Submitted to FEBS lett.*

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

Proteins are the core of life, providing functional support for all chemical processes in living cells. To perform this task, proteins must have a specific structure achieved by folding from its linear primary level to a three dimensional architecture. Disturbing this process will unavoidably leads to serious metabolic and physiological changes. Throughout evolution, molecular chaperones and complex quality control mechanisms evolved to keep protein structures functional in their lifespan. When something goes awry, misfolded proteins may aggregate, being involved in conformational diseases where Alzheimer's, Parkinson's, Andrade's syndromes and prion disease are epitomes. A legion of factors may contribute to protein misfolding, including point mutations, chemical stress, post-translational modifications and abnormal quality control mechanisms.

Protein glycation is a non-enzymatic post-translational modification where arginine and lysine side chains are irreversibly modified by carbonyl-containing molecules. Contrary to controlled post-translational modifications, like phosphorylation or glycosylation, where enzymes specifically modify proteins to produce a certain cellular effect, glycation is a chemical process and any protein or other biomolecules with free amino groups are potential targets of the Maillard reaction. Moreover, there are several carbonyl-containing molecules *in vivo*, like glucose and methylglyoxal that have the ability to irreversibly modify proteins through this process. Thus, it is expected that the extensive non-enzymatic unregulated modification of particular proteins might have a deleterious effect on protein structure and function and be associated with cell and tissue damage observed in some pathologies and aging. In fact, the observation that AGE-modified proteins accumulate in several clinical conditions links protein glycation to a sizeable amount of human diseases such as diabetes *mellitus*, age-related disorders, atherosclerosis and amyloid diseases. Due to the increase of older populations associated with the appearance of new diseases, there is a growing interest in this post-translational modification and in the development of therapies to inhibit the Maillard reaction. However, despite being extensively studied, the role of protein glycation in the development of pathological conditions and the mechanisms involved in the formation of

this post-translational modification *in vivo* are still unknown. This is likely to be related to our limited knowledge concerning the formation and biochemical effects of AGE-modified proteins *in vivo*. The elucidation of these processes could be achieved using cellular models of protein glycation allowing the study of the metabolic conditions that lead to an increase of AGE formation and its biochemical effects on protein targets and cell physiology.

The major goals of the investigations presented in this thesis focused on understanding the biochemical effects of protein glycation *in vivo* by methylglyoxal, which is considered the most relevant glycation agent in biological systems, using *Saccharomyces cerevisiae* as a cellular model. The second aim was to investigate the relationship between protein glycation and amyloid fibril formation *in vivo*. Detailed molecular and kinetic models of protein amyloidogenesis *in vivo* that accurately describe the role of glycation in this process will contribute for the design of novel therapeutic strategies for these widespread human diseases.

In Chapter I, a detailed overview of the relevant literature introduces and describes the theme. Following a brief description about the origins of the Maillard reaction, the chemical modification of proteins by carbonyl-containing compounds (protein glycation) is presented. A review of methylglyoxal metabolism in living cells including the biochemical effects of this main glycation agent *in vivo*, especially as a protein modifier through the Maillard reaction, was made. Finally, the implications of protein glycation in several human pathologies are described together with the effects of this post-translational modification in protein structure and function.

In Chapter II, protein glycation by methylglyoxal was investigated *in vivo* using *Saccharomyces cerevisiae*. In this study, we provided the first evidence that this non-enzymatic post-translational modification also affects short-lived organisms like yeast. Importantly, different glycation phenotypes were identified, which depend directly on the intracellular methylglyoxal concentration. Furthermore, the results show that, although protein glycation is a non-enzymatic process, preferential protein targets exist. This work validated *Saccharomyces cerevisiae* as a eukaryotic cell model for understanding protein glycation *in vivo*.

In Chapter III, the specific protein glycation targets were identified and the chemical nature and molecular location of MAGE on these proteins were assigned. A novel method, based on the hidden information of peptide mass fingerprint, was developed. Three glycolytic enzymes and heat shock proteins were found to be glycated *in vivo*. In addition, a cellular response to protein glycation appears to exist: the activation of the molecular chaperone pathway involving Hsp26 that reduces the detrimental effects of protein misfolding and aggregation. Enolase2, the main yeast protein target, endures a glycation-dependent activity loss due to the modification of a critical arginine residue essential for dimer stability and hence activity.

Chapter IV describes the effects of glycation *in vivo* by methylglyoxal on the structure, thermal stability and enzyme activity of yeast enolase. Our results demonstrate that glycation causes enolase unfolding and structural changes, leading to loss of biological function. Furthermore, we provided direct evidences for the existence of substantial differences between *in vivo* and *in vitro* glycation. These results raise serious doubts on the relevance of the huge amount of research work that employed *in vitro* glycated proteins to understand the glycation effects on protein structure and function.

The results described in the next two chapters (Chapter V and Chapter VI) establish a bridge between protein glycation *in vivo* and amyloid disorders. In chapter V, the analysis of methylglyoxal-derived protein glycation in transthyretin-amyloid deposits extracted from Portuguese-type FAP patients is reported. This study unequivocally revealed the presence of the MAGE argpyrimidine, suggesting that protein glycation by methylglyoxal is involved in this neurodegenerative amyloid disorder. Taking advantage of the established cellular model of protein glycation and the fact that yeast has been used to study protein aggregation in the context of prion and Parkinson's disease, different amyloidogenic human TTR variants were expressed in yeast and TTR amyloid fibril formation under glycation conditions was investigated. This work, described in chapter VI, gives the first experimental evidence that protein glycation promotes the formation of transthyretin amyloid fibrils *in vivo*.

The concluding remarks in chapter VII provide an integrative framework of the findings presented in this thesis. The relevance of this work and perspectives for further studies are also highlighted in this chapter.

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TABLE OF CONTENTS

SUMMARY

Protein glycation, the non-enzymatic and irreversible modification of amino groups by carbonyl compounds, is assuming an important role in the context of a wide range of human pathologies, including diabetes *mellitus*, age-related disorders and neurodegenerative diseases of amyloid type. Hence, there is a growing interest in this post-translational modification and, ultimately, the quest for inhibitors of protein glycation. However, despite extensive research, mainly by *in vitro* glycation studies with relevant or model proteins, the role of glycation in pathological conditions is still unknown. Therefore, cellular research models are required to investigate protein glycation *in vivo*, the resulting biochemical effects and its role in human diseases.

In the work presented in this thesis, a novel approach was developed to investigate protein glycation *in vivo* by methylglyoxal and its biochemical effects. Moreover, the role of glycation by methylglyoxal in protein amyloidogenesis was also investigated. It was found that protein glycation occurs in yeast and defined glycation phenotypes were identified. Although protein glycation was primarily associated with complex organisms and long-lived proteins, this post-translational modification also affects short-lived organisms like yeast. A direct relationship between methylglyoxal formation rate and protein glycation was observed. A kinetic model of methylglyoxal metabolism was developed to investigate the relative importance of the glyoxalase pathway, aldose reductase and methylglyoxal formation rate on the methylglyoxal steady-state concentration and their relationship with protein glycation *in vivo*. It was found that the glyoxalase system and aldose reductase enzyme are equally important as key anti-glycation defenses in yeast. A higher methylglyoxal input leads to a direct increase in methylglyoxal concentration even in the presence of the glyoxalase system and aldose reductase. In fact, challenging non-growing yeast cells with a high D-glucose medium, consequently increasing methylglyoxal formation rate, causes methylglyoxal-derived protein glycation even in the reference strain with all enzymatic defenses against methylglyoxal. Therefore, there is a subtle balance between methylglyoxal metabolism and the accumulation of MAGE-modified proteins, in which cells can prevent MAGE formation only until anti-glycation defenses are overcome.

Interestingly, glycation in yeast appears to be a targeted process, whereby only a few proteins are modified. Protein identification, MAGE assignment and location were determined by mass spectrometry. The heat shock proteins Hsp71/72 and Hsp26 were found to be glycated *in vivo*. Hsp26, a critical element in the unfolding stress response, was only detected in the soluble form upon glycation. This finding shows that Hsp26 is most likely activated in glycation conditions, similarly to what happens in thermal stress. Three glycolytic enzymes were also glycated in yeast, namely phosphoglycerate mutase, aldolase and enolase, being the latter the main glycation target. This discovery prompted a deeper study of the biochemical implications of enolase glycation *in vivo*. Despite the observed glycation-dependent activity loss of enolase, glycolysis and cell viability remained unchanged, hinting that yeast cells evolved to cope with high glycation levels. To investigate the effects of glycation on enolase structure and enzymatic activity, the protein was purified from yeast cells under native and *in vivo* glycation conditions. For the first time, a protein was studied while enduring glycation in physiological conditions, allowing a direct comparison between *in vivo* and *in vitro* protein glycation. Significant differences were found between these distinct experimental conditions. *In vivo* glycation appears to be a specific process with only a few amino acid residues consistently modified with the same MAGE. Structural changes, evaluated by circular dichroism spectroscopy and thermal denaturation, showed that glycation mainly decreases α-helical content and increase unordered structure while enolase structural rigidity increases. *In vitro*, a greater molecular heterogeneity was observed with different MAGE occurring at the same molecular locations. α-Helical content also decreased, but *in vitro* glycation markedly increases β-sheet content and structural rigidity was further enhanced. It was also observed that glycation causes enolase unfolding and dimer dissociation.

Based on the identification of MAGE location by mass spectrometry, a molecular model for enolase inactivation upon glycation was developed. Glycation occurs at R414, a critical residue for dimer stability. Modification of R414 disrupts electrostatic interactions with E20 on the other enolase chain that stabilize the enolase dimer, leading to its dissociation and consequent formation of inactive monomers.

The molecular location of MAGE in enolase suggests that the tri-dimensional structure may directly influence glycation reaction. The modified-arginine residues were

mainly found in an arginine-rich crevice located at the dimer interface, but solvent accessible. This arginine-rich cave could create a favourable environment for methylglyoxal-derived glycation reactions, sequestering free methylglyoxal that evaded from its catabolic routes. Hence, the high enolase reactivity towards methylglyoxal-induced protein glycation could scavenge methylglyoxal, preventing changes in the biochemical functionalities of other proteins. Upon glycation, enolase unfolds but cells activate the refolding chaperone pathway to counteract enolase misfolding and to limit the harmful effects associated with extensive protein misfolding and aggregation.

Yeast cells emerged as a living test tube to investigate the biochemical effects of glycation on protein structure and function *in vivo*. So, taking advantage of this finding, the link between protein glycation and amyloid disorders, was investigated. Using an improved procedure for the extraction of amyloid fibrils from FAP patients, we provided the first unequivocal evidence that methylglyoxal-derived advanced glycation end-products are present in transthyretin amyloid deposits of FAP patients. Thus, we studied the effect of protein glycation in transthyretin amyloid fibril formation in yeast. For this purpose, TTR variants with different amyloidogenic potentials (TTR-wt, TTR-L55P and TTRd-D) were expressed in *Saccharomyces cerevisiae* and amyloid deposits were detected by fluorescence microscopy. It was observed that TTR is glycated when yeast cells are exposed to glycation conditions. Furthermore, the formation of transthyretin-amyloid aggregates in cells expressing the amyloidogenic TTR-L55P variant is induced. These results provide the first direct evidence that glycation causes protein aggregation *in vivo* in the context of human amyloid disorders.

The presented results and conclusions are of great value not only to increase our knowledge about protein glycation and its biochemical effects *in vivo*, but also to assign a clear role of this non-enzymatic process in the development of amyloid disorders. In this context, yeast cells will certainly be useful as a eukaryotic model to study these processes at a cellular level. This is of vital importance in the design of novel or improved therapeutic strategies to inhibit protein glycation and counteract its harmful effects.

Keywords: Protein glycation, methylglyoxal, misfolding diseases, anti-glycation defenses, *Saccharomyces cerevisiae*

RESUMO

A glicação de proteínas é uma modificação pos-traducional, tendo como consequência a modificação irreversível de grupos amina por compostos contendo grupos carbonilo. Esta modificação tem sido implicada em diversas patologias humanas, tais como diabetes *mellitus*, doenças relacionadas com o envelhecimento e doenças neurodegenerativas do tipo amilóide. Neste contexto, a glicação de proteínas tem sido alvo de interesse na comunidade científica, com o objectivo final de desenvolver estratégias para inibir esta modificação pos-traducional. No entanto, apesar de intensivamente investigada *in vitro* com proteínas modelo ou clinicamente relevantes, o papel da glicação de proteínas no desenvolvimento de diversas condições patológicas não é ainda conhecido. Assim, é de extrema importância desenvolver modelos celulares para investigar a glicação de proteínas *in vivo*, os seus efeitos bioquímicos e finalmente a sua importância nas diversas doenças humanas em que está envolvida.

Neste trabalho, foi desenvolvida uma nova abordagem para investigar a glicação *in vivo* pelo metilglioxal e os seus efeitos bioquímicos. Para além disso, foi também investigado o papel da glicação de proteínas pelo metilglioxal na formação de fibras amilóides derivadas de uma proteína amiloidogénica. Observou-se a acumulação de proteínas glicadas pelo metilglioxal na levedura *Saccharomyces cerevisiae*, com diferentes fenótipos de glicação. Apesar da glicação de proteínas ter sido associada a organismos complexos e proteínas com baixo *turnover*, esta modificação pos-traducional também afecta microrganismos como a levedura. Foi observado que existe uma relação directa entre a velocidade de formação de metilglioxal e a ocorrência de glicação. Um modelo cinético do metabolismo do metilglioxal em *S. cerevisiae* foi construído para investigar a importância da via dos glioxalases, do aldose reductase e da velocidade de formação de metilglioxal na concentração em estado estacionário deste α-oxoaldeído. Com esta análise, verificou-se que o sistema dos glioxalases e o aldose reductase são igualmente importantes como defesas anti-glicação pelo metilglioxal. Observou-se também que existe uma relação directa entre a velocidade de formação de metilglioxal e a sua concentração em estado estacionário, mesmo na presença do sistema dos glioxalases

e do aldose reductase. De facto, um aumento da formação de metilglioxal, através da exposição de células a uma concentração elevada de D-glucose, provoca a glicação de proteínas pelo metilglioxal na estirpe de referência que apresenta todas as defesas enzimáticas contra o metilglioxal. Em condições fisiológicas, as células previnem a acumulação de proteínas modificadas pelo metilglioxal, mas só até as suas defesas serem ultrapassadas.

Na levedura *S. cerevisiae*, a glicação é um processo específico, onde apenas algumas proteínas são modificadas. Estas proteínas foram identificadas por espectrometria de massa, assim como a natureza e localização dos produtos avançados de glicação derivados do metilglioxal (MAGE, *Methylglyoxal Advanced Glycation End-products*). As proteínas de choque térmico Hsp71/72 e Hsp26 foram identificados e apresentam modificações pos-traducionais derivadas da glicação pelo metilglioxal. A Hsp26, um elemento crítico na resposta celular a condições de stress de *unfolding*, foi apenas detectada na forma solúvel após glicação. Esta observação indicia que a Hsp26 é activada em condições de glicação, tal como se verifica em condições de stress térmico. Para além destas proteínas, três enzimas glicolíticos estão também glicados *in vivo*: o fosfoglicerato mutase, o aldose e o enolase, sendo este último o principal alvo de glicação. Um estudo detalhado das implicações bioquímicas da glicação *in vivo* do enolase foi realizado. Apesar de ter sido detectada uma diminuição da actividade deste enzima devido à glicação, a via glicolítica e a viabilidade celular permaneceram inalteradas, sugerindo que a levedura evoluiu de forma suportar elevados níveis de glicação. Para averiguar o efeito da glicação na estrutura e actividade enzimática do enolase, esta proteína foi purificada em condições nativas e em condições de glicação. Pela primeira vez, os efeitos da glicação *in vivo* na estrutura e função de uma proteína, foram investigados. Para além disso, este estudo permitiu efectuar uma comparação directa entre a glicação de proteínas *in vivo* e *in vitro*, tendo sido encontradas diferenças significativas. *In vivo*, a glicação aparenta ser um processo específico, em que apenas alguns resíduos de aminoácidos são consistentemente modificados pelo mesmo MAGE. Nestas condições, a glicação induz alterações estruturais, observadas por dicroismo circular, com uma diminuição do conteúdo em hélice α e um aumento da estrutura desordenada e da rigidez estrutural. *In vitro*, foi observado uma elevada heterogeneidade

molecular, com o mesmo resíduo de aminoácido modificado por diferentes MAGE em diferentes moléculas de proteína. Foi também detectada uma diminuição do conteúdo em hélice α, mas a glicação *in vitro* induz um aumento significativo de folha β. Em ambas as condições experimentais, a glicação causa a desnaturação do enolase, com a consequente dissociação do dímero, a forma activa do enzima.

Com base na identificação da localização molecular dos MAGE por espectrometria de massa, foi proposto um modelo para a inactivação do enolase pela glicação. Esta modificação pos-traducional ocorre em R414, essencial para a estabilidade da forma dimérica. A formação de uma hidroimidazolona neste resíduo de arginina destrói as interacções electrostáticas com E20 da outra cadeia que estabilizam o dímero, levando à sua dissociação e consequente formação de monómeros inactivos.

A localização molecular dos MAGE no enolase sugere que a estrutura tridimensional da proteína influencia as reacções de glicação. As modificações ocorrem maioritariamente numa cavidade rica em resíduos de arginina localizada na interface do dímero. Este local pode criar um ambiente favorável a reacções de glicação pelo metilglioxal, eliminando assim o metilglioxal não catabolisado pelos sistemas enzimáticos. Assim, a elevada reactividade do enolase para reacções de glicação derivadas do metilglioxal pode diminuir a concentração intracelular deste α-oxoaldeído, prevenindo assim a alteração da função de outras proteínas celulares. Após glicação, o enolase sofre alterações estruturais resultando na desnaturação da proteína e a célula activa a via de *refolding* para neutralizar os efeitos nocivos associados a processos de *misfolding* proteico e agregação.

Os estudos apresentados nesta tese revelaram que a levedura *S. cerevisiae* constitui um excelente modelo para investigar *in vivo* os efeitos bioquímicos da glicação na estrutura e função de proteínas. Assim, o papel da glicação de proteínas em doenças amilóides foi estudado na levedura *S. cerevisiae* como modelo celular. Utilizando um método aperfeiçoado de extracção de fibras amilóides de pacientes com polineuropatia amiloidótica familiar (FAP, *Familial Amyloidotic Polyneuropathy*), foi detectada inequivocamente a presença da argipirimidina (um produto avançado de glicação derivado do metilglioxal) nos depósitos amilóides de transtirretina (TTR). Esta observação indica que a glicação de proteínas deverá estar envolvida nesta doença

amilóide. Para investigar o papel da glicação na transtirretina e consequente formação de fibras amilóides *in vivo*, variantes da TTR com diferentes potenciais amilóidogénicos (TTR-wt, TTR-L55P and TTRd-D) foram expressos em *S. cerevisiae* e os depósitos amilóides foram detectados por microscopia de fluorescência. Foi observada glicação *in vivo* da TTR quando as células foram expostas a condições favoráveis à ocorrência deste processo. Nestas condições experimentais, foi observada uma indução da formação de depósitos amilóides derivados da variante amiloidogénica TTR-L55P. Esta observação constitui a primeira evidência experimental de que a glicação de proteínas induz a agregação e formação de fibras amilóides *in vivo*.

No seu conjunto, os resultados apresentados nesta tese e as conclusões inerentes são bastante importantes não apenas para compreender os mecanismos envolvidos na glicação de proteínas e consequentes efeitos bioquímicos *in vivo*, mas também para clarificar o papel deste processo não enzimático no desenvolvimento de diversas patologias humanas, como as doenças do tipo amilóide. Neste contexto, a levedura *Saccharomyces cerevisiae* constitui um excelente modelo para investigar estes processos a nível celular. Estes estudos são de vital importância para desenvolver novas abordagens terapêuticas para inibir a glicação de proteínas e minimizar os seus efeitos prejudiciais.

Palavras-chave: Glicação de proteínas, metilglioxal, doenças conformacionais, defesas anti-glicação, *Saccharomyces cerevisiae*

ABBREVIATIONS

ADP	Adenosine diphosphate
AGE	Advanced glycation end-products
ALS	Amyotrophic lateral sclerosis
AP-1	Activator protein-1
apoB	Apolipoprotein-B
BSA	Bovine serum albumin
CD	Circular dichroism
CEL	N^{ϵ}-(carboxyethyl)lysine
CML	N^{ϵ}-(carboxymethyl)lysine
CoA	Coenzyme A
C-terminal	Carboxyl-terminal
D	Aspartatic acid
DAPI	4',6-Diamidino-2-phenylindole
DHAP	Dihydroxyacetone phosphate
DHB	2,5-Dihydroxybenzoic acid
DNA	Deoxyribonucleic acid
DRA	Dyalisis-related amyloidosis
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
E	Glutamatic acid
EDTA	Ethylenediamine tetraacetic acid
ENO2	Yeast enolase2 gene
ESI-FTMS	Electrospray ionization - Fourier transform mass spectrometry
FAP	Familial amyloidotic polyneurophaty
G	Glycine
GAP	D-Glyceraldehyde 3-phosphate
GAPDH	D-Glyceraldehyde 3-phosphate dehydrogenase
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
GPX	Glutathione peroxidase

GRE3	Yeast aldose reductase gene
GSH	Glutathione (L-γ-glutamyl-L-cysteinylglycine)
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
ICAM-1	Intercellular adhesion molecule 1
IGF-I	Insulin growth factor-1
IL-1	Interleukin-1
IL-6	Interleukin-6
K	Lysine
*k*cat	Catalytic constant
*K*m	Michaelis constant
L	Leucine
LB	Luria Bertani
LC-MS	Liquid cromathography-mass spectrometry
LDL	Low-density lipoprotein
M	Methionine
MAGE	Methylglyoxal advanced glycation end-products
MALDI-FTMS	Matrix assisted laser desorption ionization - Fourier transform mass spectrometry
MALDI-TOF	Matrix assisted laser desorption ionization - time of flight
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

N	Asparagine
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
NFκB	Nuclear factor-κB
NMR	Nuclear magnetic ressonance
P	Proline
PACE	Paired basic amino acid cleaving enzymes
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PEP	Phosphoenolpyruvate
PepMix1	Peptide mixture for mass spectrometers calibration
PLAS	Power law analysis and simulation software
PMF	Peptide mass fingerprint
PMSF	Phenylmethylsulfonyl fluoride
ProMix3	Protein mixture for mass spectrometers calibration
PVDF	Polyvinylidene difluoride
Q	Glutamine
R	Arginine
RAGE	Receptor for advanced glycation end-products
RBP	Retinol-binding Protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations *per* minute
S	Serine
S-buffer	Sorbitol-containing buffer
SDLGSH	*S*-D-Lactoylglutathione
SDS	Sodium dodecyl sulphate
SOD-1	Superoxide dismutase-1
SSA	Senile Systemic Amyloidosis
SSAO	Semicarbazide-sensitive amine oxidase
T	Threonine

TAE	Tris/acetate/EDTA buffer
TBS	Tris-buffer saline
TFA	Trifluoroacetic acid
THP	Tetrahydropyrimidine
TIM	Triosephosphate isomerase
TNF-α	Tumor necrosis factor-α
Tris	Trishydroxymethylaminomethane
TTR	Transthyretin
TTRd-D	Transthretin variant with the β-strand D delected
TTR-L55P	Transthyretin variant with a substitution of a leucine per proline in position 55
TTR-V30M	Transthyretin variant with a substitution of a valine residue per methionine residue in position 30
Tween-20	Polysorbate 20 sorbitan monolaurate
Ura	Uracil
UV	Ultra violet
UV-vis	Ultra violet - visible
V	Valine
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
Wt	Wild-type
Yap1	Yeast transcription factor of the AP-1 family
YNB	Yeast nitrogen base growth medium
YPGlu	Yeast extract, peptone and D-glucose growth medium
α-CHCA	α-Cyano-4-hydroxicinamic acid
β_2M	β_2-microglubulin
% v/v	Percentage expressed in volume/volume
% w/v	Percentage expressed in weight/volume

CHAPTER I

INTRODUCTION

1. THE MAILLARD REACTION

The Maillard reaction is one of the most ubiquitous chemical reactions in food chemistry and biological systems. The expression *Reaction du Maillard* was introduced by the French chemist Louis Camille Maillard who studied the reactions between sugars and amino acids or other amine-containing compounds (Maillard, 1912). As a result of the reaction, a brown coloration was observed and therefore the Maillard reaction is also referred as the *browning reaction*. The association of the reaction with the attractive colours and flavours of cooked food has its origins at least half a million years ago when Man first employed fire to cook. During the Second World War, the preservation of military food rations focused the minds of food scientists on the Maillard reaction and showed that the reaction could also be detrimental to food quality. The research on the Maillard reaction in biological systems began with the recognition, in the 1970s, that this reaction also occurs *in vivo* in diabetic patients and healthy subjects (Bunn *et al.*, 1975). Presently, the Maillard reaction comprises a broad range of non-enzymatic reactions between carbonyl-containing compounds and amino groups leading to the formation of irreversible modifications, known as advanced glycation end-products (AGE). This term was introduced by Brownlee and co-workers (Brownlee *et al.*, 1984) to describe the brown, fluorescent and cross-link structures produced in the latter stages of the reaction between sugars and proteins *in vivo*, although some AGE have no colour or fluorescence and neither are cross-link structures. Biomolecules with free amino groups like proteins, nucleotides and basic phospholipids can be irreversibly modified by this non-enzymatic reaction.

2. PROTEIN GLYCATION

Protein glycation, the result of the Maillard reaction in proteins, is a post-translational modification whereby lysine and arginine side chains are irreversibly modified by carbonyl compounds producing AGE in a non-enzymatic process (Lo *et al.*, 1994; Westwood & Thornalley, 1995; Westwood & Thornalley, 1997). N-terminal free

amino groups and thiol groups of cysteine residues are also potential targets, although with cysteine, reversible and unstable adducts are usually formed (Lo *et al.*, 1994; Westwood & Thornalley, 1997). Moreover, the N-terminal is usually modified, leaving arginine and lysine side chains as the main glycation targets in proteins (Driessen *et al.*, 1985).

The first step in protein glycation involves the nucleophilic attack by the nitrogen atom of the protein amino group to the electrophilic carbonyl group of an aldehyde or ketone. After the elimination of a water molecule, a Schiff's base is generated, which undergoes a spontaneous rearrangement, the Amadori rearrangement, to form the Amadori product, a ketoamine (Hodge, 1955; Koenig *et al.*, 1977; Westwood & Thornalley, 1997). The reaction of glucose with lysine residues leads to specific Amadori products termed fructosamines (Njoroge & Monnier, 1989; Westwood & Thornalley, 1997) (Figure I.1). Although the Amadori product is more stable than the Schiff's base, it undergoes a complex series of chemical reactions, such as intramolecular rearrangements, oxidative and non-oxidative fragmentation and dehydratation reactions to yield an irreversible bound adduct, the AGE (Bucala & Cerami, 1992; Vlassara *et al.*, 1994; Westwood & Thornalley, 1997) (Figure I.1). In older scientific literature, protein glycation refers only to lysine modifications by glucose with the formation of fructosamines, the Amadori product. Currently, glycation is used in a more general way, comprising all the reactions leading to AGE formation.

Protein glycation depends on the concentration, specific reactivity and duration of exposure to the glycation agent (Acharya & Manning, 1980; Eble *et al.*, 1983; Farah *et al.*, 2005; McPherson *et al.*, 1988), the presence of catalytic factors (as metal and buffer ions and oxygen) (Fu *et al.*, 1996; Smith & Thornalley, 1992a; Smith & Thornalley, 1992b; Watkins *et al.*, 1987), the physiological pH and temperature (Smith & Thornalley, 1992a; Smith & Thornalley, 1992b) and the protein half-life (Schleicher & Wieland, 1986). Moreover, the location of the amino acid residue in a folded protein also influences the rate of glycation, either because the neighbouring amino acids influence the pKa value of the amino acid side chain undergoing glycation or restrict the binding of the glycation agent (Ahmed *et al.*, 2005; Westwood & Thornalley, 1997).

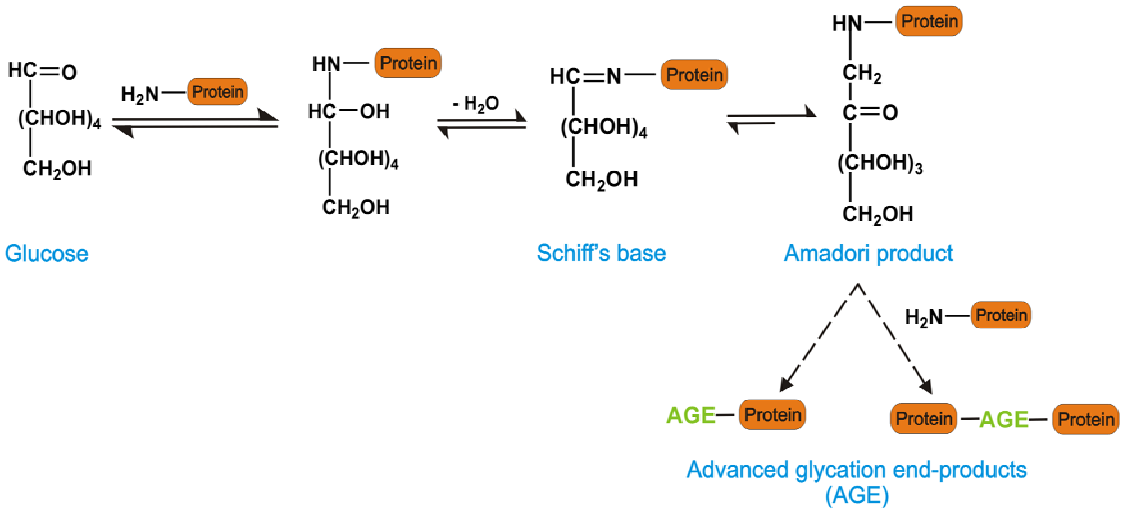


Figure I.1. Initial steps of protein glycation by glucose, including the formation of the Schiff's base and the Amadori product. The Schiff's base, which is formed by the nucleophilic attack of the amino group to the carbonyl group, undergoes the Amadori rearrangement to produce fructosamines, the Amadori product. Through a complex series of chemical reactions, irreversible protein adducts, the advanced glycation end-products (AGE), are produced. These adducts may involve protein cross-link by the reaction with another protein amino group. Adapted from (O'Brien, 1997; Westwood & Thornalley, 1997).

The first two characterized AGE were N^{ε}-(carboxymethyl)lysine (CML), a degradation product of fructosamines (Ahmed *et al.*, 1986), and pentosidine, a fluorescent lysine-arginine cross-link (Sell & Monnier, 1989) (Figure I.2). These AGE were identified *in vivo* and accumulate with age in tissue collagens and lens proteins (Ahmed *et al.*, 1986; Dyer *et al.*, 1991; Dyer *et al.*, 1993; Sell & Monnier, 1989). The early observation that CML and pentosidine formation requires oxidation reactions (Ahmed *et al.*, 1986; Baynes, 1991) led to the concept of glicoxidation, in which the Amadori product autoxidation is essential of AGE formation (Baynes, 1991). In this context, oxygen is sometimes referred as fixative of the irreversible Maillard reaction modification of proteins (Baynes, 1991; Thorpe & Baynes, 1996). This is in agreement with the original concept that the Amadori product is the central precursor to AGE formation (Hodge, 1953; Hodge, 1955). However, it was found that Schiff's base fragmentation also occurs, prior to the Amadori rearrangement (Namiki & Hayashi, 1975; Namiki & Hayashi, 1983). Furthermore, Wolff and Dean also found that metal-catalysed autoxidation of glucose may be more important than the initial attachment of glucose to

the amino group (Wolff & Dean, 1987; Wolff *et al.*, 1991). Nowadays, it is acknowledged that multiple sources and mechanisms for AGE formation *in vivo* exist. These comprise oxidative and non-oxidative reactions of reducing sugars, Schiff's bases, Amadori products and metabolic intermediates, although these processes are not yet fully understood. Importantly, glucose autoxidation, Schiff's base fragmentation and Amadori products autoxidation yield highly reactive dicarbonyl compounds, such as 3-deoxyglucosone, glyoxal and methylglyoxal (Figure I.3) (Hayashi *et al.*, 1986; Kato *et al.*, 1987; Thornalley *et al.*, 1999; Wells-knecht *et al.*, 1995). These compounds also react directly with protein amino groups forming ketoimines, similar to fructosamines, but much more reactive (Figure I.3) (Hunt *et al.*, 1993; Lo *et al.*, 1994; Westwood & Thornalley, 1997). This implies that dicarbonyl compounds are relevant glycation agents and points to the fact that other reducing sugars may also initiate glycation reactions. Since the early discovery of glycated hemoglobin *in vivo* by glucose, designated HbA_1, which increases as a function of the mean glycaemia of diabetic patients (being now used as a glycaemia biomarker), glycation by glucose has been thoroughly investigated (Bunn *et al.*, 1975). Nevertheless, any reducing sugar can initiate glycation reactions with protein amino groups. Glucose is indeed the least reactive of all sugars, being speculated that this was the main reason why glucose was selected as the major metabolic fuel during evolution (Bunn & Higgins, 1981). Several different AGE derived from glycation agents, other than glucose, were identified and different glycation agents can produce the same AGE. For instance, arabinose and ribose are precursors of pentosidine (Dyer *et al.*, 1991; Wells-knecht *et al.*, 1995). In the nucleus, ADP-ribose is an important glycation agent leading to the formation of CML and pentosidine (Cervantes-Laurean *et al.*, 1996). Fructose, ascorbic acid and glyceraldehyde, to name only a few, are also relevant glycation agents (Bakhti *et al.*, 2007; Nagaraj & Monnier, 1992; Schalkwijk *et al.*, 2004; Seidler & Seibel, 2000; Tessier *et al.*, 1999).

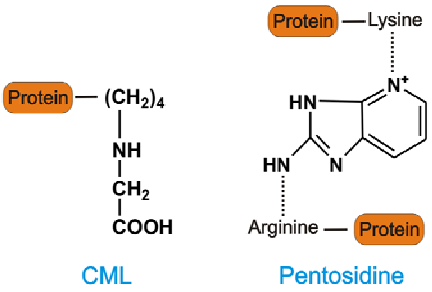


Figure I.2. Structure of CML and pentosidine, the first characterized AGE.

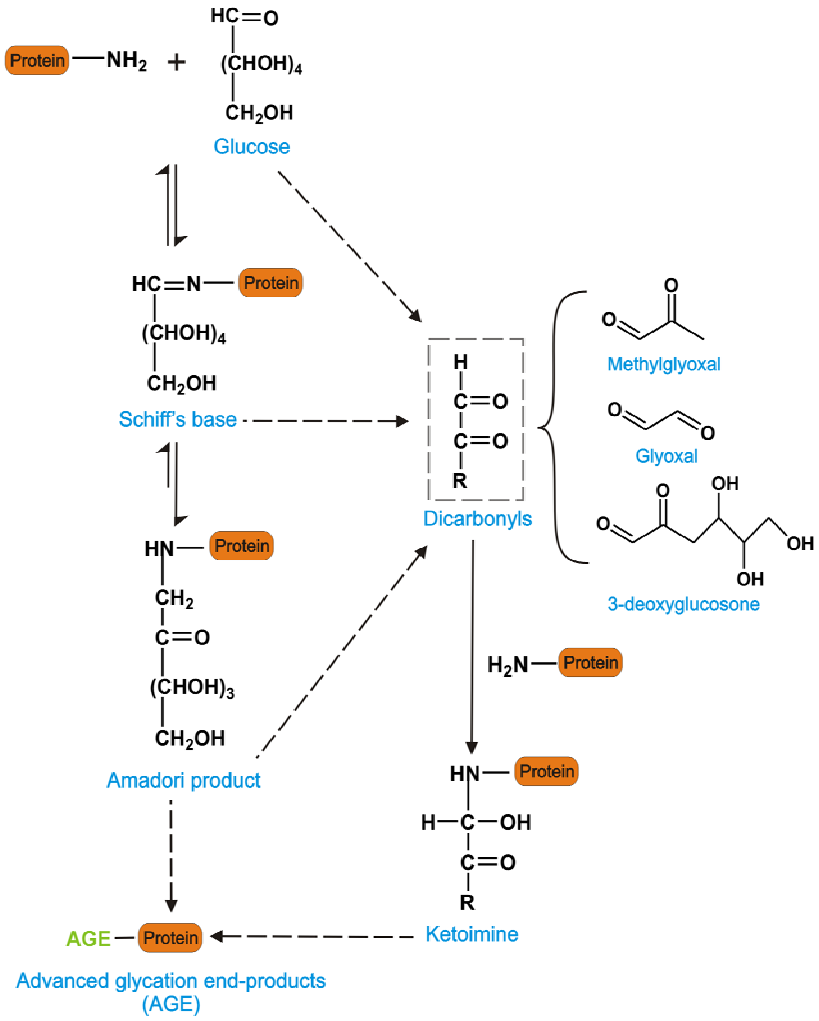


Figure I.3. Formation of dicarbonyl compounds in the early steps of protein glycation. These highly reactive compounds may derive from glucose autoxidation, Schiff's base fragmentation reactions and/or Amadori product autoxidation (Hayashi *et al.*, 1986; Thornalley *et al.*, 1999; Wells-knecht *et al.*, 1995). Methylglyoxal, glyoxal and 3-deoxyglucosone can also initiate protein glycation with the formation of a ketoimine that undergoes several chemical reactions to yield AGE (Hunt *et al.*, 1993).

Recent investigations showed that in physiological conditions, reactive dicarbonyl compounds are key intermediates of protein modification by the Maillard reaction (Chang & Wu, 2006; Thornalley, 1994; Thornalley, 1996). Although these compounds are present in very low concentrations compared to glucose, they are far more reactive. These observations focused the attention on methylglyoxal, since this α-oxoaldehyde is present

in all cells, being considered the most relevant glycation agent *in vivo*. In addition, inside living cells glucose concentration is negligible, while methylglyoxal is at a steady-state concentration in the micromolar range.

3. METHYLGLYOXAL

Methylglyoxal, a highly reactive α-oxoaldehyde, is an unavoidable product of cellular metabolism and therefore is present in all cells, either in normal or pathological conditions. The biochemical research on methylglyoxal started with the discovery of an enzymatic system that catalysed the conversion of α-oxoaldehydes, including methylglyoxal, into α-hidroxyacids - the glyoxalase system (Dakin & Dudley, 1913a; Dakin & Dudley, 1913b; Neuberg, 1913). Neuberg and his co-workers proposed that methylglyoxal was a key glycolytic metabolite and this concept was widely accepted for several years (Neuberg & Kobel, 1928). However, Lohmann established that reduced glutathione (GSH) is an essential cofactor for glyoxalase activity (Lohmann, 1932) while glycolysis does not require glutathione. Moreover, the glyoxalase-catalysed reaction produces D-lactate, instead of L-lactate, which is formed by glycolysis in muscle extracts even in the absence of GSH (Racker, 1951). These observations led to a gradual dismissal of the role of methylglyoxal as a metabolic intermediate. However, glyoxalase activity, methylglyoxal and D-lactate were detected in a wide variety of organisms, raising the question about their metabolic role (Hopkins & Morgan, 1945). The hypothesis introduced in the 1960s by Szent-Györgyi that glyoxalase I and methylglyoxal regulate cell division (the promine-retine theory) and might be involved in cancerogenesis (Szent-Gyorgyi, 1965) gave a sudden impulse to the research in this area and led to the discovery of several new pathways involved in methylglyoxal metabolism and the biochemical effects of this α-oxoaldehyde.

3.1. Methylglyoxal formation in biological systems

In living cells, methylglyoxal is formed through enzymatic and non-enzymatic pathways. Methylglyoxal may be produced by reactions catalysed by enzymes involved in L-threonine metabolism (Lyles & Chalmers, 1992; Ray & Ray, 1987) and in the catabolism of the ketone bodies acetoacetate and acetone (Aleksandrovskii, 1992; Casazza *et al.*, 1984; Koop & Casazza, 1985). The only enzyme that specifically catalyses methylglyoxal formation is methylglyoxal synthase, which appears to exist only in bacteria (Cooper & Anderson, 1970; Hopper & Cooper, 1971). Methylglyoxal is also a by-product of glycolysis, arising from the non-enzymatic decomposition of the triose phosphates dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde 3-phosphate (GAP) (Richard, 1993). Incidentally, this reaction is known since the mid-thirties (Meyerhof & Lohmann, 1934), but was considered an artefact of no physiological significance. Other non-enzymatic sources of methylglyoxal are the Maillard (Thornalley *et al.*, 1999) and lipoperoxidation (Esterbauer *et al.*, 1982) reactions (Figure I.4).

Methylglyoxal synthase (glycerone-phosphate phospho-lyase, EC. 4.2.3.3.), first purified from *Escherichia coli*, catalyses the formation of methylglyoxal from the triose phosphate DHAP (Hopper & Cooper, 1971; Hopper & Cooper, 1972). D-Lactate produced by the glyoxalase system may then be converted to pyruvate by D-lactate dehydrogenase (D-lactate:NAD^+ oxidoreductase EC. 1.1.1.28), providing a by-pass for pyruvate formation from DHAP via glycolysis (Cooper & Anderson, 1970). Methylglyoxal synthase is cooperatively inhibited by inorganic phosphate and appears to have three DHAP binding sites (Hopper & Cooper, 1972). Inorganic phosphate inhibition is reduced in an allosteric manner by DHAP (Hopper & Cooper, 1971). Thus, this enzyme is strongly inhibited by high inorganic phosphate concentrations, suitable for glyceraldehyde 3-phosphate dehydrogenase activity [GAPDH, D-glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating), EC. 1.2.1.12] and glycolysis. If the concentration of inorganic phosphate decreases sufficiently to diminish GAPDH activity, the triose phosphate concentration increases, methylglyoxal synthase activity rises and methylglyoxal is produced from DHAP (Hopper & Cooper, 1971; Hopper & Cooper, 1972). This regulation mechanism may restore the inorganic phosphate concentration

required to glycolysis, while still producing pyruvate (Cooper, 1984). Therefore, it was suggested that methylglyoxal synthase plays a role in the regulation of glycolysis depending on the availability of intracellular inorganic phosphate (Cooper, 1984). In *Desulfovibrio gigas*, this by-pass can reach about 40% of the glycolytic flux (Fareleira *et al.*, 1997). Methylglyoxal synthase was found in prokaryotes (Cooper, 1975; Cooper, 1984), but its presence in eukaryotic cells is highly controversial (Phillips & Thornalley, 1993b; Phillips & Thornalley, 1993a; Ray & Ray, 1981; Sato *et al.*, 1980). In *Saccharomyces cerevisiae*, although methylglyoxal synthase activity was reported in mutant strains (Murata *et al.*, 1985), no activity was detected in wild-type strain growing either aerobic or anaerobically with different carbon sources (Penninckx *et al.*, 1983). Also, no sequence homology to methylglyoxal synthase in *S. cerevisiae* was revealed by genomic analysis, casting even more uncertainty about the presence of this enzyme (Hodges *et al.*, 1999).

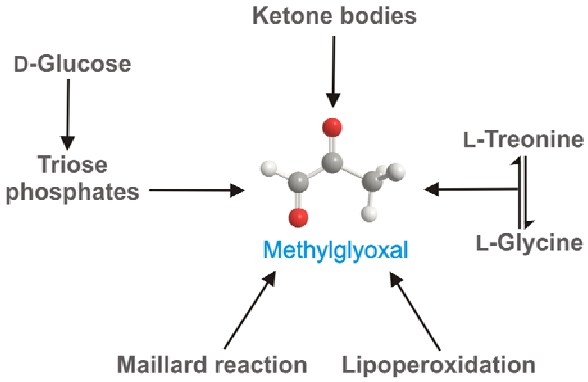


Figure I.4. Main routes of methylglyoxal formation in biological systems. This α-oxoaldehyde is an unavoidable product of cell metabolism, being produced from L-threonine and acetoacetate metabolism (Casazza *et al.*, 1984; Lyles & Chalmers, 1992). The Maillard and lipoperoxidation reactions also produce methylglyoxal. However, the most important pathway for methylglyoxal formation in eukaryotic cells is the glycolytic by-pass where the β-elimination of the phosphate group from DHAP and GAP produces this α-oxoaldehyde (Richard, 1993). In microorganisms, methylglyoxal may be enzymatically formed from dihydroxyacetone phosphate by methylglyoxal synthase (Hopper & Cooper, 1971; Hopper & Cooper, 1972).

The catabolism of L-threonine, via aminoacetone, mediated by the enzyme semicarbazide-sensitive amine oxidase [SSAO, amine:oxygen oxidoreductase (deaminating) (copper-containing), EC.1.4.3.6.] is another source of methylglyoxal (Lyles & Chalmers, 1992). L-Threonine catabolism mainly produces glycine and acetyl-CoA, through the action of an enzyme complex with aminoacetone as intermediate. In low CoA conditions, such as in diabetic ketoacidosis, where most CoA is in the form of acetyl-CoA, the formation of aminoacetone from threonine increases, enhancing methylglyoxal production by the activity of SSAO (Tressel *et al.*, 1986). Methylglyoxal may also be produced from the enzymatic oxidation of acetoacetate by myeloperoxidase (donor:hydrogen-peroxide oxidoreductase, EC. 1.11.1.7) (Aleksandrovskii, 1992). 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). Ketone bodies are likely to be an important source of methylglyoxal in pathological conditions like ketosis and diabetic ketoacidosis (Turk *et al.*, 2006).

The most important pathway for methylglyoxal formation in eukaryotic cells is the glycolytic by-pass, where the β-elimination of the phosphate group from triose phosphate intermediates DHAP and GAP produces methylglyoxal (Figure I.5) (Richard, 1993). 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 (Richard, 1984). Thus, the substrate deprotonation to an enediolate phosphate intermediate followed by the phosphate group cleavage leads to the formation of methylglyoxal (Figure I.5) (Richard, 1993). An estimative for the methylglyoxal non-enzymatic formation rate is given as 0.1 mM per day (Richard, 1993). Therefore, the stabilization of the enzyme-bound enediolate phosphate intermediate in the reaction catalysed by triose phosphate isomerase (TIM, D-glyceraldehyde-3-phosphate aldose-ketose-isomerase, EC. 5.3.1.1) is an absolute requirement to avoid the substrate degradation into methylglyoxal. In fact, the enzyme-bound enediolate phosphate intermediate is protonated approximately

10^6 folds faster than it expels the phosphate group (Richard, 1991). This is achieved by the interaction between the phosphate group of the enzyme-bound intermediate and a flexible loop of the enzyme (Alber *et al.*, 1981; Banner *et al.*, 1975).

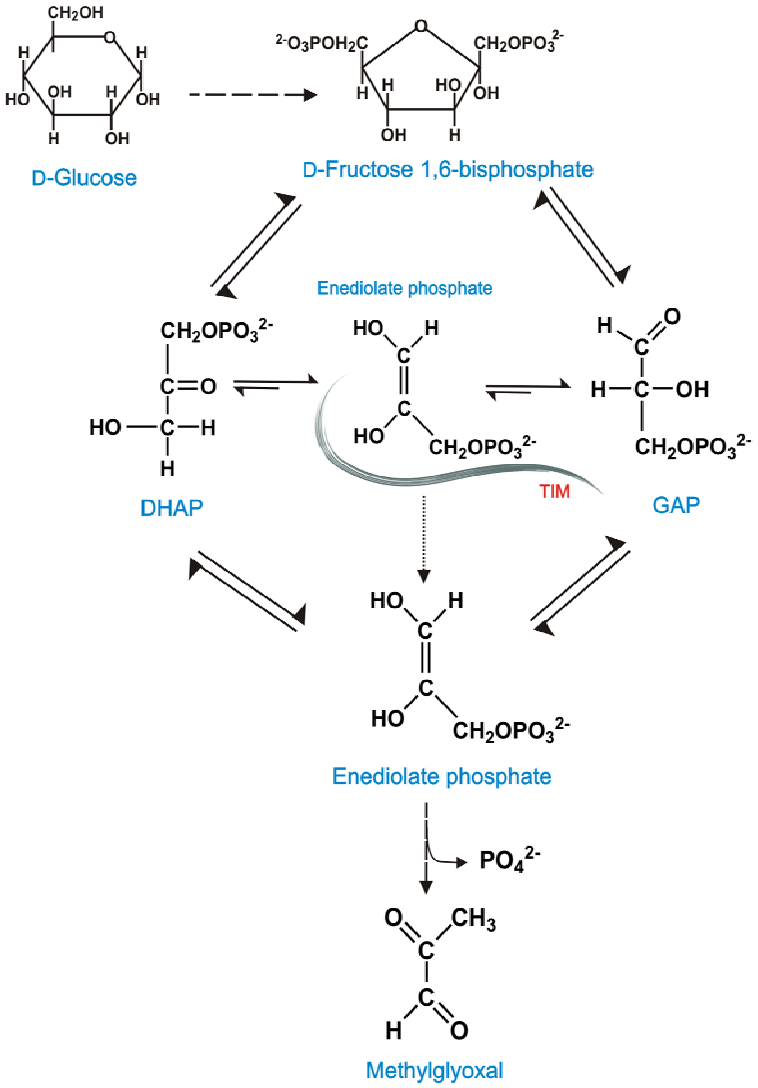


Figure I.5. Methylglyoxal formation from the triose phosphates dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP). Triose phosphates are unstable molecules and the β-elimination reaction of the phosphate group from the common enediolate phosphate intermediate irreversibly yields methylglyoxal. The stabilization of this intermediate by triose phosphate isomerase (TIM) is essential to avoid methylglyoxal formation. However, enediolate phosphate intermediate can leak from the enzyme active site forming methylglyoxal in a paracatalytic reaction. Adapted from (Richard, 1993).

With the deletion of four amino acid residues within this TIM loop, the resulting enzyme is a better catalyst of the elimination reaction (which generates methylglyoxal) than the normal isomerization reaction (Pompliano *et al.*, 1990). Unfortunately, TIM is almost perfect and the enediolate intermediate could leak from the enzyme active center producing methylglyoxal in a paracatalytic reaction (Richard, 1991) (Figure I.5). Consequently, the degradation of GAP and DHAP to methylglyoxal is observed, albeit at a very slow rate compared to the isomerization reaction catalysed by TIM (Iyengar & Rose, 1981; Webb *et al.*, 1977).

The rate of methylglyoxal formation depends on the organism, tissue, cell metabolism and physiological conditions. In most cases, it appears to be directly associated with the glycolytic flux, confirming that the glycolytic by-pass is the main pathway for methylglyoxal production. Some tumour cells show a high glycolytic flux, the Warburg effect (Altenberg & Greulich, 2004), and consequently methylglyoxal concentration is increased. Yeast cells also show a high glycolytic activity and, in *S. cerevisiae*, methylglyoxal formation accounts for 0.3% of the total glycolytic flux (Martins *et al.*, 2001a). In *Desulfovibrio gigas*, where methylglyoxal synthase activity is present, 40% of D-glucose is metabolised into methylglyoxal (Fareleira *et al.*, 1997).

3.2. Methylglyoxal catabolism

Methylglyoxal, a highly reactive and inherently toxic compound, irreversibly damages proteins and nucleic acids through the Maillard reaction (Lo *et al.*, 1994; Oya *et al.*, 1999; Westwood & Thornalley, 1997). High doses of methylglyoxal cause cell death while, with sublethal concentrations, a cell growth delay is observed (Kalapos, 1999; Maeta *et al.*, 2005b; Okado *et al.*, 1996; Ponces Freire *et al.*, 2003). Therefore, protective enzymatic mechanisms evolved to prevent the damage of biomolecules by this unavoidable product of cell metabolism. The glyoxalase system is, by far, the most investigated catabolic route for this α-oxoaldehyde. Nevertheless, since methylglyoxal can be either oxidized or reduced, some oxide-reductases and dehydrogenases are capable of using methylglyoxal as substrate (Kalapos, 1999). Several enzymes were then

implicated in methylglyoxal catabolism, namely 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$^+$ oxidoreductase, EC. 1.1.1.78) (Ray & Ray, 1984) and pyruvate dehydrogenase [pyruvate: dihydrolipoyllysine-residue acetyltransferase-lipoyllysine 2-oxidoreductase (decarboxylating, acceptor-acetylating), EC. 1.2.4.1)] (Baggetto & Lehninger, 1987). Although the real significance of each of these pathways in methylglyoxal catabolism *in vivo* and their role as anti-methylglyoxal derived glycation defense are not yet fully understood, the glyoxalase system and aldose reductase enzyme emerge as the most relevant methylglyoxal catabolic pathways.

3.2.1. The glyoxalase system

The conversion of methylglyoxal to lactic acid in animal tissues was discovered by Neuberg (Neuberg, 1913) and Dakin and Dudley (Dakin & Dudley, 1913a; Dakin & Dudley, 1913b) in independent studies. Even though it was initially believed that only one enzyme catalysed this reaction, named glyoxalase by Dakin and Dudley (Dakin & Dudley, 1913a; Dakin & Dudley, 1913b), Racker showed that in fact two enzymes, glyoxalase I and glyoxalase II, are involved in the production of lactic acid from methylglyoxal (Racker, 1951). The glyoxalase system comprises glyoxalase I (*S*-D-lactoylglutathione methylglyoxal-lyase, EC 4.4.1.5) and glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), that convert methylglyoxal to D-lactate using reduced glutathione as a specific cofactor (Racker, 1951; Thornalley, 1990). Glyoxalase I catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal, produced by the non-enzymatic reaction between methylglyoxal and GSH (Thornalley, 1990; Thornalley, 1993; Vander Jagt *et al.*, 1975). Then, glyoxalase II catalyses the thioester hydrolysis to D-lactate, regenerating GSH (Thornalley, 1990; Vander Jagt, 1993) (Figure I.6).

Interest in the mammalian glyoxalase system arised in part from evidences that associate methylglyoxal and derived AGE with the pathogenesis of diabetic complications and neurodegenerative diseases (Thornalley, 1993; Thornalley, 1996). It is clear that the glyoxalase system acts primarily as a detoxification pathway of toxic methylglyoxal. However, several studies preclude the end of the discussion about the biological functions of this enzymatic system. For instance, immature, proliferating cells and tissues have a high glyoxalase I activity but a low glyoxalase II activity, whereas in mature differentiated cells the reverse is observed (Principato *et al.*, 1982). Nevertheless, there are no evidences for a causal relationship between the glyoxalase system and cell proliferation. In *S. cerevisiae*, glyoxalase I and glyoxalase II mutants are viable, discarding any association between the glyoxalase system activity and cell survival, except when cells are challenged with methylglyoxal (Bito *et al.*, 1997; Inoue & Kimura, 1996).

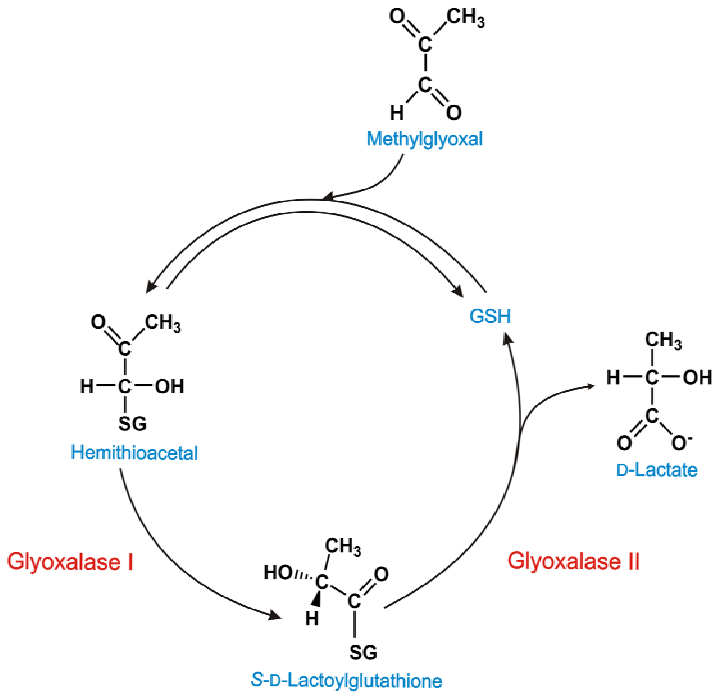


Figure I.6. The glyoxalase system. This enzymatic pathway comprises two enzymes (glyoxalase I and glyoxalase II) responsible for the GSH-dependent catabolism of methylglyoxal, producing D-lactate (Racker, 1951; Thornalley, 1990).

Glyoxalase I

Glyoxalase I activity was found in most organisms throughout the evolutionary scale, from prokaryotes to eukaryotes. It was purified and characterized at the molecular and kinetic level from various sources, namely mammalian tissues (Aronsson & Mannervik, 1977; Baskaran & Balasubramanian, 1987; Han *et al.*, 1976; Marmstal & Mannervik, 1979), plants (Deswal & Sopory, 1991; Deswal & Sopory, 1999; Norton *et al.*, 1990), *E. coli* (Clugston *et al.*, 1998), *S. cerevisiae* (Marmstal *et al.*, 1979), *Leishmania infantum* (Sousa Silva *et al.*, 2005; Vickers *et al.*, 2004) and *Plasmodium falciparum* (Deponte *et al.*, 2007).

The major physiological substrate of glyoxalase I is methylglyoxal, which accumulates markedly when this enzyme is inhibited *in situ* by cell-permeable inhibitors or GSH depletion (Abordo *et al.*, 1999; Thornalley, 1993; Thornalley *et al.*, 1996). However, glyoxal, phenylglyoxal, hydroxypiruvaldehyde and 4,5-dioxovalerate are also substrates for glyoxalase I (Jerzykowski *et al.*, 1973; Vander Jagt *et al.*, 1975; Vander Jagt *et al.*, 1972). This enzyme is highly specific to GSH and no activity is observed with L-cysteine, oxidized glutathione (GSSG), L-cysteinylglycine and γ-L-glutamylcysteine (Behrens, 1941; Carnegie, 1963; Wieland *et al.*, 1956). Glyoxalase I is selectively overexpressed in leukemia cells and a specific inhibitor of this enzyme was shown to be a potential anti-cancer agent (Sakamoto *et al.*, 2000). Overexpression of glyoxalase I prevents the formation of hyperglycemia-induced AGE in bovine endothelial cells, indicating a protective role for this enzyme in diabetic complications (Shinohara *et al.*, 1998).

The reaction mechanism of glyoxalase I is not straightforward due to the non-enzymatic hemithioacetal formation from methylglyoxal and GSH, implying the simultaneous presence of the three species in equilibrium. A steady-state kinetic analysis led to the proposal of a mechanism with the unusual feature of alternative one- and two-substrate branches, the latter involving GSH and methylglyoxal as the first and second substrate, and the former having their hemithioacetal adduct as the substrate (Figure I.7) (Mannervik *et al.*, 1974). The dissociation constant for the hemithioacetal ($3x10^{-3}$ M) is in the range of intracellular glutathione concentration (Vander Jagt, 1993)

and therefore, the fraction of methylglyoxal that exists as hemithioacetal is markedly dependent on the GSH concentration (Vander Jagt, 1993). Hence, a reduction in GSH concentration, as observed in several physiological conditions such as oxidative stress, may diminish the glyoxalase system catalytic capacity.

Human and *S. cerevisiae* glyoxalase I are Zn^{2+} metalloenzymes (Cameron *et al.*, 1997; Frickel *et al.*, 2001; Marmstal *et al.*, 1979; Ridderstrom *et al.*, 1998), while *E. coli* glyoxalase I is a Ni^{2+} dependent enzyme (Clugston *et al.*, 2004; He *et al.*, 2000). Human glyoxalase I is a homodimer and each monomer consists of two structurally equivalent domains (Cameron *et al.*, 1997). The enzyme contains two active sites located at the dimer interface and residues from both subunits contribute to each of the binding pockets (Cameron *et al.*, 1997). The essential zinc ion, which is located at the active site, is coordinated by four amino acids, two from each subunit (glutamine Q33 and glutamate E99 from one chain, and histidine H126 and Q172 from the other chain) (Cameron *et al.*, 1997). The reaction mechanism entails a base-catalysed shielded-proton transfer from C-1 to C-2 of the hemithioacetal to form an ene-diol intermediate followed by rapid ketonization to the thioester product (Thornalley, 1990; Thornalley, 2003a). Glutamate residue 172 is directly involved in the catalytic mechanism, presumably serving as the base that abstracts the proton from the hemithioacetal substrate (Ridderstrom *et al.*, 1998).

Interestingly, yeast glyoxalase I is a monomer with two copies of a segment equivalent to the human enzyme monomer, suggesting that gene duplication events occurred during the evolution of the yeast glyoxalase I gene (Cameron *et al.*, 1997; Marmstal & Mannervik, 1978; Ridderstrom & Mannervik, 1996). This observation raises the question whether yeast glyoxalase I has two active sites, like the human enzyme, in a single polypeptide chain. Indeed, two functional active sites were found in yeast glyoxalase I (Frickel *et al.*, 2001). Although unusual, other enzymes have also two functional active sites within the same polypeptide chain (Darby *et al.*, 1998; Mitsuhashi *et al.*, 2000; Sjostrom *et al.*, 1980; Wacker *et al.*, 1984). Recently, it was shown that *Plasmodium falciparum* glyoxalase I also contains two active sites in a single polypeptide chain, displaying cooperative properties (Deponte *et al.*, 2007).

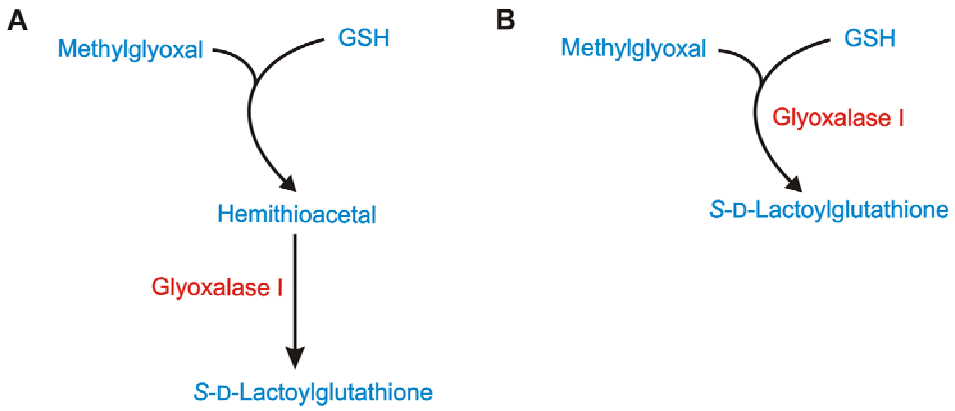


Figure I.7. Glyoxalase I-catalysed formation of *S*-D-lactoylglutathione. Glyoxalase I behaves like a one-substrate enzyme converting hemithioacetal, produced by the non-enzymatic reaction between methylglyoxal and GHS, into *S*-D-lactoylglutathione (A). This enzyme can also catalyse the formation of *S*-D-lactoylglutathione directly from methylglyoxal and GSH, being considered a two-substrate enzyme (Mannervik *et al.*, 1974).

The analysis of the amino acid sequence of yeast glyoxalase I, coded by the *GLO1* gene (YML004C) (Inoue & Kimura, 1996), revealed three potential N-glycosylation sites at asparagine amino acid residues N24, N126 and N184 (Inoue & Kimura, 1996). Actually, it was observed that yeast glyoxalase I contains 0.75% of carbohydrates, hinting for the possible glycosylation of the enzyme (Douglas *et al.*, 1986). Two of the predicted putative phosphorylation sites of human and bacterial glyoxalases I (Ranganathan *et al.*, 1993) were also found in yeast (threonine T96 and serine S144), together with a new probable phosphorylation site (T429) (Inoue & Kimura, 1996). However, the biological function of glyoxalase I phosphorylation remains unclear.

In yeast, the glyoxalase system is a defense mechanism against methylglyoxal. The glyoxalase I activity increases after methylglyoxal addition to the growth medium and in cells grown in glycerol as carbon source (Inoue & Kimura, 1996; Inoue *et al.*, 1998; Penninckx *et al.*, 1983). Glyoxalase I gene has two stress response elements in the 5´flanking region (Inoue *et al.*, 1998). An increase of *GLO1* expression is induced by osmotic stress, thereby avoiding methylglyoxal accumulation due to the enhanced glucose consumption for glycerol synthesis, used as a compatible solute for adaptation to highly osmotic conditions (Inoue *et al.*, 1998). This response is mediated by the high

osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway (Inoue *et al.*, 1998). Interestingly, cells treated with methylglyoxal also develop a stress response, inducing the expression of *GLO1* gene through Hog1p that is phosphorylated and translocated into the nucleus (Maeta *et al.*, 2005a). These observations indicate that methylglyoxal may function as a signal initiator of the HOG-MAPK pathway (Maeta *et al.*, 2005a).

Glyoxalase II

Glyoxalase II, a glutathione thioesterase, has been purified and characterized from several tissues of mammalian source (Allen *et al.*, 1993; Ball & Vander Jagt, 1981; Oray & Norton, 1980; Principato *et al.*, 1984; Uotila, 1973), plants (Maiti *et al.*, 1997; Norton *et al.*, 1990), yeast (Talesa *et al.*, 1990b) and *Leishmania infantum* (Trincao *et al.*, 2006). Glyoxalase II seems to be absent from the human spleen, mouse heart and rat skeletal muscle (Jerzykowski *et al.*, 1978), although the results may differ depending on the technique used for the glyoxalase II activity assay (Martins *et al.*, 1999). This feature can be illustrated with glyoxalase II from yeast *S. cerevisiae*; even though Penninckx and co-workers did not detect any glyoxalase II activity (Penninckx *et al.*, 1983), this enzyme is unequivocally present in this organism (Bito *et al.*, 1997; Martins *et al.*, 1999). Glyoxalase II consistently shows a specific activity of 600-900 $\mu mol.min^{-1}.mg^{-1}$, except for *S. cerevisiae* in which an extremely low specific activity (1.34 $\mu mol.min^{-1}.mg^{-1}$) was reported (Vander Jagt, 1993). However, in the same study, the *K*m value obtained (7 μM) is not in agreement with more recent studies where the *K*m value reported was 110 μM for the purified enzyme (Bito *et al.*, 1997), 360 μM *in situ* and 150 μM in cell free extracts (Martins *et al.*, 1999).

Besides cytosolic glyoxalase II, a mitochondrial isoform of the enzyme was also found (Bito *et al.*, 1997; Cordell *et al.*, 2004; Maiti *et al.*, 1997; Talesa *et al.*, 1990a; Talesa *et al.*, 1988; Talesa *et al.*, 1989). In mammals, both isoforms are coded by a single gene, being the different isoenzymes produced by alternative translation initiation of the gene transcripts (Cordell *et al.*, 2004). In *S. cerevisiae*, two different nuclear genes code

for the mitochondrial (*GLO4*, YOR040W) and the cytosolic glyoxalase II (*GLO2*, YDR272W) (Bito *et al.*, 1997). *GLO4* is only expressed on glycerol-containing medium, while *GLO2* expression is observed with both glucose and glycerol (Bito *et al.*, 1997). The amino acid sequences of both enzymes are 59.1% identical and show high similarities with human glyoxalase II (Bito *et al.*, 1997). Since glyoxalase I activity is absent from rat (Talesa *et al.*, 1988; Talesa *et al.*, 1989) and yeast mitochondria (Bito *et al.*, 1997), the presence of mitochondrial glyoxalase II is quite intriguing. Nevertheless, Scire and co-workers found *S*-D-lactoylglutathione in mitochondria, raising the hypothesis that mitochondrial glyoxalase II hydrolyses the thioester that diffused or was transported to this cellular compartment (Scire *et al.*, 2000). Furthermore, it was also suggested that *S*-D-lactoylglutathione uptake by the mitochondria and the subsequent glyoxalase II activity could function as a pathway for mitochondrial GSH import (Scire *et al.*, 2000). Even though it is a very interesting possibility, it requires further research, mainly because active GSH import to the mitochondria has been described (Martensson *et al.*, 1990). Another possibility is that mitochondrial glyoxalase II catalyses the hydrolysis of other glutathione thioesters formed in this organelle. Although *S*-D-lactoylglutathione is the preferred substrate, glyoxalase II shows a broad specificity for GSH thioesters and can catalyse the hydrolysis of *S*-D-manendolylglutathione, *S*-D-acetylglutathione, *S*-D-acetoacetylglutathione and *S*-D-glycolylglutathione, among others (Thornalley, 1990; Thornalley, 1993; Vander Jagt, 1993). The enzyme is however highly specific for glutathione moiety, since no activity was detectable with CoA esters and thioglycolate (Thornalley, 1990; Vander Jagt, 1993). Interestingly, in trypanosomatids, where glutathione is functionally replaced by trypanothione [N^1,N^8-bis(glutathionyl) spermidine], glyoxalase II is specific for trypanothione thioesters (Irsch & Krauth-Siegel, 2004; Sousa Silva *et al.*, 2005).

Although previous studies indicated that glyoxalase II was not a metalloenzyme, the crystal structure of human enzyme shows the presence of two Zn^{2+} ions per molecule (Cameron *et al.*, 1999). Intriguingly, mitochondrial glyoxalase II from *Arabidopsis thaliana* can accommodate a number of different metal centers although the predominant is $Fe^{3+}Zn^{2+}$ (Marasinghe *et al.*, 2005).

Glyoxalase II 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). The active site contains two metal-binding sites. The first consists of three histidine residues (H110, H56 and H54), a bridging aspartic acid residue (D134) and a bridging water/hydroxide ion. The second metal binding site contains two histidine residues (H173 and H59), a terminal bound aspartic acid (D58) and the same bridging aspartic acid residue (D134) and water/hydroxide ion (Cameron *et al.*, 1999). Based on these observations it was suggested that the hydroxide ion could act as the nucleophile (Cameron *et al.*, 1999), which is markedly different from the previously proposed mechanism involving a direct nucleophilic attack of a histidine residue located on the active site to the thioester substrate producing an acyl-imidazole intermediate that would rapidly hydrolyse (Thornalley, 1993; Vander Jagt, 1993). However, it is noteworthy that Cameron and co-workers used *S*-(N-hydroxy-N-bromophenylcarbamoyl)glutathione, which is a poor glyoxalase II substrate (Cameron *et al.*, 1999). This substrate analogue binds to the domain interface through hydrogen-bonding interactions between the glycine and cysteine residues of the glutathione moiety and the enzyme, probably through arginine (R249) and lysine residues (K249 and K252) (Cameron *et al.*, 1999).

Glyoxalase II has been associated with important human pathologies. Of particular interest are the studies of Willingham and co-workers, showing that among 4850 haploid yeast mutants containing deletions of non-essential genes, 52 were sensitive to Huntington fragment toxicity including a ΔGLO2 mutant strain, lacking cytosolic glyoxalase II (Willingham *et al.*, 2003). Additionally, ΔGLO4 yeast strain, lacking mitochondrial glyoxalase II, was sensitive to α-synuclein toxicity, while no toxicity was observed with ΔGLO2 mutant strain (Willingham *et al.*, 2003). Glyoxalase II was also recently implied as a pro-survival factor of p53 family of transcription factors, which include p63 and p73 (Xu & Chen, 2006). It was observed that glyoxalase II gene is up-regulated by p63 and p73 and, after overexpression, the cytosolic but not the mitochondrial form of glyoxalase II, inhibits cell apoptotic response to methylglyoxal (Xu & Chen, 2006). This may suggest that methylglyoxal can act as a signal initiator for several cellular responses.

3.2.2. Aldose reductase

Aldose reductase, a member of the aldo-keto reductase superfamily, was first described by Hers, who observed the NADPH-dependent reduction of glucose and other aldehydes to polyols by extracts of seminal vesicles and placenta (Ginsburg & Hers, 1960; Hers, 1956). Aldose reductase is the first enzyme of the so-called polyol pathway (Burg *et al.*, 1996), reducing D-glucose in a NADPH-dependent reaction to sorbitol, which is then converted to D-fructose by the NAD^+-dependent sorbitol dehydrogenase (Jeffery & Jornvall, 1983; Leissing & McGuinness, 1983) (Figure I.8). The observation that the polyol pathway activity is increased during hyperglycaemia (Gonzalez *et al.*, 1984a; Gonzalez *et al.*, 1984b), leading to several cellular damages and $NADPH/NAD^+$ depletion (Yabe-Nishimura, 1998), suggests a role for aldose reductase in the pathogenesis of diabetic complications. In fact, transgenic mice overexpressing aldose reductase in lens epithelial cells accumulate high levels of sorbitol and become highly susceptible to the development of diabetic cataracts (Lee *et al.*, 1995b). When a sorbitol dehydrogenase-deficient mutation is also present in these transgenic mice, a larger accumulation of sorbitol and further acceleration of diabetic cataracts were observed (Lee *et al.*, 1995b). Additionally, since fructose is a stronger glycation agent than glucose, an increase in AGE formation is likely to occur (Schalkwijk *et al.*, 2004). Hence, several aldose reductase inhibitors were developed as possible therapeutic agents for diabetic clinical complications (Iwata *et al.*, 2006; Ramasamy *et al.*, 1997; Yabe-Nishimura, 1998).

Consistent with the idea that aldose reductase and related enzymes may function primarily in detoxification, this enzyme exhibits a broad substrate specificity for a variety of aldehydes. In fact, compared to several physiological substrates, D-glucose is a poor substrate for aldose reductase, with a *K*m of 70 mM and a *k*cat/*K*m of 9.1×10^2 $M^{-1}.min^{-1}$ (Vander Jagt *et al.*, 1990; Vander Jagt *et al.*, 1992). So, the significance of aldose reductase in the polyol pathway may be quite limited under non-diabetic conditions. An extensive study of substrate specificity towards trioses, trioses phosphate and related three carbon aldehydes and ketones revealed that aldose reductase has a higher affinity for methylglyoxal (*K*m of 8 μM and *k*cat/*K*m of 1.8×10^7 $M^{-1}.min^{-1}$), suggesting that this

enzyme may be relevant in methylglyoxal detoxification (Vander Jagt *et al.*, 1992). The NADPH-dependent reduction of methylglyoxal by aldose reductase mainly produces acetol with only traces of D-lactaldehyde being detected, indicating that the reduction occurs primarily on the aldehyde carbonyl group (Vander Jagt *et al.*, 1992). Acetol, which is also a substrate of aldose reductase, can be further reduced to L-1,2-propanediol at expense of another NADPH molecule (Vander Jagt *et al.*, 1992). D-Lactaldehyde can also be reduced to D-1,2-propanediol by aldose reductase (Vander Jagt *et al.*, 1992) (Figure I.9).

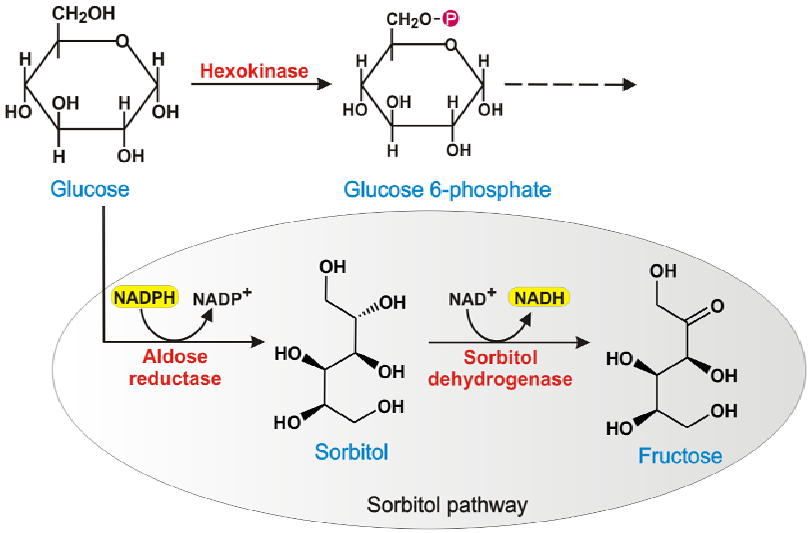


Figure I.8. The polyol pathway. In this alternative route of D-glucose metabolism, sorbitol is produced from D-glucose by aldose reductase using NADPH as cofactor. Sorbitol is subsequently converted to D-fructose by sorbitol dehydrogenase. Adapted from (Yabe-Nishimura, 1998).

Interestingly, human aldose reductase contains a putative glutathione binding site near the active site that may be relevant to methylglyoxal catabolism since *in vivo*, most of this α-oxoaldehyde is in the form of glutathione-derived hemithioacetal (Cappiello *et al.*, 1996). In the presence of glutathione, the hemithioacetal produced is also a substrate

for aldose reductase and a shift in the product distribution was observed with D-lactaldehyde being the major product compared to acetol (Vander Jagt *et al.*, 2001) (Figure I.9). In this case, aldose reductase acts like a methylglyoxal-specific ketone reductase instead of an aldehyde reductase (Vander Jagt *et al.*, 2001). At low GSH concentration, methylglyoxal reduction by aldose reductase mainly produces acetol, a poor substrate for the second reduction reaction (Vander Jagt *et al.*, 1992). In fact, acetol accumulation was observed in diabetic patients (Reichard *et al.*, 1986). The reaction catalysed by aldose reductase is reversible and, therefore, acetol may be converted back to methylglyoxal (Vander Jagt *et al.*, 2001). Hence, the reduction of this α-oxoaldehyde to acetol may be an undesirable reaction (Vander Jagt *et al.*, 2001). Although this enzyme is involved in methylglyoxal detoxification, 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.

Aldose reductase transcription is stress-inducible, playing an important protective role against methylglyoxal citotoxicity. Hydrogen peroxide induces aldose reductase mRNA synthesis (Nishinaka & Yabe-Nishimura, 2001) and methylglyoxal also promotes a dose and time-dependent increase in aldose reductase mRNA, protein levels, and enzymatic activity (Yabe-Nishimura *et al.*, 2003). In *S. cerevisiae*, aldose reductase is coded by the *GRE3* gene (YHR104W) and its expression is also up-regulated in several conditions such as osmotic and oxidative stress (Aguilera & Prieto, 2001). These type of cellular stress increases intracellular methylglyoxal concentration and yeast displays a regulatory protective mechanism that involves the induction of *GRE3* gene expression (Aguilera & Prieto, 2001; Aguilera & Prieto, 2004). The *GRE3* overexpression increases methylglyoxal tolerance and complements the glyoxalase system deficiency of a mutant strain lacking glyoxalase I (Aguilera & Prieto, 2001).

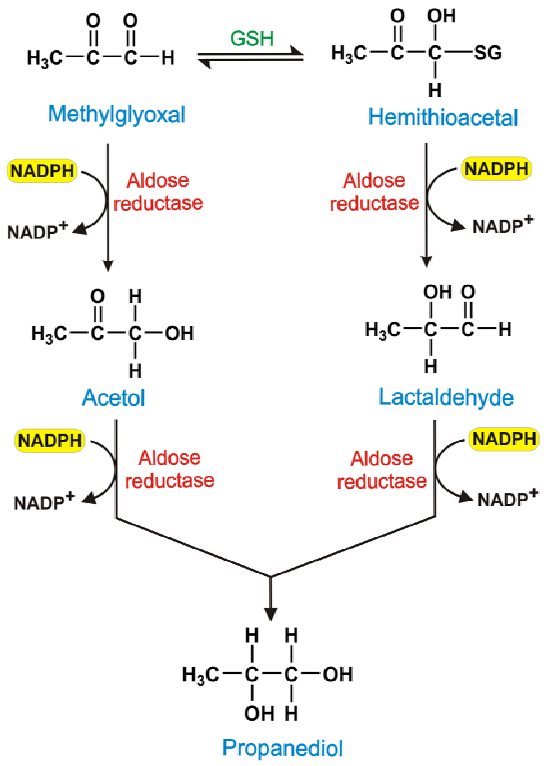


Figure I.9. Methylglyoxal catabolism by aldose reductase. Depending on the presence of GSH, aldose reductase catalyses the NADPH-dependent reduction of methylglyoxal to acetol (reduction of aldehyde group) or to lactaldehyde (reduction of ketone group). These compounds are then converted to propanediol by another NADPH-dependent reaction catalysed by aldose reductase. Adapted from (Vander Jagt & Hunsaker, 2003).

3.3. Biochemical effects of methylglyoxal

Methylglyoxal has two functional groups: a highly reactive aldehyde group and an electron acceptor ketone group. The electronic interaction between the adjacent C=O double bonds leads to a charge delocalization (Szent-Gyorgyi, 1976; Ventura & Cubas, 1992). The higher polarization of the aldehyde C=O double bound makes the aldehyde group more reactive than the ketone group (Abdulnur, 1976; Jencks, 1987). As a result, methylglyoxal is an excellent electrophile molecule, being involved in nucleophilic addition to the carbonyl group (Jencks, 1987; Szent-Gyorgyi, 1980). In biological systems, the main nucleophiles are the amine groups of proteins, nucleic acids and basic

phospholipids, which can be irreversibly modified by the Maillard reaction. Methylglyoxal also reacts with protein and GSH sulphydryl groups, but this reaction is considered to be reversible.

Methylglyoxal is a mutagenic and genotoxic agent, able to modify nucleic acids. This α-oxoaldehyde mainly reacts with guanine nucleotides (Shapiro *et al.*, 1969; Shapiro & Hachmann, 1966), being the relative reactivity towards poly-guanosine, poly-adenine and poly-uracil of 90:7:3 (Krymkiewicz, 1973). Methylglyoxal reacts with deoxyguanosine, producing a stable adduct identified as carboxyethylguanine (Papoulis *et al.*, 1995; Thornalley, 2003b). The reaction proceeds rapidly with RNA and denatured DNA but very slowly with native duplex DNA (Krymkiewicz, 1973). Glycated DNA was detected *in vivo* in human samples and cultured human smooth muscle cells and bovine aorta endothelium cells, using immunochemical techniques, HPLC and LC-MS (Bucala *et al.*, 1984; Schneider *et al.*, 2006; Seidel & Pischetsrieder, 1998). There are evidences that DNA glycation causes loss of genomic integrity associated with genotoxic effects. High methylglyoxal concentration leads to interstrand cross-links in duplex DNA (Rahman *et al.*, 1990), strand breaks (Pischetsrieder *et al.*, 1999; Rahman *et al.*, 1990) and increased mutation frequency (Cajelli *et al.*, 1987; Migliore *et al.*, 1990; Pischetsrieder *et al.*, 1999). Furthermore, Lee and co-workers found an increased mutation rate in transgenic embryos of diabetic mice that was linked to high glucose concentration (Lee *et al.*, 1995a).

Basic phospholipids (phosphatidylethanolamine and phosphatidylserine) are also potential targets of the Maillard reaction, due to the presence of free amino groups, forming lipid-linked AGE (Bucala *et al.*, 1993). This process is accompanied by oxidation of the unsaturated fatty acid side chains, with 4-hydroxyhexenal and 4-hydroxynonenal as major products (Al-Abed *et al.*, 1996; Bucala *et al.*, 1993). Glycated phospholipids were detected in rat liver, with increased levels in streptozotocin-induced diabetic animals (Pamplona *et al.*, 1995). Carboxymethylethanolamine, a lipid-linked AGE found *in vivo*, is considered a biomarker of phospholid modification by the Maillard reaction (Requena *et al.*, 1997). Recently, it was shown that diabetic patients plasma contains high amounts of glycated phospholids, identified as an Amadori-product of the reaction between phosphatidylethanolamine and glucose (Nakagawa *et al.*, 2005). Like

proteins, phospholipids amino groups may also react with other carbonyl-containing compounds like methylglyoxal. However, phospholipids glycation mechanisms and the resulting lipid-linked AGE structures are still poorly understood.

Protein glycation by methylglyoxal

In physiological conditions, methylglyoxal reacts reversibly with amino acid side chains of arginine, lysine and cysteine residues. Irreversible reactions may occur with lysine and arginine residues leading to the formation of irreversible adducts on proteins known as MAGE (methylglyoxal advanced glycation end-products). A large number of studies showed that methylglyoxal reacts to and modifies proteins, such as BSA (McLaughlin *et al.*, 1980; Lo *et al.*, 1994; Vander Jagt *et al.*, 1992), HSA (Ahmed *et al.*, 2005), aspartate aminotransferase, collagens(Bowes & Cater, 1968) and lens proteins (Riley & Harding, 1995), among others. Several MAGE were characterized through model reaction systems and identified *in vivo* (Figure I.10).

The reaction between methylglyoxal and lysine residues leads to the formation of N^{ε}-(carboxyethyl)lysine (CEL) (Ahmed *et al.*, 1997). This MAGE was detected *in vivo* in human lens proteins at a concentration similar to CML, and an increase in CEL concentration was observed with age (Ahmed *et al.*, 1997). Methylglyoxal is also responsible for protein cross-links, one of the major consequences of protein glycation (Fu *et al.*, 1994; Sell & Monnier, 1989). With lysine residues, methylglyoxal forms methylglyoxal-lysine dimers (MOLD), originally identified in model reaction systems (Nagaraj *et al.*, 1996). MOLD was also found to accumulate with age in lens proteins at significantly higher levels than pentosidine (Frye *et al.*, 1998), and also increases in diabetic patients compared to normal subjects (Nagaraj *et al.*, 1996). These observations highlight the importance of methylglyoxal as an intermediate of protein cross-link derived from the Maillard reaction. Besides the lysine cross-link, a lysine-arginine methylglyoxal-derived cross-link, termed MODIC, was also described (Lederer & Klaiber, 1999).

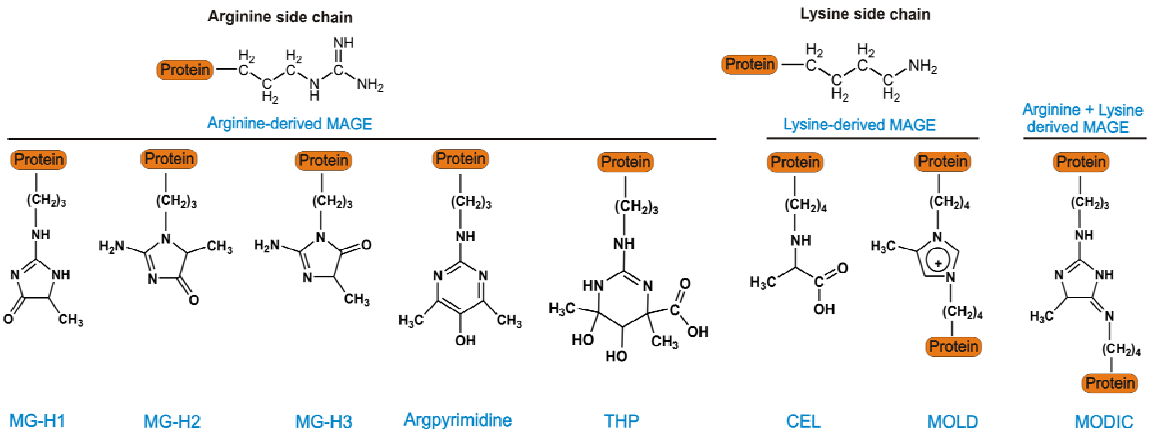


Figure I.10. Chemical structures of MAGE. Methylglyoxal reacts preferentially with arginine side chain to form hydroimidazolones, tetrahydropyrimidine (THP) and argpyrimidine, a fluorescent MAGE. Protein cross-links are also observed with lysine residues (MOLD) and with both lysine and arginine residues (MODIC). To a better understanding of the modification caused by methylglyoxal, arginine and lysine side chains are shown. Adapted from (Nemet *et al.*, 2006).

Contrary to glucose, methylglyoxal preferentially modifies arginine residues (Lo *et al.*, 1994; Oya *et al.*, 1999). The non-fluorescence protein bound imidazolone, 5-hydroimidazolones [N^{δ}-(5-methyl-imidazolone-2-yl)-ornithine] (Henle *et al.*, 1994) is produced by the reaction between methylglyoxal and arginine residues. This MAGE has been identified *in vivo* (Ahmed *et al.*, 2003; Niwa *et al.*, 1997b; Uchida *et al.*, 1997), and is present at high levels in the kidneys of streprozotocin-induced diabetic mice (Niwa *et al.*, 1997a). Hydroimidazolones, believed to be the major product of methylglyoxal-derived protein glycation, exist as three structural isomers (MG-H1, MG-H2 and MG-H3) (Ahmed *et al.*, 2002; Ahmed & Thornalley, 2002). Another non-fluorescent MAGE, tetrahydropyrimidine [THP, N^{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)ornithine], is also derived from the reaction with arginine (Oya *et al.*, 1999). Besides protein cross-link, the formation of fluorescent adducts is another important feature of protein glycation. For instance, BSA and lens proteins modified by methylglyoxal exhibit new fluorescence properties (Lo *et al.*, 1994; Riley & Harding, 1995). The MAGE responsible for this new fluorescence properties was identified as

argpyrimidine [N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine] (Oya *et al.*, 1999; Shipanova *et al.*, 1997). The emission fluorescence spectra of argpyrimidine and methylglyoxal-modified proteins are similar, suggesting that this MAGE is a relevant product and that the intrinsic proteins fluorescence associated to pathological conditions may be due in part to methylglyoxal-derived glycation (Shipanova *et al.*, 1997). Using specific antibodies towards proteins modified by methylglyoxal, which major antigenic epitope is argpyrimidine, revealed the presence of this fluorescent MAGE in the *intima* and media small artery walls of diabetic kidneys (Oya *et al.*, 1999), and in human lens proteins (Padayatti *et al.*, 2001). In the latter case, the amount of argpyrimidine was related to lens aging and cataractogenesis (Padayatti *et al.*, 2001).

Contrasting with the irreversibility of arginine and lysine modifications, interactions with cysteine residues are reversible, resulting in the formation of hemithioacetals (Lo *et al.*, 1994). Recent reports raised the possibility that the reversible cellular responses induced by methylglyoxal may involve reversible cysteine modifications. The *S. cerevisiae* transcriptional factor Yap1, a functional homologue of mammalian AP-1, is reversibly activated by methylglyoxal by a non-disulfide bounds mechanism, since a Yap1 mutant with only one cysteine residue can still be activated (Maeta *et al.*, 2004). In mammals, the formation of a specific MAGE seems to be involved in TNF-induced cell death (Van Herreweghe *et al.*, 2002). The chemical nature of this cysteine-methylglyoxal adduct is unknown due to its unstable and reversible nature (Maeta *et al.*, 2004). In this context, it was proposed that phosphorylated glyoxalase I could convert the normally reversible hemithioacetal into irreversible adducts (Van Herreweghe *et al.*, 2002). If this is true, methylglyoxal and the glyoxalase system would have an important role in cell physiology, regulating protein activity while extensive unregulated irreversible modifications could result in cell dysfunction (Van Herreweghe *et al.*, 2002). In addition, it was described that methylglyoxal inactivates the mitochondrial permeability transition pore, through a fast, reversible and specific reaction (Speer *et al.*, 2003). In this case, the authors suggested that a reversible arginine modification would be involved (Speer *et al.*, 2003).

4. PROTEIN GLYCATION IN HUMAN PATHOLOGIES: THE AGE HYPOTHESIS

Post-translational modifications are important biological tools for the production of several protein species from a single gene, which may vary in structure, function, biological half-life and display differentiated protein-protein interactions. However, the extensive non-enzymatic unregulated modification of particular proteins, by glycation for example, could have a deleterious effect on protein structure and function. These changes might be associated with cell and tissue damage observed in several pathological conditions and aging. This is markedly different from controlled post-translational modifications where enzymes modify specific sites on determined proteins to produce a given effect. In the mid 1980s, the "AGE hypothesis" was introduced in the pathophysiology of diabetes *mellitus* and related clinical complications (Bucala & Cerami, 1992; Vlassara, 1994; Vlassara *et al.*, 1994). According to this hypothesis, increased AGE formation alters the structure and function of tissue proteins, contributing to the development of diabetes *mellitus* clinical complications. The AGE hypothesis was introduced in the context of diabetes *mellitus*, since the earlier studies about protein glycation considered mainly glucose as a glycation agent. As glucose-derived protein glycation is an extremely slow process (being considered to occur only if the protein persists in the body for months to years), a large amount of research work focused on CML and pentosidine accumulation in long-lived proteins like lens crystalline and collagen (Ahmed *et al.*, 1986; Dyer *et al.*, 1991; Dyer *et al.*, 1993; Sell & Monnier, 1989). Indeed, CML and pentosidine accumulation is accelerated by hyperglycemia and correlates with the severity of clinical complications such as retinophaty, nephrophaty, neurophaty, vascular disorders, diabetic cataracts and diabetic atherosclerosis (Ahmed, 2005; Beisswenger *et al.*, 1993; Dyer *et al.*, 1991; Dyer *et al.*, 1993; Sell *et al.*, 1992). The accumulation of AGE-modified proteins was obviously associated with the high glucose concentration in the plasma of diabetic patients. Nowadays, several different aspects need to be added to this hypothesis. As stated before, glucose is the least reactive sugar (Bunn & Higgins, 1981), thus highly reactive compounds, such as methylglyoxal, are more relevant glycation agents. It was also observed that short-lived plasma proteins (Makita *et al.*, 1991; Makita *et al.*, 1992) and intracellular proteins are irreversibly

modified by glycation (Giardino *et al.*, 1994; Gugliucci & Allard, 1996). Besides its higher reactivity, methylglyoxal reacts preferentially with arginine residues and several new AGE structures were discovered and identified *in vivo* (as described in 3.3.1). It is noteworthy that the stimulation of glucose metabolism observed in diabetes *mellitus* and related clinical complications causes an increase of methylglyoxal levels (McLellan *et al.*, 1994). Another important question resulted from the observation that AGE also accumulate in other diseases like atherosclerosis in non-diabetic individuals (Kume *et al.*, 1995) and patients with dialysis-related amyloidosis (DRA) (Miyata *et al.*, 1994a; Miyata *et al.*, 1994b; Miyata *et al.*, 1993). Alzheimer's disease (Du Yan *et al.*, 1997a; Vitek *et al.*, 1994; Yan *et al.*, 1994a) and Parkinson's disease (Castellani *et al.*, 1996; Munch *et al.*, 2000) are significant examples of AGE accumulation in amyloid deposits. It is now believed that protein glycation plays a major role in these disorders although the underlying mechanisms are still unknown. Importantly, in all these pathological conditions glycaemia is normal. These observations led to the introduction of a new type of cellular stress, carbonyl stress, which is caused by a generalized increase in the concentration of reactive carbonyl AGE- precursors (like methylglyoxal), glycoxidation and lipoxidation products (Baynes & Thorpe, 1999). In line with this concept, protein glycation is not due to hyperglycaemia but to carbonyl stress that may result from an increased concentration of glycation agents and/or a decrease of their catabolism (Baynes & Thorpe, 1999). Noteworthy, carbonyl stress includes oxidative and non-oxidative pathways (Baynes & Thorpe, 1999).

Much research efforts have been focused in the glycation effects on protein function and structure and cell physiology that underlies the genesis or contribute to the development of pathological conditions. It is believed that protein glycation leads directly to changes in the function of specific proteins with consequences to normal cell physiology. Furthermore, glycated proteins promote directly oxidative stress and, by interaction with cell receptors, trigger cellular responses and inflammation that may lead to cell dysfunction.

4.1. Biochemical effects of protein glycation

4.1.1. Protein structure and function

Since the discovery that AGE-modified proteins accumulates in several human pathologies, the implications of protein modifications induced by glycation have been extensively investigated. In this context, it is of extreme importance to understand whether these processes are mechanistically related or merely associated with disease processes.

Arginine, lysine and cysteine residues are usually present in enzymes active sites. Almost all glycolytic enzymes, for example, contain arginine residues in their active site (Nelson & Cox, 2000). Consequently, the irreversible modification of these residues would alter enzyme activity. For instance, methylglyoxal inactivates Cu,Zn-superoxide dismutase with the formation of covalent cross-links, releasing copper ions from the enzyme (Kang, 2003). Methylglyoxal at just 1 μM (physiological concentration) decreases GAPDH activity by 20%, while exposure to 1 mM causes a 97% inhibition (Lee *et al.*, 2005). Likewise, glycation by methylglyoxal and other glycation agents decreases the activity of several enzymes, namely glutathione reductase, lactate dehydrogenase, GAPDH (Morgan *et al.*, 2002), aspartate aminotransferase (Seidler & Seibel, 2000), catalase (Yan & Harding, 1997), Na,K-ATPase (Garner *et al.*, 1990) and esterase activity in HSA (Ahmed *et al.*, 2005). In other cases, enzymatic activity increases upon glycation, as observed in the esterase activity of hemoglobin and myoglobin (Sen *et al.*, 2007). Interestingly, it was recently discovered that Hsp27 and α-crystallins enhance their chaperone activity upon glycation by methylglyoxal (Nagaraj *et al.*, 2003; Oya-Ito *et al.*, 2006). Glycation is also responsible for changing molecular conformation (Raabe *et al.*, 1996) with redistribution of the secondary structure elements (Bakhti *et al.*, 2007; Bouma *et al.*, 2003), enhancing hydrophobicity (Bakhti *et al.*, 2007) and altering protein stability (Luthra & Balasubramanian, 1993; Raabe *et al.*, 1996; Seidler & Seibel, 2000). In addition, protein glycation could also be the direct cause of protein misfolding with important cellular consequences in the context of amyloidotic neurodegenerative diseases (Bouma *et al.*, 2003; Ledesma *et al.*, 1994). Although some

of these glycated proteins were identified *in vivo,* in the majority of these studies, proteins were glycated *in vitro* by incubation with different glycation agents (like methylglyoxal, glyoxal, ribose, glucose and triose phosphates) and, in most cases, in non-physiological conditions. So, the relevance of the observed results is arguable considering that any protein with lysine and arginine residues is a target for glycation. Indeed, chemical modifications by methylglyoxal and phenylglyoxal have been used for about forty years to identify critical residues involved in enzyme catalysis (Raess *et al.*, 1985; Takahashi, 1968; Takahashi, 1977a; Takahashi, 1977b).

Glycation of specific proteins could alter different cellular functions in several ways. In organisms from yeast to mammals, many proteins are translated as inactive precursors. These protein precursors, such as hormones, neuropeptides, adhesion molecules, growth factors, receptors, matrix metalloproteases, and plasma proteases, are cleaved to generate biologically active proteins. The recognition sequences for many proteolytic cleavage reactions are often paired basic amino acid residues like arginine-arginine or arginine-lysine (Rehemtulla *et al.*, 1992). One example is the paired basic amino acid cleaving enzymes (PACE), a serine protease that recognizes and cleaves arginine-arginine or arginine-X-lysine sequences (where X is any other amino acid residue) (Rehemtulla *et al.*, 1992; Seidah & Chretien, 1997; Wise *et al.*, 1990). Trypsin only cleaves the carboxyl side of lysine and arginine residues and thrombin hydrolyses arginine-glycine bonds on specific peptide chains. While the effect of methylglyoxal on these cleavage reactions is not completely understood, the presence of phenylglyoxal-arginine adducts in RNase-A inhibits proteolytic cleavage by trypsin and chymotrypsin (Takahashi, 1968). Glycated proteins are in fact highly resistant to proteolysis (Fu *et al.*, 1992; Fu *et al.*, 1994). A reduction in proteolytic cleavage reactions may prevent the final processing of many proteins, resulting in their eventual degradation (Dorner *et al.*, 1992). Many signal sequences that dictate the final intracellular protein localization usually contain lysine and arginine residues (Pohlschroder *et al.*, 2005). One example is the twin arginine translocation pathway in which the recognized peptide signal sequence contains an almost invariant twin arginine surrounded by a less conserved motif (Pohlschroder *et al.*, 2005).

Cysteine, lysine and arginine residues are also generally located in putative active site of the tyrosine kinase receptor family and therefore glycation of these proteins will interfere with cell signalling (Portero-Otin *et al.*, 2002).

A direct link between glycation of specific protein targets and cellular dysfunction *in vivo* has been described, mainly in the context of diabetes *mellitus* and related clinical complications. The interference on the normal physiology of low-density lipoproteins (LDL) particles is perhaps the best understood. The glycation process occurs both on the apolipoprotein B (apoB) (Bucala *et al.*, 1994; Bucala *et al.*, 1995) and phospholipids (Bucala *et al.*, 1993) LDL components, leading to a diminished recognition of glycated LDL particles by the LDL receptor (Bucala *et al.*, 1994; Bucala *et al.*, 1995) (Figure I.11). Glycation of LDL apoB occurs mainly on a positively charged lysine residue within the putative receptor binding domain, essential for the specific recognition by the LDL receptor (Bucala *et al.*, 1995). Therefore, *in vivo* clearance of glycated LDL is greatly reduced (Bucala *et al.*, 1984; Steinbrecher & Witztum, 1984). Additionally, glycation increases LDL susceptibility to oxidative modifications (Bucala *et al.*, 1993) (Figure I.11) and oxidized LDL are preferentially recognized by the macrophage scavenger receptor (Klein *et al.*, 1995). Hence, glycation promotes directly an increased LDL uptake by the scavenger receptor enhancing foam cell formation, an earlier step in the development of atherosclerosis (Dominiczak, 1997).

Portero-Otín and co-workers reported another example of receptor signalling impairment by glycation, in which methylglyoxal and glyoxal inhibit the activation of the epidermal growth factor receptor that regulates multiple cellular processes such as cell growth, mobility, differentiation, survival and death as a response to the epidermal growth factor (Portero-Otin *et al.*, 2002). Interestingly, endothelial cells exposed to high glucose concentration show a mitogenic activity reduction of 70% due to glycation of the basic fibroblast growth factor (Giardino *et al.*, 1994). Methylglyoxal also disturbs the interaction of endothelial cells with extracellular matrix by reacting within a short colagenous region containing arginine-glycine-aspartate (R-G-D) sequence that mediates endothelial cell adhesion to type IV collagen (Pedchenko *et al.*, 2005). Glucose has no effect on cell adhesion, illustrating once more the relevance of methylglyoxal as the main protein glycation agent *in vivo* (Pedchenko *et al.*, 2005). It was also reported that

glycation induces functional changes on the membrane attack complement regulatory protein CD59, which inhibits the activity of the complement system (Acosta *et al.*, 2000). CD59 glycation promotes its inactivation, stimulating the proliferation of fibroblast and smooth muscle cells induced by the formation of the membrane attack complement in blood vessels and the consequent release of growth factors and cytokines (Acosta *et al.*, 2000).

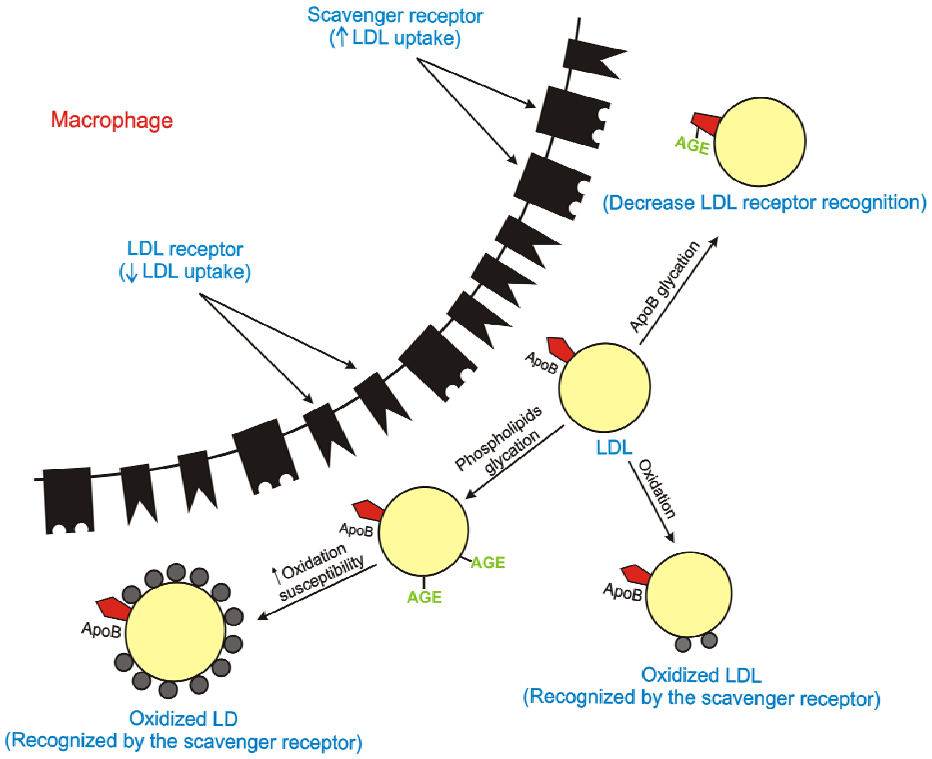


Figure I.11. LDL glycation and atherosclerosis. Formation of AGE-modified LDL particles increases the susceptibility for oxidative modifications. Oxidized LDL is recognized by the macrophage scavenger receptor instead of the LDL receptor. Glycation apoB may also reduce LDL recognition by the LDL receptor. LDL uptake by the scavenger receptor leads to foam cell formation that promotes atherosclerosis. Adapted from (Aronson & Rayfield, 2002).

The real significance of protein glycation is not yet perceived, mainly in other diseases besides diabetes *mellitus*, where it is becoming evident that protein glycation plays an important role.

4.1.2. AGE:RAGE interaction

AGE-modified proteins also exert cellular effects via interaction with specific AGE receptors, including the macrophage scavenger receptor (el Khoury *et al.*, 1994) and the receptor for AGE (RAGE), the most extensively investigated receptor (Neeper *et al.*, 1992). RAGE, a member of the multi-ligand immunoglobulin superfamily of receptors (Neeper *et al.*, 1992), is composed by three extracellular domains, namely a V-type domain with ligand binding properties and two C-type immunoglobulin domains (Neeper *et al.*, 1992; Schmidt *et al.*, 1992; Schmidt *et al.*, 2001). Besides the extracellular region, this receptor also includes a single transmembrane-spanning domain that anchors RAGE to the membrane, and a short highly charged, cytosolic domain at the C-terminal (Neeper *et al.*, 1992; Schmidt *et al.*, 1992; Schmidt *et al.*, 2001). The latter intracellular domain probably binds to signal transduction molecules in the cytoplasm to recruit cellular effector mechanisms after RAGE:ligand interaction. The C-truncated isoform of RAGE, termed soluble RAGE, lacks the transmembrane-anchoring domain and consequently is found in plasma (Malherbe *et al.*, 1999). This cell surface receptor is found in smooth muscle cells, monocyte-derived macrophages, endothelial cells and neurons (Brett *et al.*, 1993; Schmidt *et al.*, 2001). Although expressed at low levels in normal tissues, it becomes up-regulated where its putative ligands accumulate (Brett *et al.*, 1993; Li *et al.*, 1998; Li & Schmidt, 1997; Tanaka *et al.*, 2000).

RAGE acts as a signal transduction receptor not only for CML-modified proteins (Kislinger *et al.*, 1999), but also for MAGE modifications (Westwood *et al.*, 1994). It was reported that methylglyoxal-modified arginine residues could function as a specific signal for receptor-mediated recognition (Westwood *et al.*, 1997). Similar cellular effects were observed in human-cultured mesangial cells exposed to BSA glycated by glucose, glyceraldehyde or glycoaldehyde (Yamagishi *et al.*, 2002).

The AGE:RAGE interaction elicits a wide range of cellular responses, including increased endothelial permeability (Wautier *et al.*, 1996), 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; Yamagishi *et al.*, 1997), cell proliferation (Kirstein *et al.*, 1992; Kirstein *et al.*, 1990; Seki *et al.*, 2003) and inflammatory processes (Basta *et al.*, 2002; Sainhas *et al.*, 1998; Schmidt *et al.*, 2001). Although the precise mechanism of some of these responses is not yet fully understood, it is likely to involve the release of mediators like the pro-inflamatory cytokine interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) (Vlassara *et al.*, 1988), interleukin-6 (IL-6) (Schmidt *et al.*, 1994), the growth factors platelet-derived growth factor (PDGF) (Kirstein *et al.*, 1990) and insulin growth factor-1 (IGF-1) (Kirstein *et al.*, 1992). Moreover, monocyte chemoattractant protein-1 (MCP-1) (Yamagishi *et al.*, 2002) and vascular cell adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1) (Schmidt *et al.*, 1995) and intercellular adhesion molecule 1 (ICAM-1) (Basta *et al.*, 2002), are also induced by AGE:RAGE interaction (Figure I.12). Altogether, these responses could induce cellular dysfunction, being involved in several pathological conditions. Increased endothelium permeability, for example, leads to increased lipid entry into the subendothelium, relevant in the pathogenesis of atherosclerosis (Dominiczak, 1997). Furthermore, through RAGE interaction, AGE-modified proteins may prime pro-inflammatory mechanisms, thereby amplifying the inflammatory response (Basta *et al.*, 2002).

The interaction between RAGE and AGE-modified proteins enhances cellular oxidative stress, which appears to mediate this receptor signal transduction by activating the transcriptional factor NFκB (Yan *et al.*, 1994b). One known RAGE-dependent cellular signal pathway involves the induction of oxidative stress with $p21^{ras}$ activation that will subsequently activate MAP kinases and ultimately NFκB, resulting in the transcription of target genes (Lander *et al.*, 1997; Schmidt *et al.*, 1995; Yan *et al.*, 1994b). Noteworthy, RAGE gene has a putative NFκB binding site (Li & Schmidt, 1997). Therefore, AGE:RAGE interaction will trigger a positive feedback in which the increase of RAGE expression enhances the ability of this cell receptor for subsequent binding of more ligands molecules (Schmidt *et al.*, 1999; Tanaka *et al.*, 2000).

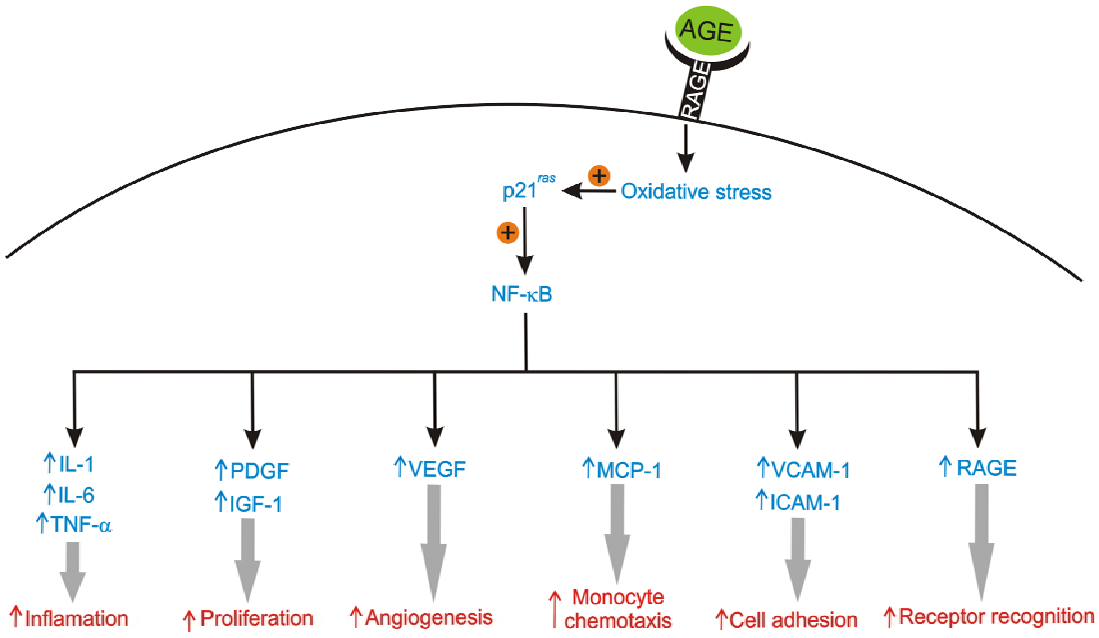


Figure I.12. Cellular responses to AGE:RAGE interaction. These responses are mediated by NF-κB, which is activated by the oxidative stress-dependent $p21^{ras}$ pathway. By these mechanisms, AGE can mediate cell toxicity.

RAGE is a multi-ligand receptor interacting with other ligands besides AGE. The observation that this receptor is highly expressed during development, especially in the central nervous system, led to the discovery that RAGE is a cellular binding site for amphoterin, a protein associated with basement membranes and abundant in the developing central nervous system (Hori *et al.*, 1995). RAGE-amphoterin interaction promotes neurite outgrowth in rat cortical neurons cultures (Hori *et al.*, 1995; Huttunen *et al.*, 1999). This observation indicates that RAGE has physiological relevant ligands distinct from AGE, being involved in normal physiological processes outside the context of AGE related disorders. Other important RAGE ligands are the amyloid β-sheet fibrils composed of amyloid β-peptide, structures found in Alzheimer's disease (Du Yan *et al.*, 1997b; Yan *et al.*, 1996). The interaction between RAGE and amyloid β-peptide also promotes NFκB activation and the production of macrophage-colony stimulating factor (Du Yan *et al.*, 1997a). Amyloid forming β-sheet fibrils of amylin, serum amyloid A and

prion protein are also ligands for this receptor (Yan *et al.*, 2000). Also, transthyretin amyloid fibrils are recognized by RAGE, eliciting a cellular response (Sousa *et al.*, 2001b; Sousa *et al.*, 2000). These observations raised the question whether RAGE may function as a signal transduction receptor for β-sheet fibrils. Coincidence or not, protein glycation leads to a β-sheet content increase (Bouma *et al.*, 2003) and several evidences raised the hypothesis that glycation directly promotes amyloid fibril formation (Bouma *et al.*, 2003; Ledesma *et al.*, 1994).

4.1.3. Oxidative stress induced by glycation

The association between glycation and oxidative stress is well documented, being both processes mutually stimulated. The previously referred glycoxidation theory implies that oxygen and oxidation reactions play an important role in the Maillard reaction (Baynes, 1991; Elgawish *et al.*, 1996; Fu *et al.*, 1994). Due to their chemical properties, AGE-modified proteins can cause oxidative stress *per se* (Elgawish *et al.*, 1996). The transition metal-catalysed autoxidation of glucose and oxidative degradation of Schiff's base and protein-bound Amadori products yield superoxide, hydroxyl-radicals and hydrogen peroxide (Hunt *et al.*, 1993; Wolff, 1993; Wolff & Dean, 1987). So, in addition to the oxidative stress' role as a modulator of AGE formation, glycated proteins can also enhance oxidative stress. For example, LDL glycation, increases LDL susceptibility to oxidative damage (Bucala *et al.*, 1993). *In vivo*, it was shown that AGE deposits in the arterial wall could themselves generate free radicals capable of oxidizing vascular wall lipids and accelerate atherogenesis in hyperglycaemic diabetic patients (Mullarkey *et al.*, 1990). Mitochondrial protein glycation is also associated with mitochondria-induced oxidative stress (Rosca *et al.*, 2005).

In earlier investigations, free radicals formation was observed upon the reaction of methylglyoxal with proteins (McLaughlin *et al.*, 1980; Sakurai & Tsuchiya, 1988). Using L-alanine as a model, Yim and co-workers found three types of free radical species produced upon methylglyoxal incubation: a cross-link radical cation, methylglyoxal radical anion and superoxide radical anion (Yim *et al.*, 1995). Transition metals or

oxygen are not required to form of the cross-link radical cation and methylglyoxal radical anion. However, oxygen can accept an electron from the methylglyoxal radical anion generating superoxide radical and initiating damaging chain reactions (Yim *et al.*, 1995). BSA glycated by methylglyoxal also generates protein-bound cross-link free radicals, like the ones observed for alanine (Lee *et al.*, 1998). Moreover, glycated BSA is capable of catalysing oxidative modification of macromolecules, which suggests that *in vivo* accumulation of glycated proteins provides a stable locus for free radicals production, with consequent cellular damage (Lee *et al.*, 1998) (Figure I.13).

The increased oxidative stress, caused by AGE-modified proteins or through RAGE interaction, may be directly involved in a wide range of pathological conditions.

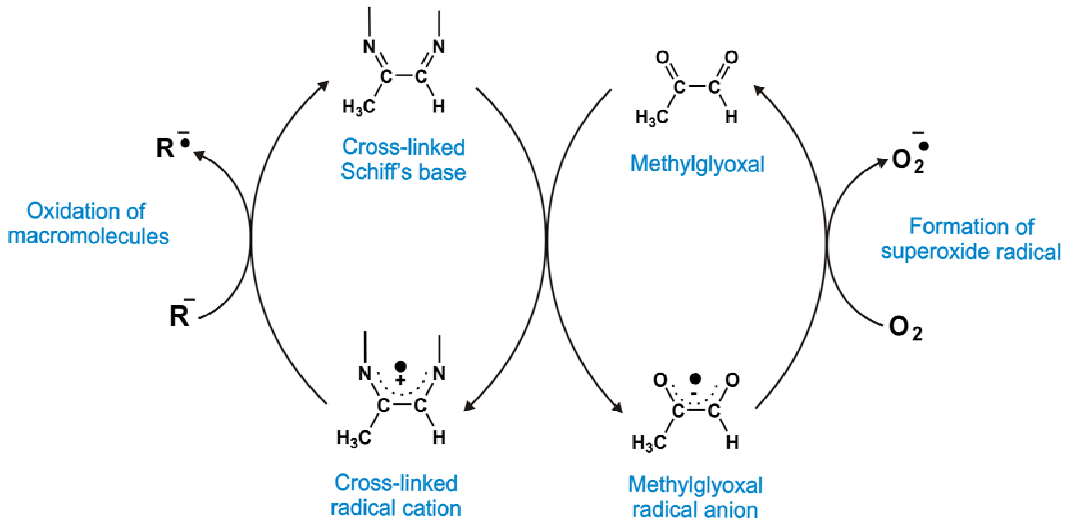


Figure I.13. Generation of oxidative stress during methylglyoxal-derived protein glycation. The reaction between methylglyoxal and protein amino groups leads to the formation of cross-link Schiff's base which can donate an electron directly to methylglyoxal producing two radicals: cross-link radical cation and methylglyoxal radical anion. Oxygen can accept an electron from the anion to generate a superoxide radical anion. Importantly, glycated proteins can oxidize macromolecules contributing to oxidative damage. Adapted from (Lee *et al.*, 1998).

4.2. Glycation in amyloid diseases

Amyloidosis is a generic term used to designate a group of clinical and biochemically diverse diseases characterized by protein deposition into insoluble fibrils with a characteristic structure, termed amyloid deposits (Ghiso *et al.*, 1994; Sipe, 1992). There are over 16 biochemically distinct amyloidosis, each one characterized by the deposition of a particular amyloidogenic protein (Ghiso *et al.*, 1994; Sipe, 1992). Several types of amyloidosis and their amyloid protein precursors were identified (Ghiso *et al.*, 1994). Alzheimer's and Parkinson's diseases are both neurodegenerative disorders of amyloid type affecting the central nervous system (Ross & Poirier, 2004). Alzheimer's disorder is characterized by extracellular deposits of amyloid β-peptide and intracellular amyloid deposits of tau protein (Ghiso *et al.*, 1994), whereas α-synuclein amyloid deposits are characteristic of Parkinson's disease (Lucking & Brice, 2000). In familial amyloidotic polyneurophaty (FAP), a neurodegenerative amyloidosis affecting the peripheral nervous system, amyloid deposits are mainly composed of transthyretin (Andrade, 1952; Costa *et al.*, 1978). Although there is no obvious sequence homology between the different amyloidogenic proteins, all amyloid deposits share particular biochemical features such as high insolubility and proteolysis resistance, a β-pleated sheet structure and similar tinctorial properties like apple-green birefringence under polarized light after Congo red staining and yellow-green fluorescence with thioflavin S (Ghiso *et al.*, 1994; Sipe, 1992) (Figure I.14). These similarities suggest that common mechanisms are involved in this type of disorders. Since amyloidogenic proteins are normally innocuous it is believed that they undergo several structural modifications that eventually result in amyloid deposit formation. Numerous point mutations are associated with the amyloidogenic behaviour of several proteins like transthyretin (Saraiva, 2001), Aβ protein (Haass *et al.*, 1994) and α-synuclein (Pankratz & Foroud, 2004; Valente *et al.*, 2004). In FAP, more than 80 transthyretin point mutations were associated with amyloid fibril formation (Saraiva, 2001). However, non-mutated transthyretin can also form amyloid deposits (Westermark *et al.*, 1990), hinting for the complexity of amyloid fibril formation pathways where several factors beyond genetic determinants may play an important role.

The abnormal proteolytic processing and/or post-translational modifications including oxidation, phosphorylation, glycosylation, methylation and glycation, are probably involved in amyloidogenesis. Due to the biochemical similarities between glycated proteins and neurophatological amyloid lesions, Colaco and Harrington proposed that protein glycation might account for amyloid formation *in vivo* (Colaco & Harrington, 1994; Harrington & Colaco, 1994). Glycated proteins and amyloid deposits are highly insoluble, protease resistant, with characteristic cross-link structure, fluorescent and brown-coloured compounds (Colaco & Harrington, 1994; Harrington & Colaco, 1994). Moreover, glycation may cause protein aggregation and cross-link (Eble *et al.*, 1983) producing detergent-insoluble, protease resistant aggregates similar to those isolated from the brain of Alzheimer's disease patients (Ledl & Schleicher, 1990). In agreement with this hypothesis, AGE-modified proteins were detected in amyloid deposits from several amyloidosis such as Alzheimer's (Smith *et al.*, 1994; Yan *et al.*, 1994a) and Parkinson's diseases (Castellani *et al.*, 1996; Munch *et al.*, 2000) and dialysis-related amyloidosis (DRA) (Miyata *et al.*, 1993).

The contribution of protein glycation to amyloid formation and toxicity has been investigated in the context of DRA and Alzheimer's disease. In DRA, amyloid deposits are derived from β_2-microglobulin (β_2M), which renal clearance is decreased in this pathology (Gejyo *et al.*, 1986). β_2M isolated from amyloid fibrils is markedly different from native β_2M showing a more acidic pI, brown colour and fluorescence, all biochemical characteristics of AGE-modified proteins (Miyata *et al.*, 1993). Indeed, β_2M in amyloid plaques is strongly labelled with anti-AGE antibody, indicating that AGE-modified β_2M is the dominant component of DRA amyloid deposits (Miyata *et al.*, 1993). The α-amino group of isoleucine residue from the N-terminal is the primary site for β_2M AGE modification (Miyata *et al.*, 1994b). Contrary to the native protein, AGE-β_2M purified from long-term haemodialysis patients induces monocyte chemotaxis and macrophage activation (Miyata *et al.*, 1994a), which could explain the observation that DRA amyloid deposits are surrounded by macrophages and other inflammatory cells (Depierreux *et al.*, 1988). By RAGE interaction, AGE-β_2M can initiate a local inflammatory response with the release of IL-1, TNF-α and IL-6, leading to the connective tissue degeneration and bone and joint destruction, characteristic of DRA

(Miyata *et al.*, 1996; Miyata *et al.*, 1994a). Contrary to diabetes *mellitus* and related clinical complications, enhanced AGE content is attributed to an increase of carbonyl compounds (carbonyl stress) found in chronic renal failure (Miyata *et al.*, 1997; Miyata *et al.*, 2000). Oxidative stress increase associated with uraemia (Miyata *et al.*, 1997) may also promote AGE formation.

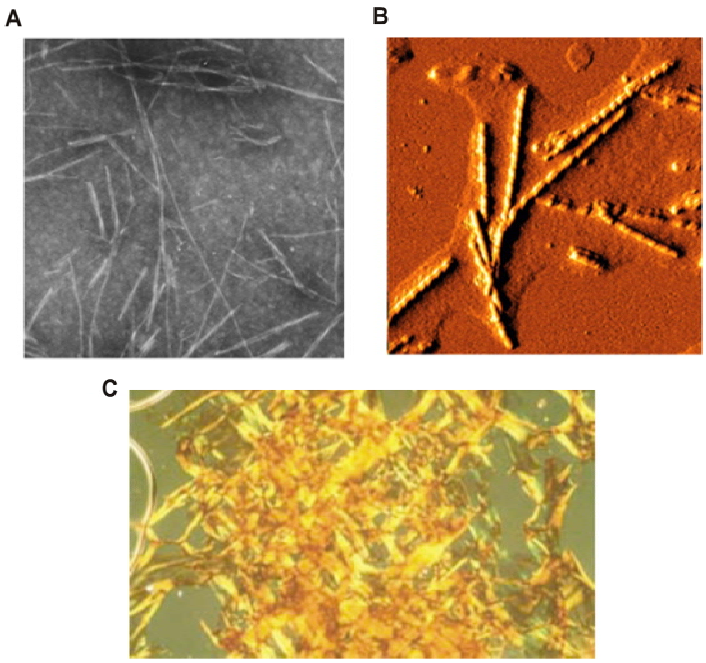


Figure I.14. Amyloid deposits. (A) Electron microscopy of β_2-microglobulin-derived amyloid deposits. (B) Atom force microscopy of β_2-microglobulin amyloid fibril. Images were obtained from (Chatani *et al.*, 2006; Kihara *et al.*, 2006). (C) Amyloid fibrils show unique tinctorial properties, such as apple-green birefringence under polarised light upon staining with Congo-red. TTR-amyloid fibrils are shown. Image obtained from (Saraiva, 2002).

In Alzheimer's disease, both extracellular Aβ-peptide amyloid plaques and intracellular neurofibrillary tangles of tau protein are highly modified with AGE (Smith *et al.*, 1994; Vitek *et al.*, 1994; Yan *et al.*, 1994a). It was therefore suggested that protein modifications through the Maillard reaction could stabilize the amyloid deposits, accounting for their high insolubility and protease-resistance (Smith *et al.*, 1994). AGE-

modified proteins in neurofibrillary tangles are associated with oxidative stress, based on histochemical evidence from increased levels of malondialdehyde epitopes and heme oxigenase antigens (Yan *et al.*, 1994a). Likewise, recombinant tau protein glycated *in vitro* generates reactive oxygen species and higher molecular mass aggregates, and induces oxidative stress in neuroblastoma cells (Yan *et al.*, 1994a). Tau protein isolated from neurofibrillary tangles contains two major post-translational modifications, phosphorylation and glycation (Ledesma *et al.*, 1994). Both these modifications are necessary for the formation of protein aggregates similar to those found *in vivo* (Perez *et al.*, 2002). Like in DRA, it is believed that protein glycation occurs due to carbonyl stress increase (Munch *et al.*, 2003; Perez *et al.*, 2002). Glycation of β-amyloid protein also promotes the nucleation and precipitation of this protein, suggesting an additional mechanism by which protein glycation may accelerate the progression of Alzheimer's disease (Vitek *et al.*, 1994). Protein cross-link derived from glycation *in vivo* might stabilize the specific β-amyloid peptide conformation and promoting further aggregation (Vitek *et al.*, 1994). Besides the structural changes induced by glycation, AGE-modified tau induces neuronal oxidative stress, resulting in an increased expression of cytokine genes and amyloid precursor protein as well as the release of Aβ peptide (Yan *et al.*, 1995).

The mechanisms underlying the toxicity of amyloid fibril formation are not yet known. In Alzheimer's disease, it is believed that neurons are subjected to the deleterious cytotoxic effects of activated microglia and astroglia (Wong *et al.*, 2001), to exacerbate inflammatory processes (Gasic-Milenkovic *et al.*, 2003) and increase oxidative stress (Dickson, 2004). Reactive oxygen species can be generated during AGE modifications (Lee *et al.*, 1998; McLaughlin *et al.*, 1980; Sakurai & Tsuchiya, 1988) and the interaction with cellular receptors can also induce oxidative stress (Lander *et al.*, 1997; Schmidt *et al.*, 1995; Yan *et al.*, 1994b), as well as the production of pro-inflamatory citokynes (Schmidt *et al.*, 1994; Vlassara *et al.*, 1988). Hence, protein glycation could be directly involved in the toxicity of amyloid deposits. The intracellular glycation of tau protein could also generate intracellular ROS, modulating cellular functions in a sustained fashion (Yan *et al.*, 1994a). In Parkinson's disease, oxidative stress is also related to

AGE-modified proteins found in Lewy bodies of α-synuclein deposits (Castellani *et al.*, 1996).

The presence of the MAGE argpyrimidine in amyloid deposits isolated from FAP patients was unequivocally identified by chromatographic methods (Gomes *et al.*, 2005a). Just like in DRA, Alzheimer's and Parkinson's diseases, carbonyl concentrations are significantly higher in amyloid-rich tissues of FAP patients compared to control subjects (Ando *et al.*, 1997). Importantly, a clear relationship between transthyretin amyloid deposits and RAGE, the AGE receptor, was shown (Matsunaga *et al.*, 2002). These observations hint for a possible implication of protein glycation in this neurodegenerative amyloidosis.

Even though glycation is involved in amyloidosis, it is still controversial whether glycation of susceptible proteins could be an initial event in amyloid fibril formation or merely a result of amyloid fibril accumulation due to the longevity of the protein components which, as a result of their extreme insolubility and protease resistance, persist in the body for long periods of time. Nevertheless, several lines of evidence suggest that glycation directly promotes or accelerates abnormal protein deposition into β-fibrils structures characteristic of these pathologies. Münch and co-workers found AGE in very early Lewy bodies suggesting its involvement in protein cross-link and the formation of insoluble, non-degradable aggregates (Munch *et al.*, 2000). In addition, glycation of tau protein leads to the formation of amyloid-like structure (Ledesma *et al.*, 1994). Regardless of the exact chronology of AGE accumulation, glycation increases protein insolubility and protease resistance (Fu *et al.*, 1992; Fu *et al.*, 1994), decreasing its turnover (Figure I.15). The accumulation of AGE-modified proteins also leads to inflammation and propagation of tissue damage by several mechanisms like oxidative stress increase and release of pro-inflammatory cytokines mediated by AGE:RAGE interaction (Figure I.15). Since amyloidosis are multifactor diseases, glycation may well be one progression factor superimposed on a pre-existent pathologic state in which this post-translational modification accelerates and determines the course of neuronal disease (Yan *et al.*, 1994a). It is becoming evident that AGE in these deposits are not only static by-products of disease, but rather dynamic participants in neuronal dysfunction, inducing several cellular responses that lead to cell dysfunction and death.

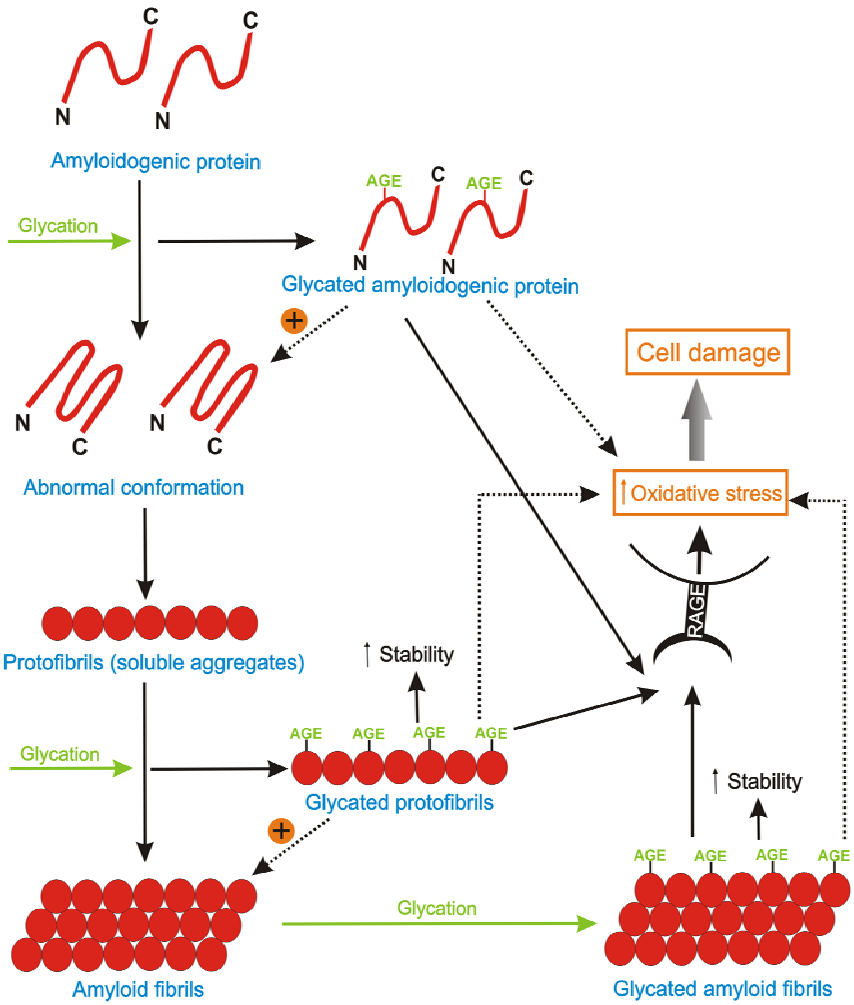


Figure I.15. Potential role of protein glycation in amyloid diseases. Glycation of the amyloidogenic protein may induce conformational changes, and may also be involved in the transition of soluble aggregates to amyloid fibrils. The glycation of amyloid fibrils will increase their stability, just like glycation of the soluble aggregates. In each case, protein glycation may be involved in cellular damage by increasing oxidative stress or by activating different cellular responses such as inflammation through by AGE:RAGE interaction.

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. SUMMARY

Methylglyoxal is the most important intracellular glycation agent, formed non-enzymatically 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 polyneuropathy) and in the clinical complications of diabetes *mellitus*. 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 non-growing yeast cells to a higher D-glucose concentration (250 mM) increases methylglyoxal formation rate and argpyrimidine-modified proteins appear within 1 h. A kinetic model of methylglyoxal metabolism in yeast, comprising its non-enzymatic 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 (Brownlee, 1995; Bucala & Cerami, 1992). 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 (Bouma *et al.*, 2003; Du Yan *et al.*, 1997a). In Alzheimer's disease (β-amyloid deposits) and FAP (transthyretin deposits) glycation is present in extracellular amyloid deposits (Chen *et al.*, 2004; Gomes *et al.*, 2005a; Vitek *et al.*, 1994). Intracellular protein glycation also occurs in amyloid fibrils in Alzheimer's disease (τ deposits) and Lewy inclusion bodies of α-synuclein in Parkinson's disease (Castellani *et al.*, 1996; Yan *et al.*, 1994a). 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.*, 2005a).

Because AGE 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).

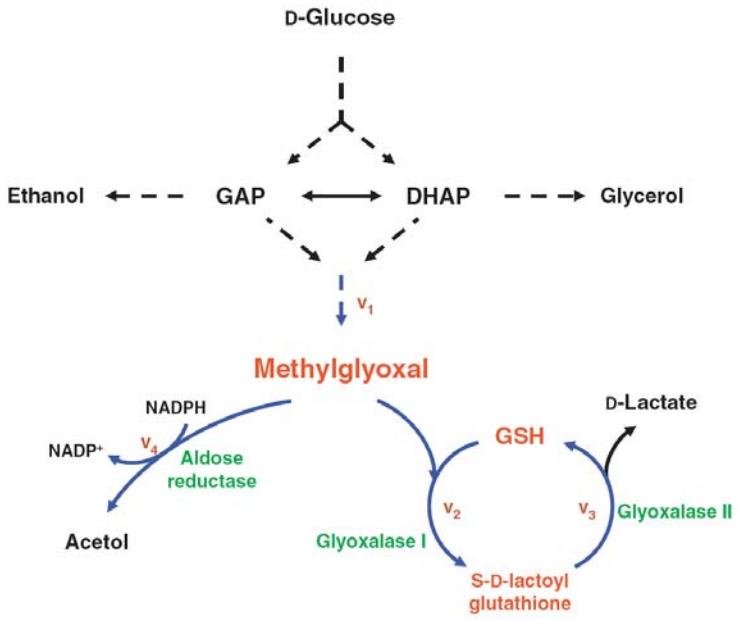


Figure II.1. Methylglyoxal metabolism in *S. cerevisiae*. Methylglyoxal is formed non-enzymatically from DHAP and GAP during glycolysis. It is converted into D-lactate by the glyoxalase system or acetol through aldose reductase. This metabolic map was used to build a mathematical model comprising the 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.

Yeast cells growing on D-glucose show a high glycolytic flux and a high rate of methylglyoxal formation (Martins *et al.*, 2001a), 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.

3. MATERIAL AND METHODS

Reagents and materials

Peptone, yeast extract and agar were obtained from Difco while D-glucose (microbiology grade) was from Merck. Mes, potassium dihydrogen phosphate, methylglyoxal 1,1-dimethyl acetal and monobromobimane were acquired from Fluka. Digitonin was obtained from CalBiochem. Coomassie brilliant blue G, Ponceau S, dithiothreitol, phenylmethylsulfonyl fluoride (PMSF), glass beads (452–600 microns), *S*-D-lactoylglutathione (SDLGSH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 1,2-diaminobenzene were obtained from Sigma. 2,3-Dimethylquinoxaline was purchased from Aldrich while NADPH and GSH were obtained from Roche. Solvents were of HPLC grade while all other reagents were of analytical grade.

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 ºC 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.

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).

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 ºC. 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 $ml.min^{-1}$.

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 ºC 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 ºC 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 ºC). 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.

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 ºC, in an orbital shaker incubator (Infors HT). Enzyme activities were determined at 30 ºC 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.*, 2001b). Kinetic parameters were determined by varying SDLGSH initial concentration between 0.1 and 1.5 mM.

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.*, 2001b; 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

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.

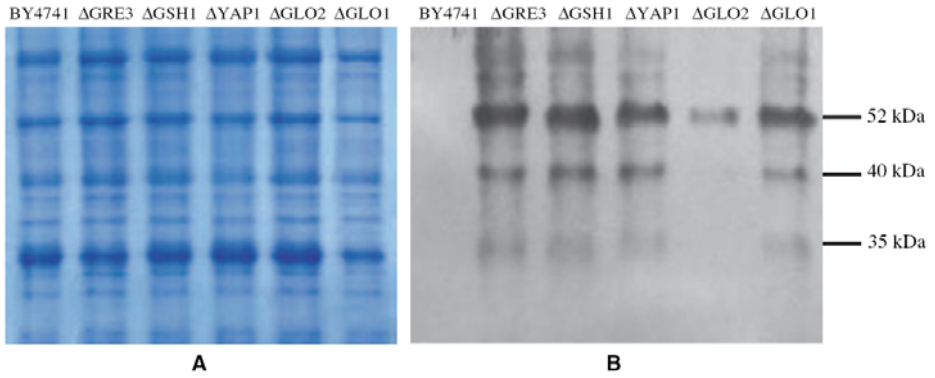


Figure II.2. Protein glycation in yeast cells. (A) Total protein Coomassie blue stained gel of the reference strain (BY4741) and mutant strains (ΔGRE3, ΔGSH1, ΔYAP1, ΔGLO2 and ΔGLO1). (B) Argpyrimidine formation in intracellular proteins from the same yeast strains as in (A), probed by western blotting 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.

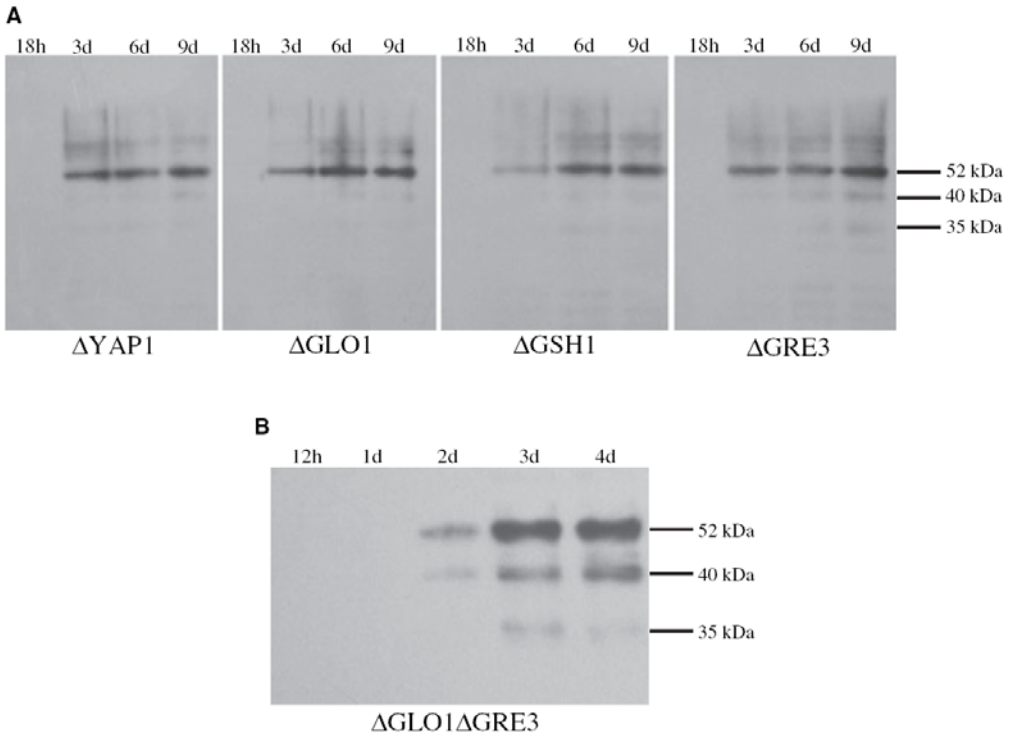


Figure II.3. Time course of argpyrimidine formation in yeast. (A) Time course of argpyrimidine formation in single gene deletion strains. Yeast strains and growth time are shown. (B) Time course of argpyrimidine formation in a double mutant ΔGRE3ΔGLO1, lacking glyoxalase I and aldose reductase. Argpyrimidine 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). AGE-modified proteins were detected by western blot as described. Representative 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.

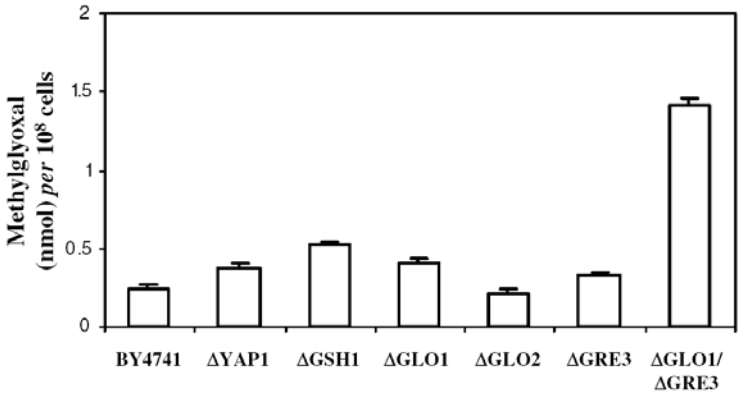


Figure II.4. Methylglyoxal concentration in yeast cells at the end of the exponential phase (18 h of growth). Methylglyoxal was quantified by HPLC as 2-methylquinoxaline after derivatization with 1,2-diaminobenzene. Yeast strains showing glycation present higher levels of methylglyoxal, compared with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

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
$v_2 = \frac{V_1 \times \mathrm{MGO} \times \mathrm{GSH}}{(Km_1 + \mathrm{MGO})(Km_2 + \mathrm{GSH})}$ $v_3 = \frac{V_2 \times \mathrm{SDLGSH}}{Km_3 + \mathrm{SDLGSH}}$ $v_4 = \frac{V_3 \times \mathrm{NADPH} \times \mathrm{MGO}}{(Km_4 + \mathrm{MGO})(Km_5 + \mathrm{NADPH})}$	$\frac{d\,\mathrm{MGO}}{dt} = v_1 - v_2 - v_4$ $\frac{d\,\mathrm{SDLGSH}}{dt} = v_2 - v_3$ $\frac{d\,\mathrm{GSH}}{dt} = v_3 - v_2$	$v_1 = k_1\,\mathrm{GAP} + k_2\mathrm{DHAP}$ $= 2.41 \times 10^{-3}$ 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.

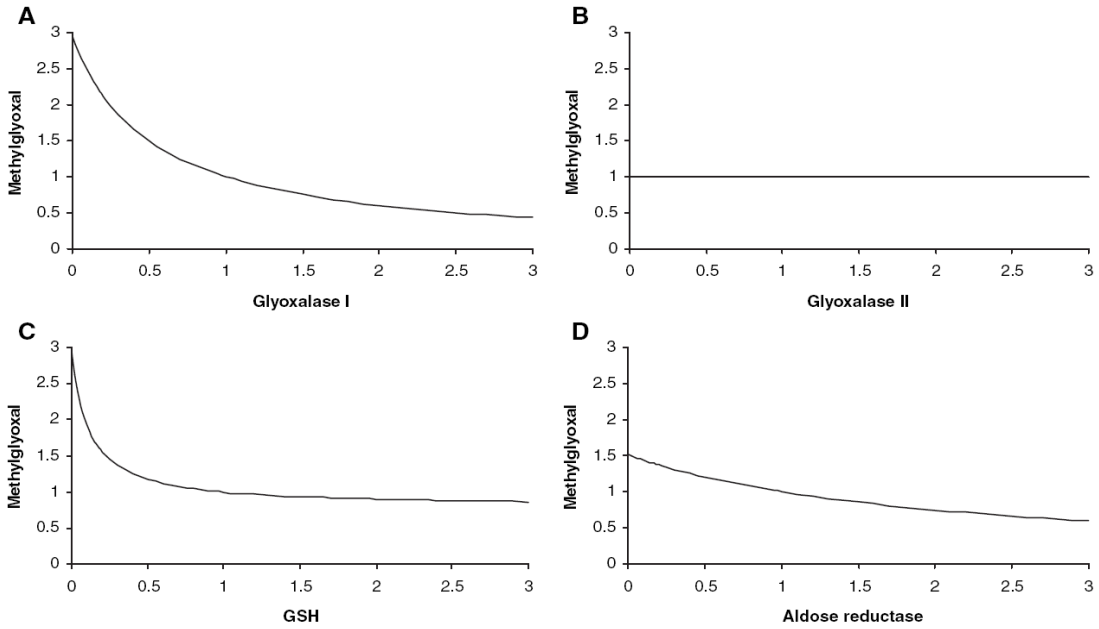


Figure II.5. Sensitivity analysis of methylglyoxal metabolism in *S. cerevisiae*. Single parameter variation. The effects of system parameters on the methylglyoxal intracellular steady-state concentration were investigated by finite parameter changes (between zero- and threefold) around the reference 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).

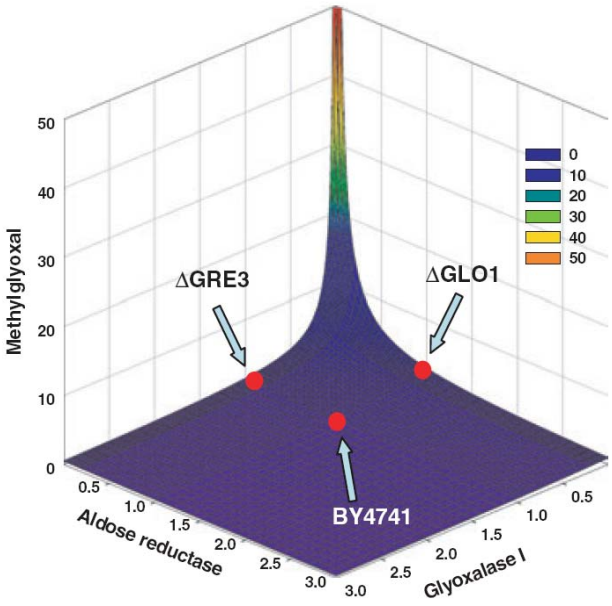


Figure II.6. Sensitivity analysis of methylglyoxal metabolism in *S. cerevisiae*. Synergistic effects of glyoxalase I and aldose reductase activities on methylglyoxal steady-state concentration. The reference strain BY4741 (glyoxalase I and aldose reductase reference activities) and the mutants ΔGRE3 (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).

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 (Aguilera & Prieto, 2001; Aguilera & Prieto, 2004; Inoue *et al.*, 1998), 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). 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).

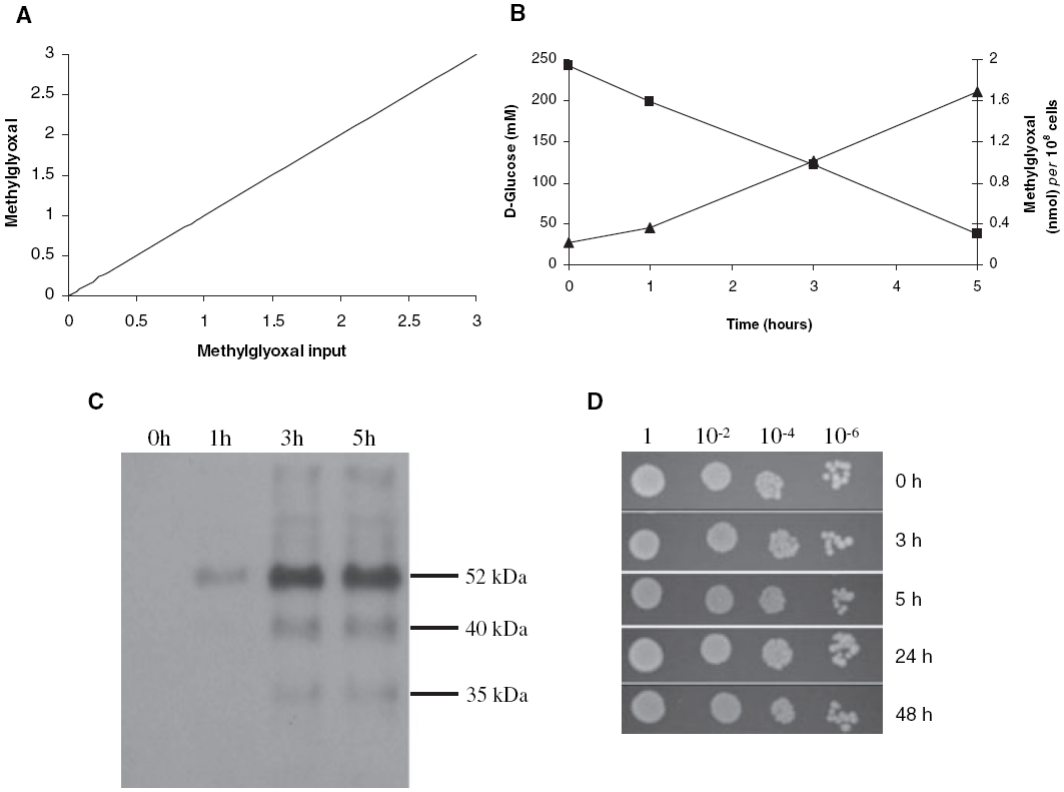


Figure II.7. Predicting glycation phenotype: increasing methylglyoxal concentration causes the formation of argpyrimidine-modified proteins within 1 h in *S. cerevisiae*. (A) Simulated effect of finite changes of methylglyoxal input in methylglyoxal steady-state concentration. All values are fold variations 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). AGE-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.

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 AGE 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.

AGE 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 AGE 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. ACKNOWLEDGMENTS

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.* ***In press****.*

1. SUMMARY

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 (AGE). Glycation is equivalent to a point mutation, exerting profound effects on protein structure, stability and function. AGE 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 τ deposits in Alzheimer's disease (Chen *et al.*, 2004; Vitek *et al.*, 1994; Yan *et al.*, 1994a), in Lewy inclusion bodies of α-synuclein in Parkinson's disease (Castellani *et al.*, 1996) and in transthyretin amyloid deposits in FAP (Gomes *et al.*, 2005a). In all these amyloid pathologies, β-sheet fibril structure and the presence of AGE 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.*, 2005b). 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.*, 2005b). 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.*, 2005b).

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. MATERIAL AND METHODS

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 form 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.

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 ºC 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].

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 ºC 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.

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.*, 2005b). 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 ºC 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 AGE 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.

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 a protein sequence coverage greater than 10%.

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.

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 ºC, 160 r.p.m. in an orbital shaker incubator. Enzyme activities were determined at 30 ºC 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 started 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.

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 *et al.* (Gomes *et al.*, 2005b), which comprises the glyoxalase pathway, aldose reductase and methylglyoxal formation from the triose phosphates. 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).

Protein structure

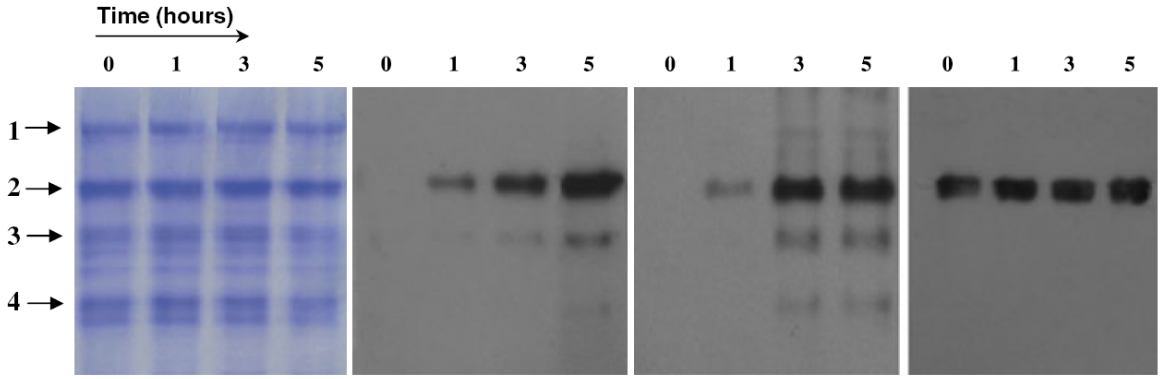
Enolase dimer structure was represented by PDB entry 1ebh, containing Mg. It has 95% identity and 4% homology with enolase2. Molecular graphics images were produced using the UCSF CHIMERA package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (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

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. 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 an AGE, 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.



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 a protein sequence coverage greater than 10%.

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 a CEL modification (Figure III.2B). 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. 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.

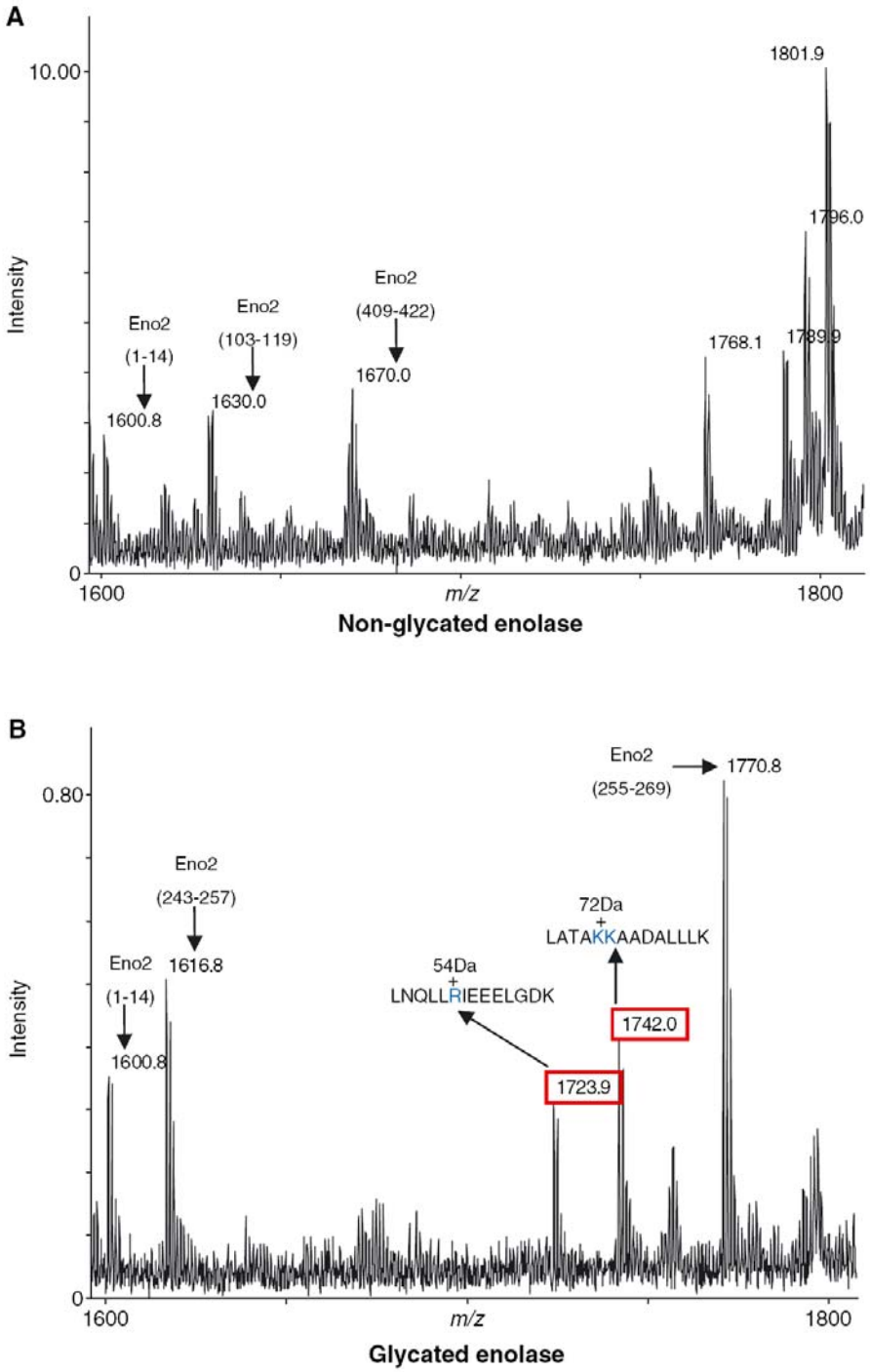


Figure III.2. Detection and molecular location of MAGE in glycated enolase *in vivo*. The figure shows a section of a MALDI-TOF spectrum of tryptic digests of glycated and non-glycated enolase. (A) Mass spectrum of non-glycated enolase. (B) Same section of MALDI-TOF spectrum from glycated enolase. New 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.

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

Identified protein	Observed mass (Da)	Theoretical peptide mass (Da)	Peptide sequence	Mass increase (Da)	MAGE	Glycated resídue
Enolase	1723.92	1669.91	LNQLL**R**IEEEL GDK (409-422)	54	MG-H	R414
	1741.96	1669.03	IATAIE**KK**AA DALLLK (330-345)	72	CEL	K336 or K337
	2178.09	2124.05	SVYDS**R**GNPT VEVELTTEK (9-27)	54	MG-H	R14
Aldolase	1082.66	1002.54	VWV**R**EGEK (332-339)	80	Argp.	R335
Hsp71/72	1736.68	1664.92	IAS**K**NQLESIA YSLK (532-546)	72	CEL	K535
	2058.94	2004.92	**R**LIG**R**NFNDPE VQGDMK (69-85)*	54	MG-H	R69 or R73

* Specific peptide from Hsp72

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 appear, 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).

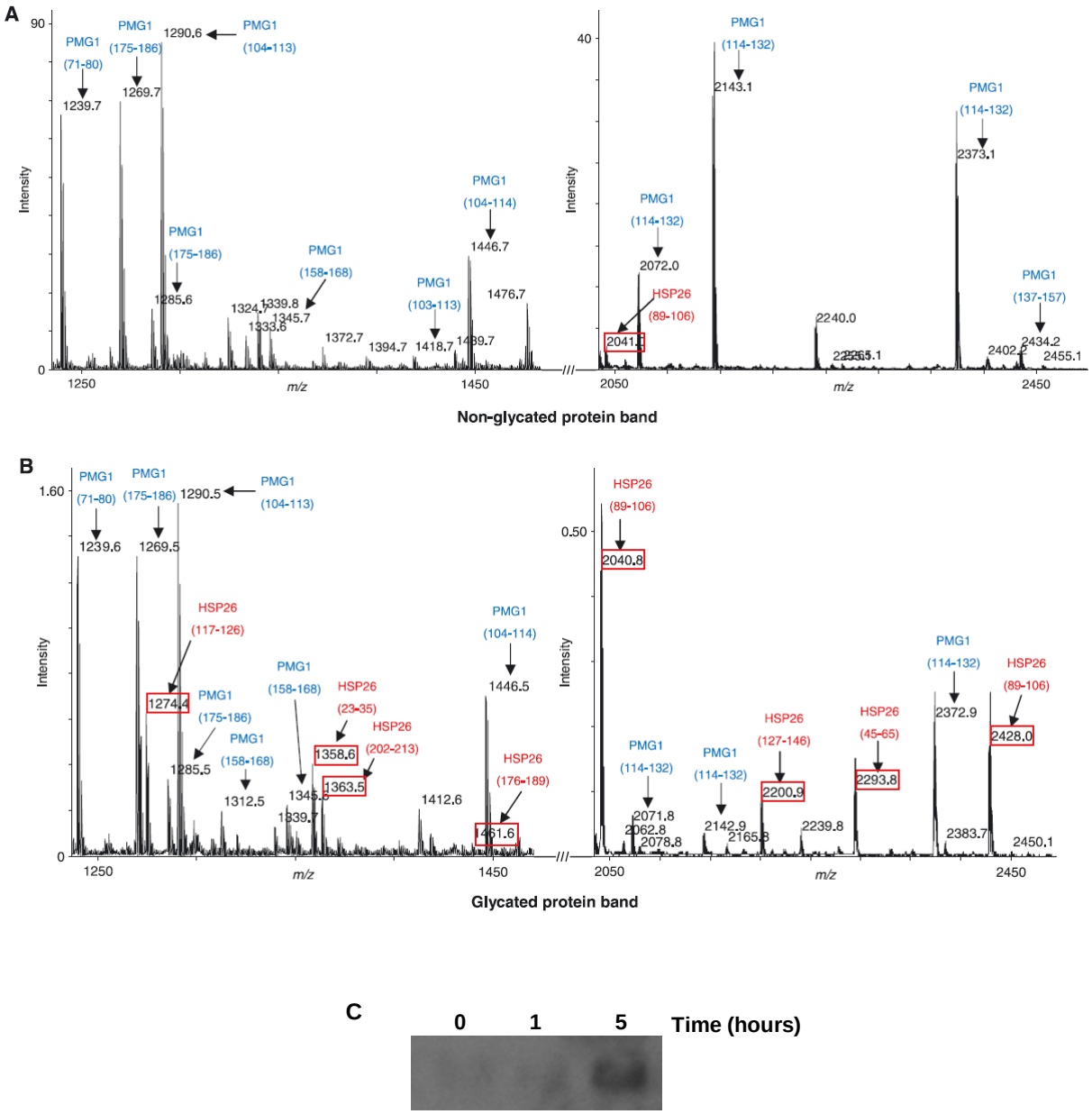


Figure III.3. Detection of Hsp26 upon glycation. (A) MALDI-TOF spectrum of tryptic digestion of the non-glycated protein band 4 (Figure III.1), mainly showing peptides from phosphoglycerate mutase and one peptide, with low intensity, from Hsp26. (B) In glycation conditions, more peptides from Hsp26, 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.

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 bold. MG-H, hydroimidazolone; Argp., argpyrimidine; THP, tetrahydropirimidine.

Identified protein(s)	Observed mass (Da)	Theoretical peptide mass (Da)	Peptide sequence	Mass increase (Da)	MAGE	Glycated resídue
Phosphogly-cerate mutase	1320.48	1239.69	AD**R**LWIPVNR (71-80)	80	Argp.	R73
	1500.55	1446.71	FGEEKFNTY**R**R (104-114)	54	MG-H	R113
	1574.58	1429.72	LNE**R**HYGDLQG K(84-95)	144	THP	R87
	2360.94	2280.28	DLLSGKTVMIA AHGNSL**R**GLVK (169-190)	80	Argp.	R186
Hsp26	1412.63	1358.75	LLGEGGL**R**GYA PR(23-35)	54	MG-H	R30

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.*, 2005b). Strains BY4741, ΔGLO1 and ΔGRE3, with different glycation levels, were challenged with 250 mM D-glucose, and enolase activity was determined *in situ*.

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.

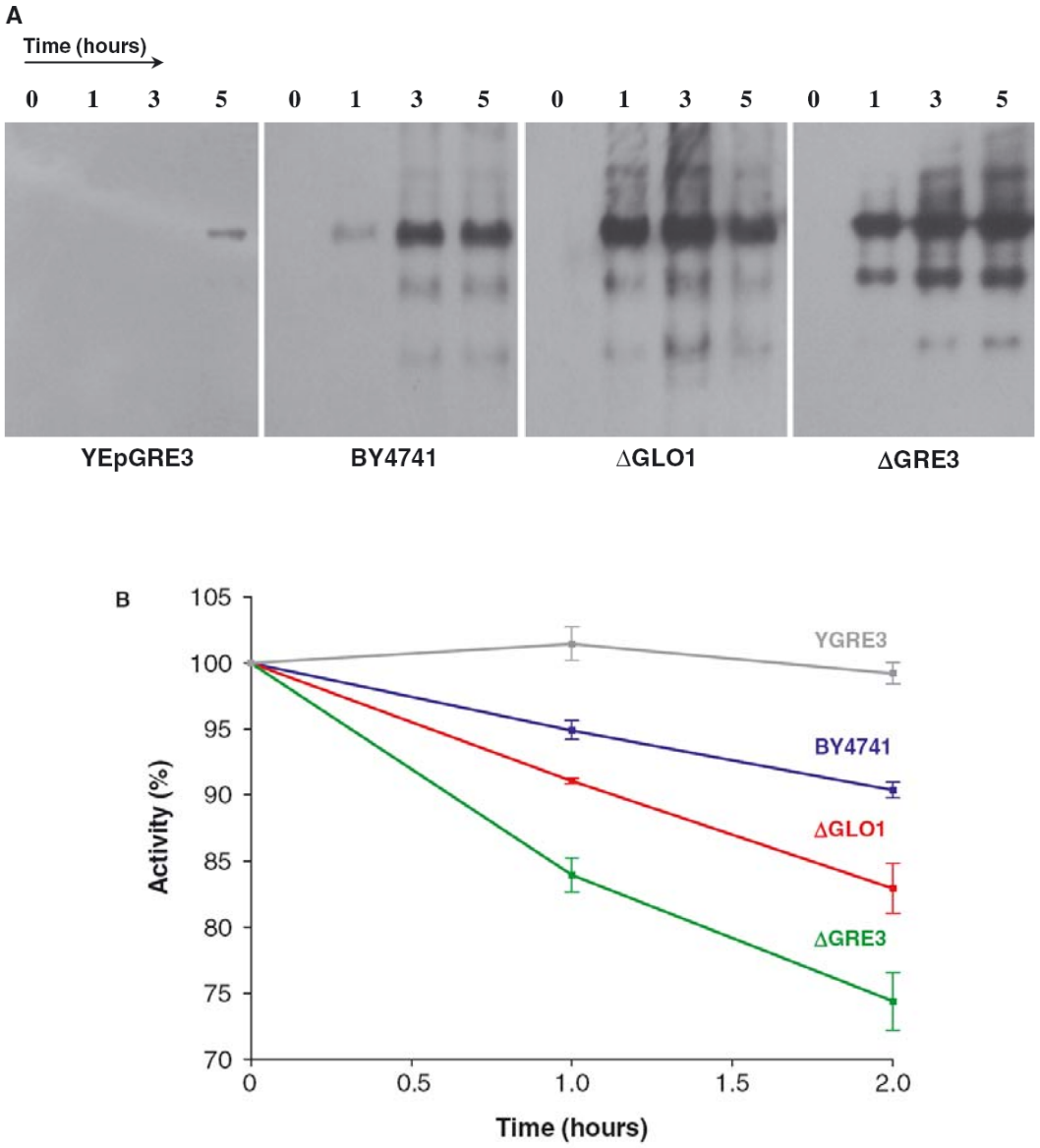


Figure III.4. *In vivo* glycation of enolase causes a decrease of its enzyme activity, directly related to glycation levels. Cells from different yeast strains, incubated with 250 mM D-glucose were sampled at indicated times. Glycated proteins were detected by western blotting using a specific anti-argpyrimidine 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.*, 2005b) 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.

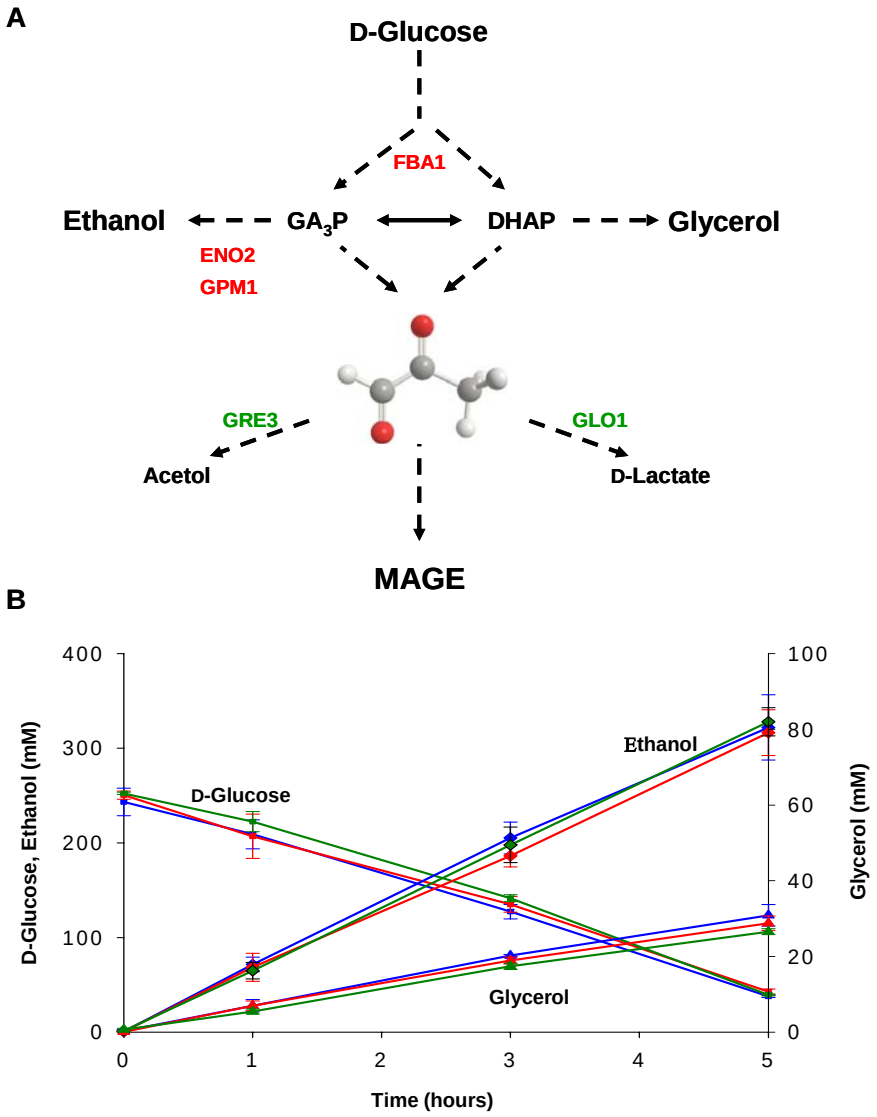


Figure III.5. Glycolysis, methylglyoxal metabolism and glycated proteins in yeast. (A) The well-known glycolytic pathway forms methylglyoxal as a non-enzymatic and unavoidable by-product. The triose phosphates are chemically unstable and suffer an irreversible β-elimination reaction of the phosphate 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.

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 loosing 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.

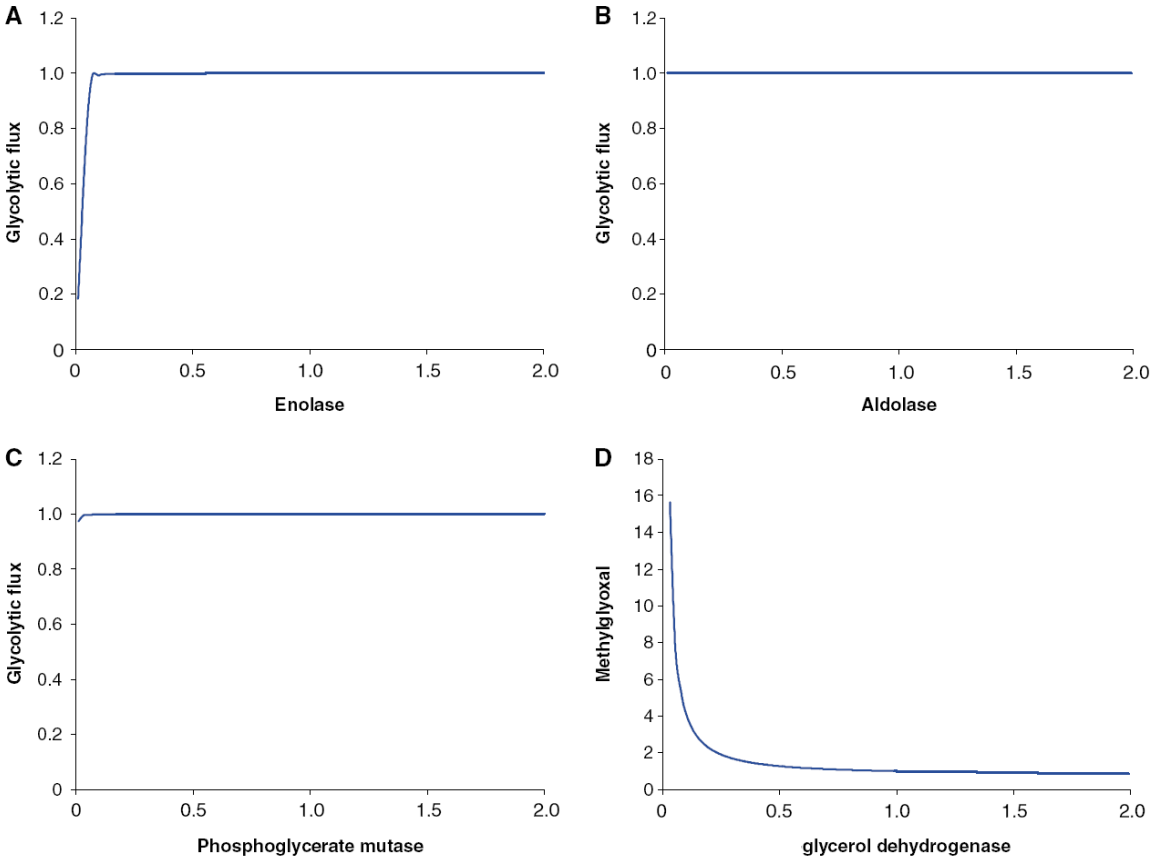


Figure III.6. Sensitivity analysis of glycation effects on glycolytic flux by modeling and computer simulation. Single, finite parameter changes (between zero-fold and two-fold) around the reference steady state were performed. All values are fold variations relative to the reference state (normalized 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 AGE formation (Castellani *et al.*, 1996; Yan *et al.*, 1994a). Tumour cells also show a high dependence on glycolysis, the Warburg effect (Altenberg & Greulich, 2004). In these cells, expression of glyoxalase I is increased (Davidson *et al.*, 1999; Di Ilio *et al.*, 1995), suggesting that an increase in methylglyoxal and AGE 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.*, 2005b), 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.

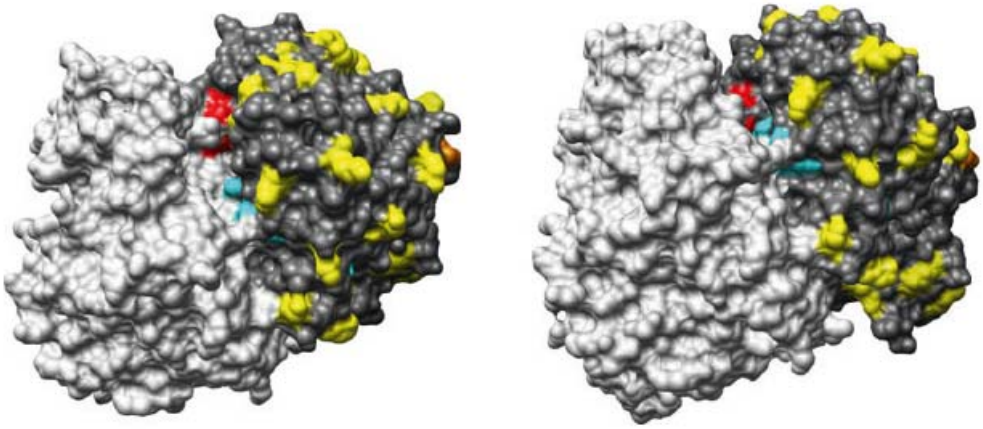


Figure III.7. Surface landscape of dimeric yeast enolase, showing solvent-exposed lysine (yellow) and arginine (cyan) residues. For greater clarity, the surface of one of the subunits is shown in light gray. Two views of the same molecule are shown, rotated clockwise by 180º along the molecule's horizontal 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%.

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.

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 AGE formation only until anti-glycation defenses are overcome (Gomes *et al.*, 2005b). 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.

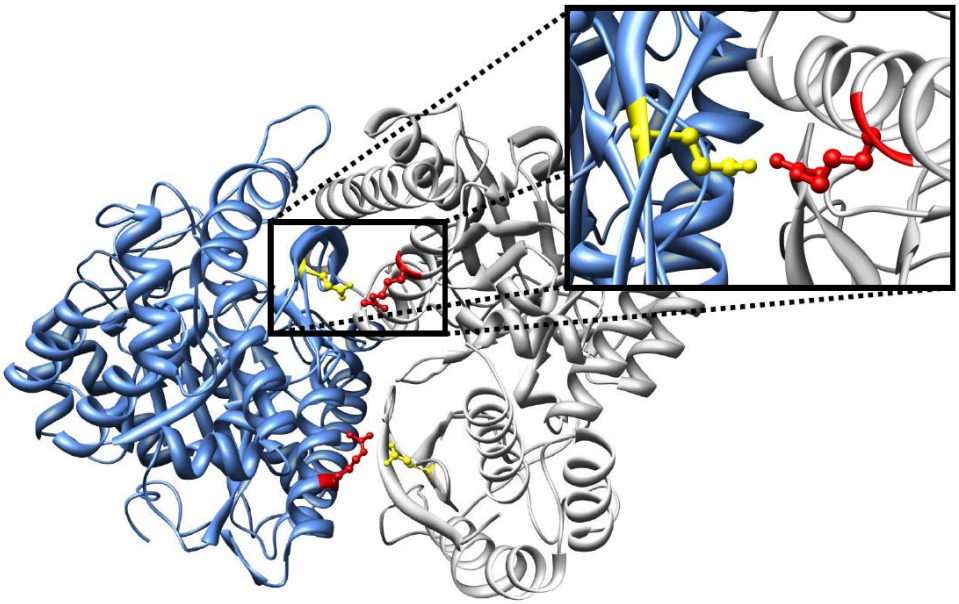


Figure III.8. Surface structure representation of enolase showing the glycation of the critical arginine residues (R414, red) located in a deep cleft at the dimer interface. Once glycated, a critical salt bridge with glutamate residue 20 is disrupted (E20-R414, magnified view) and the dimer dissociates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

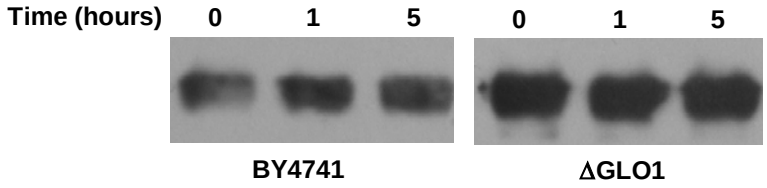


Figure III.9. Western blot analysis of enolase. Cells were incubated with 250 mM of D-glucose and samples were taken at defined times, as indicated. Enolase amount in the soluble cytosolic fraction does not vary with time. However, the glyoxalase I null mutant (ΔGLO1) shows a higher constitutive level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

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CHAPTER IV

IN VIVO PROTEIN GLYCATION: STRUCTURAL AND FUNCTIONAL EFFECTS ON YEAST ENOLASE

Gomes RA, Oliveira L, Silva M, Sousa Silva M, Costa C, Coelho AV, Ascenço C, Quintas A, Cordeiro C, Ponces Freire A. 2007. *Submitted to FEBS lett.*

1. SUMMARY

Protein glycation is an irreversible non-enzymatic post-translational modification associated with major structural and functional changes on cellular proteins, which could have an effect on cell physiology. Indeed, protein glycation was implied in a wide range of human pathological conditions like diabetes *mellitus*, age related disorders and neurodegenerative diseases of amyloid type, although the precise role of this post-translational modification on these diseases remains elusive. To investigate the effects of glycation on protein structure and function, most lines of research deploy model proteins glycated *in vitro*. The major drawback of this approach is the difference between the glycation process inside a living cell or organism and the glycation of a model protein by glycation agents in non-physiological conditions, on a buffer solution within a text tube where cellular responses are absent.

Yeast was shown to be an ideal model to investigate glycation *in vivo* since different glycation phenotypes, controlled through experimental design, and specific glycation targets exist. The glycolytic enzyme enolase is the major target, enduring a glycation-depend activity loss.

To get further insights into the biochemical effects of glycation *in vivo*, we investigated the effects of glycation on enolase structure, stability and function. A mass spectrometry analysis of *in vivo* and *in vitro* glycated enolase was also performed. It was found that glycation leads to structural changes with an increase in random coil structure, extensive dimer dissociation with monomer unfolding, and an increase of protein melting temperature. Concomitantly, a decrease of enolase enzymatic activity was observed. Striking differences between glycation *in vivo* and *in vitro* were also detected, with *in vitro* glycation causing more severe effects. Mass spectrometry analysis revealed that glycation *in vivo* appears to be a specific process. Altogether, these results highlight the importance of investigating protein glycation in model systems such as yeast.

2. INTRODUCTION

Protein glycation is a specific non-enzymatic post-translational modification where arginine and lysine side chain amino groups are irreversibly modified by carbonyl compounds, forming AGE (Westwood & Thornalley, 1997). Methylglyoxal is the most significant glycation agent *in vivo*, considering its high reactivity and continuous formation mainly by the irreversible β-elimination of the phosphate group of DHAP and GAP (Richard, 1984; Richard, 1993; Thornalley, 1996). Albeit non-enzymatic, this is a physiological process that happens simultaneously with glycolysis, hence in all living cells. The glyoxalase pathway and aldose reductase are the main responsible systems for its catabolism (Racker, 1951; Thornalley, 1990; Vander Jagt *et al.*, 1992), but a stable methylglyoxal steady state always exists and glycation reactions will unavoidably occur (Gomes *et al.*, 2005b). Not surprisingly, increased protein glycation is associated with several human pathologies (Brownlee, 1995; Bucala & Cerami, 1992; Castellani *et al.*, 1996; Harrington & Colaco, 1994; Miyata *et al.*, 1993; Yan *et al.*, 1994a).

It is of paramount importance to understand how glycation exerts its effects on target proteins. Glycation consequences on protein structure and function have been investigated mainly *in vitro* in non-physiological conditions regarding the concentration of the glycation agent and reaction conditions. In most cases, glycation agents were deployed in millimolar to molar concentrations for extended periods of time, from days to years (Bakhti *et al.*, 2007; Bouma *et al.*, 2003; Luthra & Balasubramanian, 1993; Raabe *et al.*, 1996; Seidler & Kowalewski, 2003). In addition, protein interactions, which limit the accessibility of the glycation agent to reactive amino acid residues, are not taken into account. These studies are in sharp contrast to what happens in a living cell, where the concentration of glycation agents, namely methylglyoxal, is in the nanomolar to micromolar range and protein concentration is much higher. Consequently, to investigate the effects of glycation on protein structure and function *in vivo*, cellular models must be sought in conditions where target proteins exist and glycation can be controlled. Yeast offers an ideal cell model to investigate glycation *in vivo*, now that some glycation phenotypes and protein targets were identified (Gomes *et al.*, 2006; Gomes *et al.*, 2005b).

Among these, enolase2 (2-phospho-D-glycerate hydrolase, EC 4.2.1.11), shows a glycation-dependent enzyme activity loss (Gomes *et al.*, 2006).

In this work we expand our knowledge on the biochemical effects of methylglyoxal-mediated glycation *in vivo* on the structure, thermal stability and enzyme activity of yeast enolase. A comparison between the effects of glycation *in vivo* and *in vitro* was also performed. Moreover, a bottom-up mass spectrometry analysis of enolase glycation was performed by MALDI-TOF and FTMS.

3. MATERIAL AND METHODS

Reagents and materials

Peptone, yeast extract and agar were from Difco while D-glucose (microbiology grade), KCl and $MgSO_4$ were obtained from Merck. Ammonium sulphate, NaH_2PO_4, Na_2HPO_4, NaCl, NaF, dithiothreitol, iodoacetamide and TFA were obtained from Sigma. Tris, SDS 20% (w/v) and glycine were obtained from BioRad. EDTA was obtained from BDH chemicals LTD while phosphoenolpyruvate and methylglyoxal 1,1-dimethyl acetal and 2,5 dihydroxybenzoic acid (DHB) were acquired from Fluka. Modified trypsin was obtained form Promega. GELoader tips were obtained from Eppendorf. POROS 10 R2 reversed-phase chromatography medium was obtained from PerSeptive Biosystems. α-Cyano-4-hydroxicinamic acid (α-CHCA), sinapinic acid (3,5 dimethoxy-4-hydroxycinnamic acid), PepMix1 (mass spectrometry peptide standards) and ProMix3 (mass spectrometry protein standards) were obtained from LaserBiolabs. Amicon filters were purchased from Millipore.

Solvents acetonitrile and methanol were HPLC-grade obtained from Riedel de Häen; ultrapure water (type I) was obtained from a Millipore Milli-Q system.

Yeast strains and growth conditions

Saccharomyces cerevisiae strains, Euroscarf collection (Frankfurt, Germany), were: BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and ΔGLO1 (isogenic to BY4741 with YML004c::KanMX4). Strains were kept in YPGlu agar slopes [0.5% (w/v) yeast extract, 1% (w/v) peptone, 2% (w/v) agar and 2% (w/v) D-glucose] at 4 ºC and cultured in liquid YPGlu medium. To induce protein glycation, ΔGLO1 strain was cultured for 9 days to reach the stationary phase of growth. The reference BY4741 strain was collected at the end of the exponential phase of growth (18 hours).

Enolase purification

Native enolase was purified from BY4741 yeast cells at the end of the exponential phase of growth (18 hours) while glycated enolase was purified from ΔGLO1 culture at the stationary phase (9 days). Purification was achieved by anion exchange chromatography and size-exclusion chromatography after ammonium sulfate protein precipitation from crude extracts, based on a previously described method (Kustrzeba-Wojcicka & Golczak, 2000). Cells were disrupted by sonication (5 cycles of 1 min at 100 watts with 1 min cooling on ice). The extract was centrifuged at 40000 *g* at 4 °C for 30 min to eliminate cell debris and adjusted to 50% saturation of ammonium sulphate. Saturation was subsequently adjusted to 67% by solid ammonium sulphate addition. After centrifugation at 40000 *g* at 4 °C for 30 min, the supernatant was made 100% saturated and centrifuged again. The pellet, containing enolase, was suspended in 20 mM Tris/HCl pH 8.2, containing 5 mM $MgSO_4$ and 1 mM EDTA. Sample was dialyzed overnight at 4 ºC against the same buffer to remove ammonium sulfate and applied to an ion-exchange chromatography column DEAE-Sephadex A-50 column (Pharmacia, 1-15 cm^2) equilibrated with 20 mM Tris/HCl, pH 8.2, containing 5 mM $MgSO_4$ and 1 mM EDTA. Proteins were eluted with a linear NaCl gradient (0-0.5 M) at a flow rate of 1 $ml.min^{-1}$ and proteins were monitored at 280 nm. Protein-containing fractions were collected and probed by dot blot analysis using an anti-yeast enolase antibody (a kind gift from Dr. Park, Department of Microbiology, Chungnam National University, Korea). Fractions containing enolase were collected, concentrated by ultrafiltration using Amicon filters and applied to a size-exclusion column CM-Sephadex C-50 column (Pharmacia, 1-15 cm^2) equilibrated with 50 mM NaH_2PO_4:Na_2HPO_4 buffer, pH 7.4 containing 150 mM NaCl, 5 mM $MgSO_4$ and 1 mM EDTA. Proteins were eluted with the same buffer at a flow rate of 1 $ml.min^{-1}$. Again, fractions-containing enolase, as probed by dot blot, were collected and combined. In the purification of glycated enolase, the protein fractions were probed by dot blot with anti-MAGE antibody (a king gift from Dr. Ram Nagaraj, Case Western University, Cleveland, Ohio, U.S.A.) and with anti-enolase antibody. Enolase purity was assessed by SDS-PAGE.

In vitro glycation of purified enolase by methylglyoxal

Methylglyoxal was prepared by acid hydrolysis of methylglyoxal 1,1-dimethylacetal as reported by Kellum (Kellum *et al.*, 1978) and purified by fractional distillation under reduced pressure in nitrogen atmosphere (McLellan *et al.*, 1992). Once prepared, methylglyoxal solutions were standardised by enzymatic assay with glyoxalase I and II, as described (Racker, 1951). Purity was verified by HPLC and NMR analysis on a Bruker Avance 400.

Purified native enolase (5 μM) was incubated with 10 mM methylglyoxal in 100 mM potassium phosphate buffer, pH 7.4 at 30 ºC for 5 days in sterile conditions. Enolase concentration was determined spectrophotometrically (ε_{280} = 0.89 $ml.mg^{-1}.cm^{-1}$) (Huang & Dong, 2003) in a UV-vis spectrophotometer Jasco V-530.

Western blot and HPLC analysis

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. For the dot blot assay, purified proteins were added directly to PVDF membranes previously activated with methanol and equilibrated with transfer buffer. The membranes were blocked overnight at 4 ºC in 1% (v/v) blocking solution (Roche) in TBS (50 mM Tris/HCl with 150 mM NaCl, pH 7.5). The anti-MAGE antibody was used diluted 1:5000 in 0.5% (v/v) blocking solution in TBS for 3 hours, while the anti-enolase antibody was used diluted 1:10000 in the same conditions. Washes, secondary antibody and detection procedures were performed using the BM Chemiluminescence Western Blotting Kit (Roche) following manufacturer instructions.

Detection of glycation-induced fluorescence was monitored by reversed phase HPLC on a Beckman-Coulter System Gold equipped 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 fluorescence detector FP-2020 Plus (Jasco). The mobile phase consisted of 0.08% (v/v) TFA in type I water (solvent A) and 0.08% (v/v) TFA in acetonitrile (solvent B), and the elution gradient program was: 10% to 80% solvent B in 30 min; 80% to 10% solvent B in 10 min. Separation was achieved on a reversed phase analytical column (LiChrospher 100 Merck RP-18, 5 μm) at a flow rate of 1 $ml.min^{-1}$. Eluting species were monitored by the fluorescence signal at $\lambda_{em.}/\lambda_{exc.}$ of 320/385 nm, characteristic of argpyrimidine.

Mass spectrometry analysis

MALDI-TOF mass spectra were acquired in a Voyager-DE STR MALDI-TOF (Applied Biosystems). FTMS mass spectra were acquired in a Bruker Apex Ultra with a 7 Tesla magnet. For intact protein mass measurement, sinapinic acid (20 $mg.ml^{-1}$) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA was used as matrix and MALDI-TOF spectra were obtained in positive linear mode. To identify the purified proteins and assign the glycated amino acid residues, peptide mass fingerprint was performed. Proteins 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, concentrated by R2 pore microcolumns (Gobom *et al.*, 1999) and eluted directly to the MALDI plate with 0.8 μl of recrystalized matrix α-CHCA (10 $mg.ml^{-1}$) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA. Monoisotopic peptide masses were used to search for homologies and protein identification with Peptide Mass Fingerprint of MASCOT (http://www.matrixscience.com). The identification of glycated amino acid residues was performed as described (Gomes *et al.*, 2006). Briefly, a glycated enolase peptide should have a miscleavage associated with a defined mass increment of a specific MAGE. Moreover, an arginine modification should have a miscleavage in an arginine residue and the same stands for lysine modifications (Gomes *et al.*, 2006). The analysis of glycated peptides was also performed

by FTMS. In this case, besides the in gel digestion, proteins were trypsin digested in solution, essentially as described (Olsen & Mann, 2004). The resulting peptide mixture was also purified using PerfectPure C-18 tips (Eppendorf) and diluted in 50% (v/v) acetonitrile with 0.1% (v/v) TFA for ESI-FTMS analyses. The peptide mixture was also analysed by MALDI-FTMS, using DHB matrix [10 $mg.ml^{-1}$ prepared in 70% (w/v) acetonitrile with 0.1% (v/v) TFA] in an Anchorchip MALDI target.

Structure and stability analysis

Structural analysis was performed by circular dichroism (CD) spectroscopy and size-exclusion chromatography. Prior to CD analysis, individual protein species were separated by size-exclusion chromatography on an analytical column (Amersham-Pharmacia Superdex™ 75 10/300 GL) with 10 mM phosphate buffer pH 7 containing 100 mM NaF as the mobile phase at a flow rate of 0.4 $ml.min^{-1}$ (LKB Bromma 2150 isocratic pump with a UV detector JASCO 2075). Eluting peaks were monitored at 280 nm and individual protein fractions were collected for further analysis.

Secondary structure analysis was performed by far-UV CD (185-240 nm) in a Jasco J810 spectropolarimeter at 25 ºC (Julabo F25 temperature control unit) with 0.1 cm path length. CD spectra were deconvoluted using the CDSSTR algorithm (Johnson, 1999) on Dichroweb (http://www.cryst.bbk.ac.uk/cdweb/html/home.html) (Lobley *et al.*, 2002; Whitmore & Wallace, 2004). The molar ellipticity was calculated on the basis of a mean residue mass of 107.13 Da. All the spectra were solvent baseline-corrected.

Conformational stability measurements were performed by thermal-induced protein unfolding. CD denaturation curves were constructed by raising the temperature from 20 to 85 ºC and measuring the ellipticity at 222 nm. *Tm* value (temperature at which 50% of denaturation occurs) of native and glycated enolase was calculated as previously described (Pace *et al.*, 1990).

Enolase activity assay

In all assays, enolase activity was determined at 30 ºC in a 1.5 ml reaction volume, 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 (PEP) consumption at 240 nm and its concentration was calculated using $\varepsilon = 1.42\ mM^{-1}.cm^{-1}$ determined in this work. The reaction mixture, containing 50 mM Tris/HCl (pH 7.4), 100 mM KCl, 1 mM $MgSO_4$, 0.01 mM EDTA and known amount of protein, was pre-incubated for 10 min and the reaction was started by the addition of phosphoenolpyruvate.

Protein structure

Enolase dimer structure was represented by PDB entry 1ebh. 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 Gerstein (Gerstein, 1992).

4. RESULTS

Characterization of enolase glycation by mass spectrometry

Native enolase was purified from the BY4741 reference strain at the end of the exponential phase of growth and glycated *in vitro* by incubation with 10 mM methylglyoxal in 100 mM potassium phosphate buffer pH 7.4, a common glycation condition. Native enolase was also used as a control regarding enzyme activity, secondary structure composition and thermal stability. *In vivo* glycated enolase was purified from the ΔGLO1 strain at the stationary phase of growth, where glycation was previously observed (Gomes *et al.*, 2005b). Protein purity and identity were verified by SDS-PAGE and Western blot analysis with anti-yeast enolase antibody, respectively. Protein identity was further confirmed by peptide mass fingerprinting after in gel trypsin digestion and MALDI-TOF analysis of the resulting peptide mixture (Table IV.1).

Table IV.1. Protein identification 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 a protein sequence coverage greater than 10%.

	Swiss Prot. code	Identified protein	Peptides matched	Mascot score	Sequence coverage
Native	P00925	Eno2	30	131	62 %
Glycated *in vivo*	P00925	Eno2	22	112	49 %
Glycated *in vitro*	P00925	Eno2	17	66	45 %

As previously shown, peptide mass fingerprint data contains information regarding MAGE nature and location (Gomes *et al.*, 2006). Since only lysine and arginine residues are modified, tryptic digestion of glycated proteins would then produce peptides with at least one miscleavage associated to a defined mass increase corresponding to a specific MAGE (Figure IV.1). Proteins were also trypsin hydrolyzed in solution and the resulting peptide mixtures were analysed by MALDI-TOF and ESI/MALDI FTMS. In all cases, 60 to 70% sequence coverage was obtained.

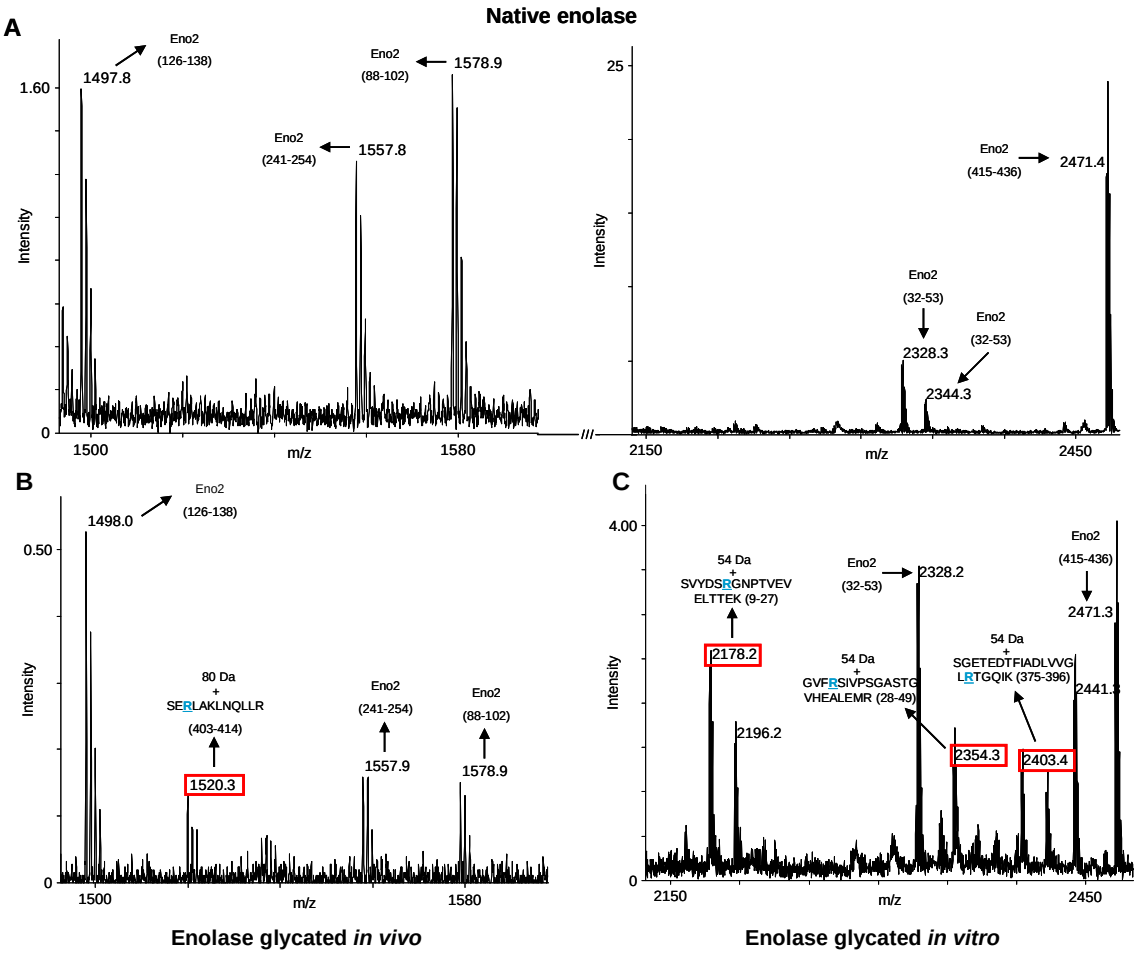

Figure IV.1. Chemical detection and molecular location of MAGE in enolase. Glycated peptides show miscleavages associated with specific mass increments characteristics of a given MAGE (Gomes *et al.*, 2006). Figure shows sections of MALDI-TOF mass spectrum where the appearance of new peptides with a mass increment of a specific MAGE in enolase glycated *in vivo* (B) and *in vitro* (C) in comparison with the native protein (A) are observed. The complete analysis of the mass spectrometry data is present on tables IV.2 and IV.3.

When enolase is glycated *in vitro*, 10 out of 14 arginine residues are modified by methylglyoxal in the form of hydroimidazolones while only one lysine residue, either K336 or K337, is modified to CEL (Table IV.2). Some arginine residues even form different MAGE, like R14 which may be modified as hydroimidazolone [observed mass of 2178.056 Da, corresponding to enolase peptide (9-27) with m/z of 2124.045 plus 54 Da of a hydroimidazolone modification] or as THP [observed mass of 2268.088 corresponding to the same enolase peptide (9-27) plus 144 Da of a THP modification] (Table IV.3).

Table IV.2. Chemical identification and molecular localization of MAGE in enolase by mass spectrometry. Glycated residues are shown in bold. MG-H, hydroimidazolone; Argp., argpyrimidine; CEL, N^{ε}-(carboxyethyl)lysine.

Glycation	Observed mass (Da)	Theoretical peptide mass (Da)	Peptide sequence	Mass increase (Da)	MAGE	Glycated residue
In vitro	1269.67	1215.61	VYA**R**SVYDSR (5-14)	54	MG-H	R8
	1310.63	1256.71	TGAPA**R**SE**R**LAK (397-408)	54	MG-H	R402 or R405
	1520.14	1440.86	SE**R**LAKLNQLLR (403-414)	80	Argp.	R405
	1724.02	1669.92	LNQLL**R**IEEELGDK (409-422)	54	MG-H	R414
	1741.92	1669.03	IATAIE**KK**AADALLLK (330-345)	72	CEL	K336 or K337
	2010.19	1956.07	LGANAILGVSMAAA**R**AAAAEK (105-125)	54	MG-H	R119
	2020.12	1965.99	TFAEAM**R**IGSEVYHNLK (178-194)	54	MG-H	R184
	2178.18	2124.05	SVYDS**R**GNPTVEVELTTEK (9-27)	54	MG-H	R14
	2354.32	2300.18	GVF**R**SIVPSGASTGVHEALEMR (28-49)	54	MG-H	R31
	2403.42	2349.23	SGETEDTFIADLVVGL**R**TGQIK (375-396)	54	MG-H	R391
	2635.57	2581.42	TAGIQIVADDLTVTNPA**R**IATAIEK (312-336)	54	MG-H	R329
In vivo	1310.63	1256.71	TGAPA**R**SE**R**LAK (397-408)	54	MG-H	R402 or R405
	1520.23	1440.86	SE**R**LAKLNQLLR (403-414)	80	Argp.	R405
	1741.90	1669.03	IATAIE**KK**AADALLLK (330-345)	72	CEL	K336 or K337
	2252.20	2171.20	SKLGANAILGVSMAAA**R**AAAAEK (103-125)	54	MG-H	R119
	1750.00‡	1669.96	LNQLL**R**IEEELGDK (409-422)	80	Argp.	R414
	1654.83‡	1600.84	AVSKVYA**R**SVYDSR (1-14)	54	MG-H	R8

‡ Observed by MALDI-FTMS

The same MAGE replacement was observed for R119 and R184 (Table IV.3). This molecular heterogeneity can be seen in a MALDI-TOF mass spectrum of *in vitro* glycated enolase, showing a large mass increase compared to the molecular mass of native enolase and peak broadening (Figure IV.2). These results are consistent with previous studies that point to hydroimidazolones as the most abundant modifications. The formation of different MAGE on the same amino acid residues also shows that glycation is not specific *in vitro*.

Table IV.3. Identification and localization of MAGE in enolase glycated *in vitro* by methylglyoxal using ESI-FTMS. Glycated residues are shown in bold. Noticeably, the same glycated amino acid residue appears with different modifications. MG-H, hydroimidazolone; THP, tetrahydropirimidine.

Observed m/z	Charge	$[M+H]^+$	Peptide sequence	Mass Increase (Da)	MAGE	Glycated residue
742.388	+3	2225.207	SKLGANAILGVSMAAA**R**AAAAEK (103-125)	54	MG-H	R119
1005.508	+2	2010.080	LGANAILGVSMAAA**R**AAAAEK (105-125)	54	MG-H	R119
772.375	+3	2315.239	SKLGANAILGVSMAAA**R**AAAAEK (103-125)	144	THP	R119
1089.478	+2	2178.056	SVYDS**R**GNPTVEVELTTEK (9-27)	54	MG-H	R14
726.67	+3	2178.056	SVYDS**R**GNPTVEVELTTEK (9-27)	54	MG-H	R14
879.771	+3	2637.315	SVYDSRGNPTVEVELTTEKGVFR (9-31)	54	MG-H	R14
756.68	+3	2268.088	SVYDS**R**GNPTVEVELTTEK (9-27)	144	THP	R14
1010.459	+2	2019.996	TFAEAM**R**IGSEVYHNLK (178-194)	54	MG-H	R184
1055.471	+2	2110.027	TFAEAM**R**IGSEVYHNLK (178-194)	144	THP	R184
703.995	+3	2110.027	TFAEAM**R**IGSEVYHNLK (178-194)	144	THP	R184

A different scenario emerges when enolase is glycated *in vivo*. Only five arginine residues are modified: R402 or R405, R119 and R8 as hydroimidazolones and R405 and R414 as argpyrimidine (Table IV.2). Only one lysine residue was found to be modified as CEL, either K336 or K337. These modifications appear to be specific since no other MAGE were found at these positions. Molecular mass increase is negligible and no peak broadening was observed, consistent with a homogeneous distribution of enolase molecular species (Figure IV.2). Argpyrimidine appears to be as abundant as hydroimidazolones while THP was not observed. Considering that the reaction mechanism for the formation of argpyrimidine is very complex, involving two methylglyoxal molecules per arginine residue, reaction conditions for argpyrimidine formation appear to be more favourable *in vivo*.

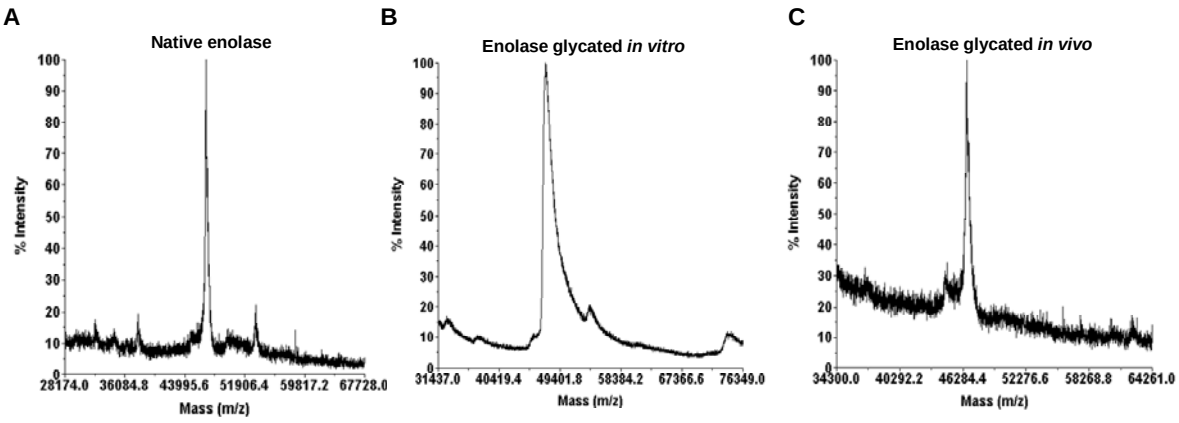

Figure IV.2. Analysis of intact protein mass by linear mode MALDI-TOF of (A) native enolase, (B) *in vitro* glycated enolase and (C) *in vivo* glycated enolase. For all mass spectra, a peak with m/z similar to theoretical yeast enolase2 molecular mass (46 782 Da) was obtained. With glycation *in vitro*, a broadening of the peak is observed indicating higher sample heterogeneity.

To get insights on the susceptibility of arginine residues towards methylglyoxal-derived glycation, we calculated its partial solvent exposure according to Gerstein (Gerstein, 1992) (Table IV.4). For arginine modifications, no obvious relationship exists between the partial solvent exposure of amino groups and the susceptibility towards glycation. It is quite interesting to notice that arginine residues with

no surface exposure of both side chain amino groups, like arginine 119, 391, 405 and 414, are glycated. Meanwhile, the two arginine residues that show the highest surface exposure (R200 and R288) were not found to be glycated. By contrast, solvent exposure appears to be a determinant factor for glycation of lysine residues since K336 or K337 shows the highest solvent exposure (data not shown).

Table IV.4. Surface exposure of the amino groups of arginine side chain in yeast enolase. Glycated and non-glycated arginine residues are shown. The surface exposure was calculated according to Gerstein (Gerstein, 1992).

Glycated arginine residues	Surface exposure	
	NH_1	NH_2
R8	0.60	0
R14	16.93	6.33
R31	3.04	15.88
R119	0	0
R184	0	15.23
R329	11.37	1.70
R391	0	0
R402	0.07	0
R405	0	0
R414	0	0
Non-glycated arginine residues	**Surface exposure**	
	NH_1	NH_2
R49	0	1.11
R200	7.51	23.11
R288	23.43	7.40
R374	0	3.78

Glycation effects on enolase folding, structure and enzyme activity

The effects of glycation on enolase structure were evaluated by far-UV circular dichroism spectroscopy after size-exclusion chromatography separation of each molecular forms of enolase in solution. Size-exclusion chromatograms clearly show two major peaks, one eluting at 20-25 min and the other at 35-40 min (Figure IV.3). The first peak corresponds to enzymatically active enolase dimer (90 kDa) with native secondary structure elements (Figure IV.4). The second peak immunoreacts with anti-yeast enolase antibody, but shows no CD signal and no enzyme activity, indicating the presence of unfolded, inactive enolase (Figure IV.4). Lack of absorption at 222 nm and 208 nm in the CD spectrum indicates a complete loss of regular secondary structural elements, consistent with the absence of enzyme activity. Similar results were obtained for folded and unfolded protein fractions of glycated enolase (data not shown). A comparison of the size-exclusion chromatograms shows that, when glycation occurs, a higher fraction of unfolded inactive enolase is observed relatively to the dimeric, folded enzyme (Figure IV.3). This feature is easily observable by comparing, within the same chromatogram, the fraction of folded active (fraction I) and unfolded inactive enolase (fraction II). For native enolase, the unfolded to folded area ratio is about 1 while a two-fold increase of this ratio is observed for *in vivo* glycated enolase. When enolase is glycated *in vitro*, an even higher amount of unfolded enolase is observed, a nine-fold increase relatively to the native enzyme (Figure IV.3).

If glycation promotes protein dissociation and unfolding, both protein fractions should be glycated. To confirm this hypothesis, these fractions were separated by size-exclusion chromatography and analysed by reversed-phase HPLC with fluorescence detection at $\lambda_{ex.320}\lambda_{em.385}$ (argpyrimidine) and by western blotting using an anti-MAGE antibody. Indeed, both protein fractions from enolase glycated *in vivo* and *in vitro* contain argpyrimidine and other MAGE (Figure IV.5).

These results clearly show that enolase glycation causes protein unfolding and since glycation is an irreversible process, unfolded protein may not be refolded back to the active enzyme form. The effect is far more severe when the protein is glycated *in vitro*.

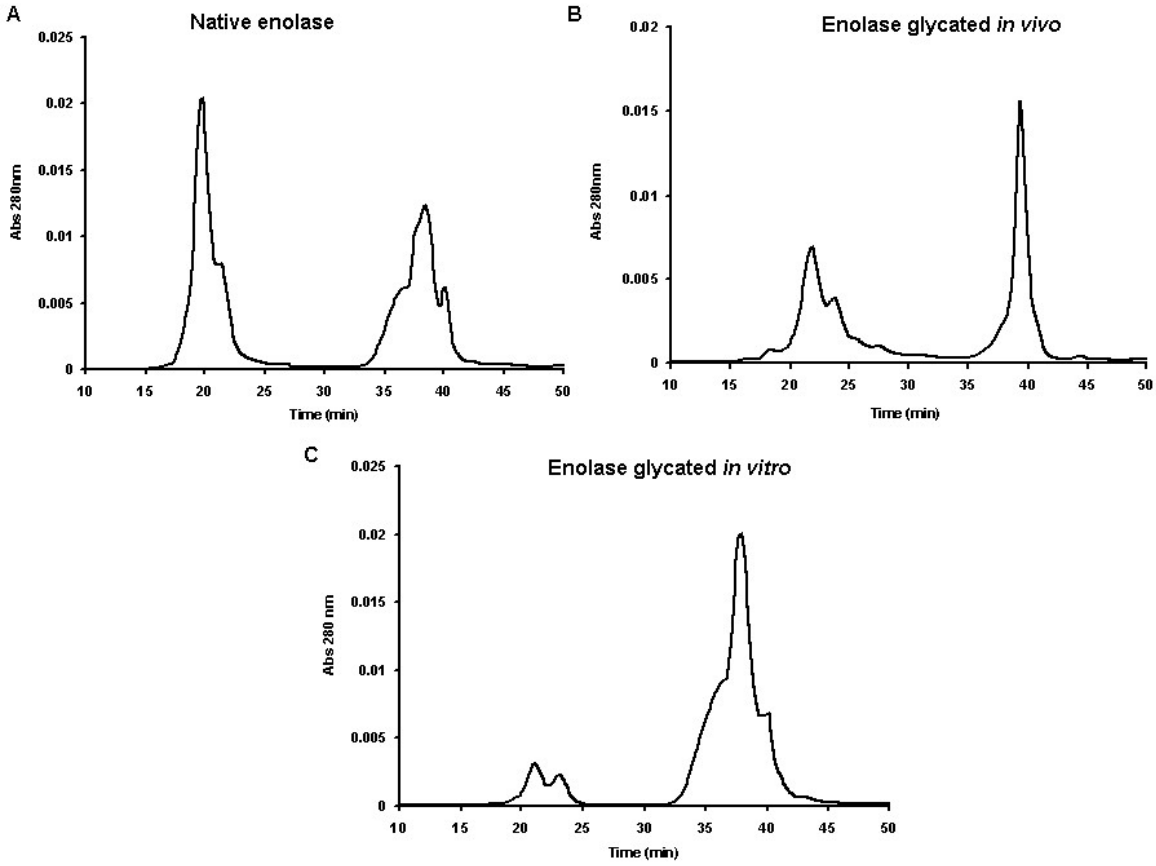

Figure IV.3. Size-exclusion chromatography of native enolase (A), glycated *in vivo* and (B) glycated *in vitro* (C). In the three chromatograms, two main peaks are observed. Upon glycation, an increase of the second peak with an elution time between 35 and 40 min is detected.

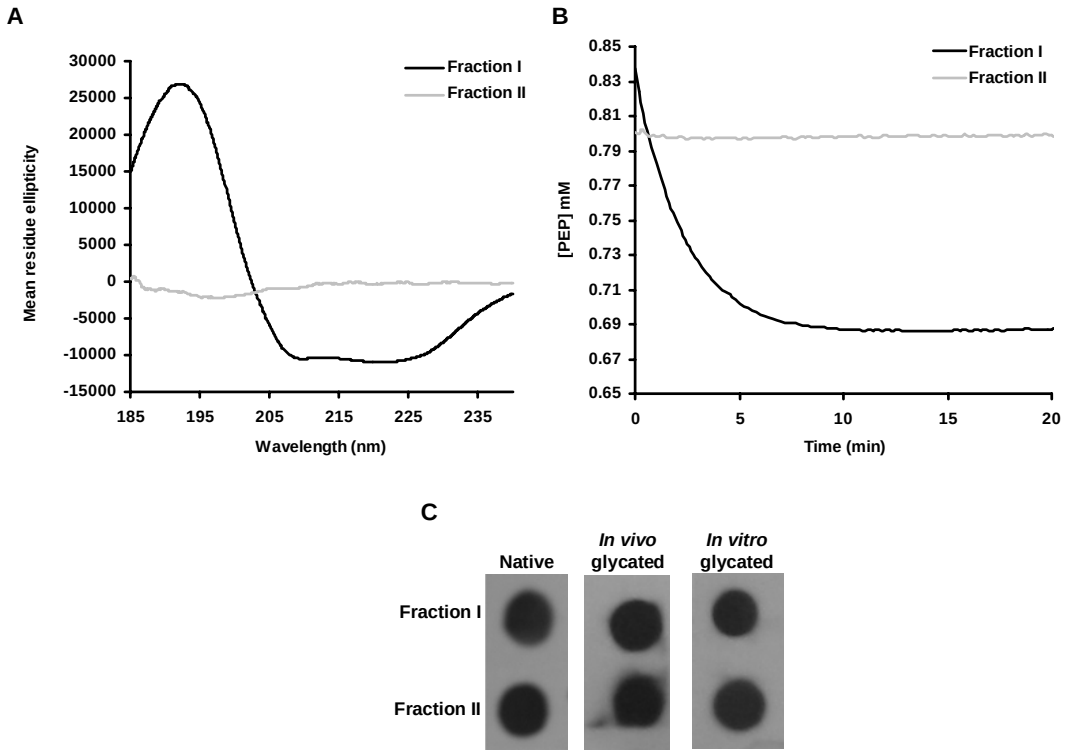

Figure IV.4. Characterization of the two main protein fractions separated by size-exclusion chromatography. (A) Far-UV CD spectrum of fraction I and fraction II collected from size-exclusion chromatography of native enolase. Contrary to fraction I, no CD signal was observed in fraction II, showing a complete loss of secondary structure elements. (B) Enolase activity assays of fraction I and II. Consistent with the lack of secondary structure, no enzyme activity was detected in fraction II. (C) Dot blot analysis of fraction I and II with anti-yeast enolase antibody. In all cases, positive signals confirm protein identity as yeast enolase. For clarity, only the results from fractions I and II of native enolase are shown in (A) and (B).

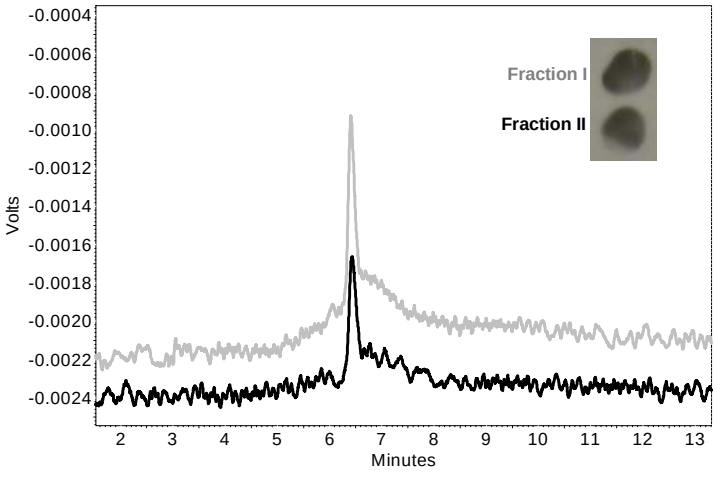

Figure IV.5. Glycation analysis of fraction I (gray) and fraction II (black) from enolase glycated *in vivo* by HPLC and dot blot. Both fractions shows a fluorescent peak around 6 min at wavelengths characteristic of argpyrimidine ($\lambda_{ex.320}\lambda_{em.385}$) indicating that both fractions are glycated. The positive signal obtained by dot blot analysis with anti-MAGE antibody also indicates that both fractions are glycated. Similar results were obtained from enolase glycated *in vitro* (not shown)

Once the different enolase molecular species were separated by size-exclusion chromatography, samples were analysed by far-UV CD spectroscopy. Striking differences were observed, particularly within the region of 195 nm, 208 nm and 222 nm (Figure IV.6). In native enolase, the α-helical content was 40%, the β-sheet was 20%, the turns were 21% and unordered structure was 19% (Table IV.5). This result is in agreement with the values of 37.6% for α-helix, 21% of β-sheet, 26% of turns and 15.4% unordered structures (Huang & Dong, 2003). X-Ray crystallography analysis of yeast enolase, estimated an α-helical content of 37.3% and 17.2% of β-sheet (Stec & Lebioda, 1990).

In vivo glycated enolase shows little or no secondary structure loss. A redistribution of secondary structure elements is evident, with an increase of unordered structure from 19% to 25% and a reduction of the α-helical content from 40% to 35%. β-Sheet content remains unchanged (Table IV.5). When enolase is glycated *in vitro*, a distinct scenario emerges. There is a much higher loss of α-helix, from 40% to 17%, and a dramatic increase of β-sheet, from 20% to 32%, compared to the differences observed for *in vivo* glycated enolase (Table IV.5). Unordered structure elements also increase, from 19% to 32 %.

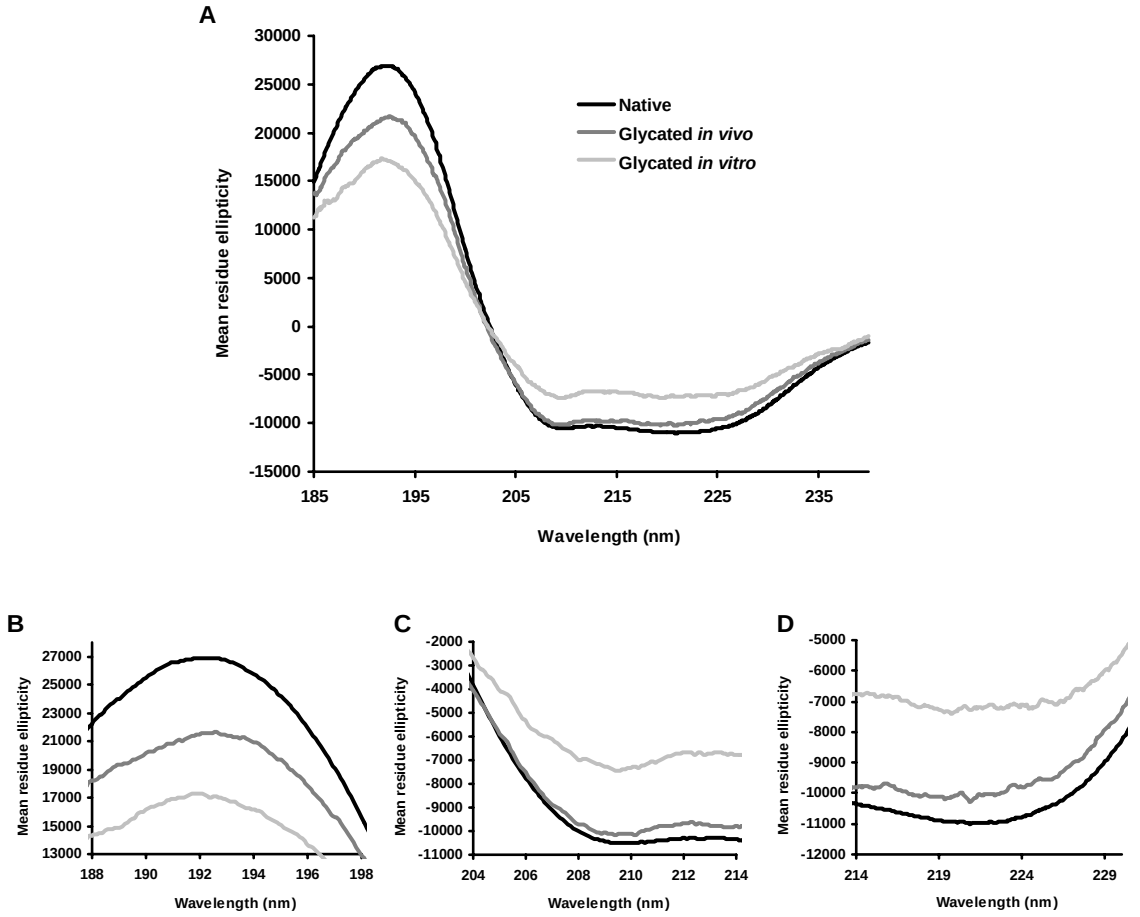

Figure IV.6. Far-UV CD spectra of native, *in vivo* glycated and *in vitro* glycated enolase. (A) CD spectra between 185 and 240 nm. Insets of the positive bands between 188 and 198 nm (B) and negative bands between 204 and 214 nm (C) and between 214 and 230 (D) are shown.

To evaluate the glycation effects on the structural stability of enolase, thermal denaturation of the native, *in vivo* and *in vitro* glycated enolase was monitored by CD spectroscopy. Glycation shifts the thermal denaturation curve of enolase to higher temperatures, indicating an increased resistance to thermal unfolding (Figure IV.7). In fact, the *Tm* for native enolase is 53.6 ºC while *in vivo* glycated enolase shows a *Tm* of 58.6 ºC and the *in vitro* glycated enolase an even higher *Tm* of 61.4 ºC. Enolase thermal denaturation is an irreversible process, as confirmed by occurrence of protein aggregation and lack of secondary structure when the temperature was returned to 25 ºC (data not shown), hence the determination of thermodynamic parameters could not be performed.

Table IV.5. Distribution of secondary structure elements for native, *in vivo* and *in vitro* glycated enolase, obtained by CD spectra deconvolution with the CDSSTR algorithm (dichroweb; http://www.cryst.bbk.ac.uk/cdweb/html/home.html). The NRMSD parameter is the normalized root mean square deviation.

Structural elements	α-Helix	β-Sheet	β-Turns	Unordered structure	NRMSD
Native	0.40	0.20	0.21	0.19	0.011
Glycated *in vivo*	0.35	0.20	0.20	0.25	0.017
Glycated *in vitro*	0.17	0.32	0.19	0.32	0.026

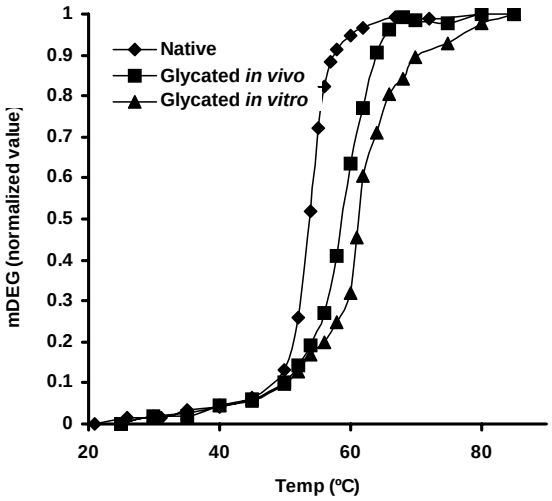

Figure IV.7. Thermal denaturation of native, *in vivo* and *in vitro* glycated enolase. Upon glycation, a shift towards higher melting temperatures occurs.

Secondary structure changes are associated with protein function modifications. Therefore, glycation-induced conformational changes are likely to have pronounced effects on enolase activity. Indeed, we observed a marked decrease in enolase activity upon glycation (Table IV.6). *In vivo* glycated enolase shows a 65% activity loss compared to native enolase. When enolase is glycated *in vitro* an even more severe activity loss is observed (84%). As glycation causes enolase denaturation with a consequent enzyme inactivation, it could be argued that the loss of enzyme specific activity may be solely explained by the higher amount of unfolded inactive protein in the sample. This implies that, if the activity of glycated folded enolase remains the same, the specific activity would decrease. To test this possibility, folded and unfolded fractions

from *in vivo* and *in vitro* glycated enolase were separated by size-exclusion chromatography and enzyme activity was determined for each individual fraction (fraction I being folded active and glycated enolase while fraction II is unfolded glycated enolase). In both cases, no enolase activity was detected in fraction II, which is consistent with the lack of secondary structure (Figure IV.4). In fraction I, enolase activity was detected, albeit the specific activity was again much lower than that of the native enzyme (Table IV.6).

Table IV.6. Glycation effects on enolase activity. The activity determined for native enolase was considered to be 100%. Fraction I (I) indicates folded active enolase. Data shown are averages from three independent activity assay ± SD.

Enolase	Enzyme activity ($\mu M.\ min^{-1}.mg^{-1}$)	Remaining activity (%)
Native	200.5 ± 55.7	100
Glycated *in vivo*	69.6 ± 12.1	34.7
Glycated *in vitro*	31.3 ± 4.6	15.6
Glycated *in vivo* (I)	78.9 ± 10.3	39.3
Glycated *in vitro* (I)	41.1 ± 3.8	20.5

5. DISCUSSION

Arginine residues have a probability of about 20% to be located in ligand and substrate binding sites of proteins (Gallet *et al.*, 2000). Hence, methylglyoxal-derived arginine glycation is expected to have significant effects on protein structure and function, contributing to protein misfolding and changes in biological activity. Therefore, this post-translational modification has been the subject of a deeply research, where *in vitro* glycation of clinically relevant and model proteins are investigated. These AGE-modified proteins were used to understand the consequences of glycation on protein structure and function. The major drawback of this approach lies on the dramatic differences between glycation conditions *in vitro* when compared to AGE formation *in vivo*. First, non-physiological concentrations of glycation agents are used, in millimolar and even in molar concentrations (Bakhti *et al.*, 2007; Bouma *et al.*, 2003; Luthra & Balasubramanian, 1993; Raabe *et al.*, 1996; Seidler & Kowalewski, 2003). Additionally, protein interactions are not taken into account, which may limit the accessibility of reactive amino acid residues to the glycation agents or modify their reactivity. Also, the action of chaperones, some of which being activated upon glycation, is not considered (Gomes *et al.*, 2006; Nagaraj *et al.*, 2003; Oya-Ito *et al.*, 2006). Furthermore, cells have the ability to recover or degrade damaged proteins and synthesize new molecules, and this protein turnover is absent from *in vitro* experiments. These differences highlight the importance of investigating protein glycation mechanisms and their biochemical effects *in vivo*. For this purpose, suitable research models are an absolute requirement. Our previous studies validated yeast as an ideal eukaryotic cell model to investigate protein glycation *in vivo* (Gomes *et al.*, 2005a; Gomes *et al.*, 2006). Glycation in yeast by methylglyoxal can be controlled, either by using mutant strains or varying D-glucose concentration (Gomes *et al.*, 2005b). Moreover, well-defined protein glycation targets exist and glycation occurs in a time scale of a few hours to about a week, most suitable for laboratory investigations (Gomes *et al.*, 2005b). Enolase, the major glycation target, endures a glycation-dependent activity loss, providing us an important model to study the glycation effects *in vivo* (Gomes *et al.*, 2006). To get further insights into this issue, a

bottom-up mass spectrometry analysis was also performed for enolase glycated *in vivo* and *in vitro*.

In agreement with our hypothesis that differences exist between *in vivo* and *in vitro* glycation, the results from mass spectrometry analysis indicate that enolase glycation *in vivo* seems to be a site-specific process, whereby only a few residues are consistently modified with the same MAGE. By contrast, glycation *in vitro* is a very heterogeneous process resulting in the formation of a complex population of enolase molecules with different glycation profiles. Far more arginine residues were modified *in vitro* (10 out of 14) when compared to *in vivo* glycation (5 out of 14). *In vitro*, different MAGE may be present in the same arginine residue, in different protein molecules. Although this may simply reflect the most favourable glycation conditions *in vitro*, it hints for a specificity of the glycation *in vivo* where hotspot arginine residues exist. In both cases, some arginine residues are resilient to glycation and do not appear to be modified.

The specificity of glycation *in vivo* seems to be unrelated to the partial solvent exposure of each arginine residues since some glycated arginine residues show high solvent exposure whereas others less exposed are also modified. Moreover, arginine residues with a high solvent exposed were not found to be glycated. In a recent work we suggested that the arginine-rich deep crevice in enolase protein structure, accessible to the solvent and located at the dimer interface, may be a favourable hotspot for the occurrence of glycation reactions (Gomes *et al.*, 2006). In fact, several glycated arginine residues identified after *in vitro* glycation and almost all glycated arginines *in vivo* are located in this cleft (Figure IV.8A and B). Interestingly, glycation was not detected in the two most exposed arginine residues (R200 and R288), which are not located in this cleft (Figure IV.8C).

Consistent with the idea that glycation induces changes on protein structure, *in vivo* glycated enolase shows a rearrangement of secondary structure elements with an increase of unordered structure. α-Helical content decreases in comparison with native enolase and *Tm* increases with glycation, suggesting that glycated enolase may exist in a more compact and rigid conformation. Additional effects were observed when enolase is glycated *in vitro*. In this case, besides an enhanced increase of unordered structure and a

decrease of α-helix, a marked gain of β-sheet was also observed. The *Tm* increase is more pronounced, consistent with a more rigid structure, probably due to higher β-sheet content. Since an increase in β-sheet content is an important step towards amyloidogenesis, it is crucial to understand the differences between glycation *in vivo* and *in vitro.*

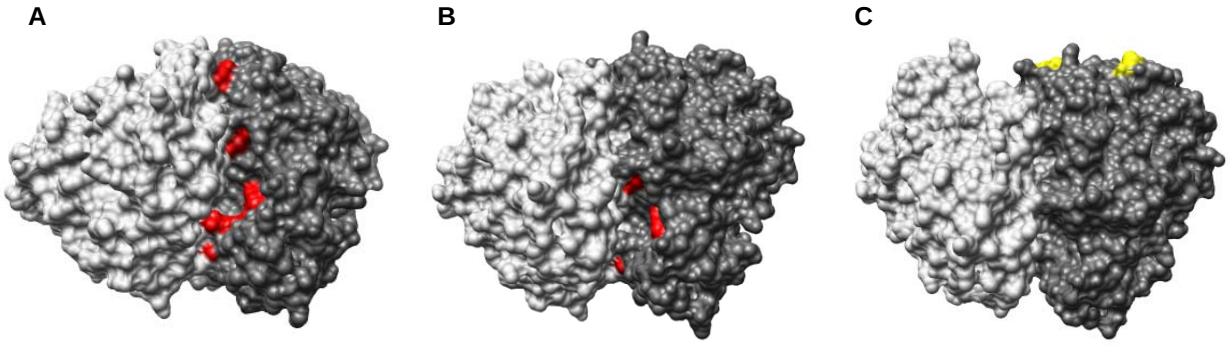

Figure IV.8. Surface landscape of dimeric yeast enolase, showing the glycated (red) and non-glycated (yellow) arginine residues. For greater clarity, the surface of one of the subunits is shown in light gray. (A) Enolase glycated *in vitro* showing glycated arginine residue (red) in a cleft at the dimer interface (B). *In vivo* glycated enolase, showing four out of five glycated arginine residues in the cleft. (C) Enolase structure showing the highest solvent exposed arginine residues R200 and R288 that were not found to be glycated (yellow). Interestingly, these arginine residues are not located at the arginine-rich crevice located at the dimer interface.

Glycation leads to enolase unfolding, probably related to the dimer dissociation and subsequent monomer unfolding. This is in agreement with our previous model of glycation-induced enolase inactivation in which the glycation of the critical arginine residue R414 disrupted an ionic pair formed with glutamate residue 20, essential for dimer stability (Gomes *et al.*, 2006). A modification of R414 was observed in this study, both consequence of *in vivo* and *in vitro* glycation. Enolase unfolding might be related with Hsp26 activation under glycation conditions (Gomes *et al.*, 2006). Hsp26 is involved in the recovery of misfolded proteins by rendering aggregates more accessible to the heat shock protein system composed by Hsp104/Hsp71/Hsp40 (Cashikar *et al.*, 2005).

Therefore, by promoting protein unfolding, glycation increases unfolding stress and elicits a cellular response. Methylglyoxal modifies Hsp26 and the formation of specific MAGE may be involved on its activation. When enolase is glycated *in vitro* its unfolding is much more pronounced. This can be due to the higher glycation extent or, more likely, because the molecular chaperone pathway which is activated by glycation *in vivo* is absent from a test tube.

The observed changes in protein structure and stability are related to the glycation-dependent activity loss. While a 65% inactivation occurs upon glycation *in vivo*, 85% of activity loss is observed after *in vitro* glycation.

The results presented suggest that important differences exist between glycation *in vivo* and *in vitro* in the conditions used, which may be related to diverse glycation specificities. This observation highlights the importance of investigating protein glycation *in vivo* in a model system such as yeast, already validated in the research of amyloidotic neurodegenerative diseases (Outeiro & Lindquist, 2003).

6. ACKNOWLEDGEMENTS

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CHAPTER V

ARGPYRIMIDINE, A METHYLGLYOXAL-DERIVED ADVANCED GLYCATION END-PRODUCT IN FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

Gomes RA; Sousa Silva M; Quintas A; Cordeiro C; Freire A; Pereira P; Martins A; Monteiro E; Barroso E; Ponces Freire A. (2005) Biochem J **385**:339-345.

1. SUMMARY

FAP (familial amyloidotic polyneuropathy) is a systemic amyloid disease characterized by the formation of extracellular deposits of transthyretin. More than 80 single point mutations are associated with amyloidogenic behaviour and the onset of this fatal disease. It is believed that mutant forms of transthyretin lead to a decreased stability of the tetramer which dissociates into monomers prone to unfolding and aggregation, latter forming β-fibrils in amyloid deposits. This theory does not explain the formation of β-fibrils nor why they are toxic to nearby cells. Age at disease onset may vary by decades for patients with the same mutation. Moreover, non-mutated transthyretin also forms the same deposits in SSA (senile systemic amyloidosis), suggesting that mutations may only accelerate this process, but are not the determinant factor in amyloid fibril formation and cell toxicity. We propose that glycation is involved in amyloidogenesis, since amyloid fibrils present several properties common to glycated proteins. It was shown recently that glycation causes the structural transition from the folded soluble form to β-fibrils in serum albumin.

In this work, we identified for the first time a methylglyoxal-derived advanced glycation end-product, argpyrimidine [N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine] in amyloid fibrils from FAP patients. Unequivocal argpyrimidine identification was achieved chromatographically by amino acid analysis using dabsyl (4-dimethylaminoazobenzene-4'-sulphonyl) chloride. Argpyrimidine was found at a concentration of 162.40 ± 9.05 pmol/mg of protein in FAP patients, and it was not detected in control subjects. The presence of argpyrimidine in amyloid deposits from FAP patients supports the view that protein glycation is an important factor in amyloid diseases.

2. INTRODUCTION

Familial amyloidotic polyneuropathy (FAP) is a systemic amyloid disease characterized by the extracellular deposition of transthyretin (TTR) in several tissues, particularly in the peripheral nervous system (Costa *et al.*, 1978). The main clinical symptom is a progressive polyneuropathy affecting both the peripheral and autosomal nervous systems (Andrade, 1952). In all amyloidoses, including FAP, fibrils are insoluble, highly stable, resistant to enzymatic proteolysis and show an extensive β-sheet structure (Ghiso *et al.*, 1994). Transthyretin is a homotetrameric protein of 55 kDa found in the plasma and cerebrospinal fluid, and is responsible for the transport of thyroxine and retinol, the latter by the association with the retinol-binding protein (Kanai *et al.*, 1968; Raz *et al.*, 1970). In certain forms of FAP, amyloid fibrils are mainly constituted by variants of transthyretin. In Portuguese-type FAP, substitution of methionine for valine at position 30 occurs (Saraiva *et al.*, 1984). Although all FAP patients have identical concentrations of variant TTR in the plasma and cerebrospinal fluid, age at onset varies widely. Therefore, despite the identification of mutations in TTR associated with FAP, the process of fibril formation and their toxicity remain to be elucidated. The observation that non-mutated TTR also forms amyloid fibrils as in SSA (Benson, 1989; Westermark *et al.*, 1990) implies that other factors besides genetic modifications must be considered in the pathogenesis of FAP. Since the first symptoms in FAP appear much earlier than in SSA, the point mutations in TTR seem to accelerate fibril formation by increasing the TTR amyloidogenicity. Moreover, different amyloidotic proteins with no sequence homology form similar amyloid fibrils (Ghiso *et al.*, 1994). Besides amyloidogenesis, the mechanisms by which the extracellular deposits are toxic to cells are not well understood. One important observation is the presence of oxidative stress markers associated with amyloid fibrils, suggesting that interactions with specific receptors may occur (Sousa *et al.*, 2001c).

At the present, there is evidence that protein glycation is involved in the pathogenesis of several amyloid diseases, like Alzheimer's disease (Du Yan *et al.*, 1997a; Vitek *et al.*, 1994; Yan *et al.*, 1994a) and dialysis-related amyloidosis (Miyata *et al.*, 1994a). Protein glycation may be equivalent to a point mutation since amino acid side

chains are modified. In living cells, the effects of glycation are countered by high turnover and short half-life of most cellular proteins. In contrast, long-lived extracellular proteins accumulate glycation adducts with age (Ahmed *et al.*, 1997; Frye *et al.*, 1998; Riley & Harding, 1995). Like amyloid fibrils, glycated proteins are resistant to proteolysis *in vivo* and toxic to animal cells (Dyer *et al.*, 1991; Iversen *et al.*, 1995; Monnier & Cerami, 1981). The cellular effects of advanced glycation end-products (AGE) occur by interacting with specific cellular receptors, like the receptor for AGE (RAGE), the best characterized (Neeper *et al.*, 1992; Schmidt *et al.*, 1992). The AGE:RAGE interaction *in vivo* generates a significant cellular oxidative stress (Lander *et al.*, 1995).

One of the most powerful glycation agents *in vivo* is methylglyoxal formed in all living cells mainly from dyhidroxyacetone phosphate and D-glyceraldehyde 3-phosphate in glycolysis (Richard, 1993). Methylglyoxal irreversibly modifies lysine and arginine residues in proteins. However, methylglyoxal arginines AGE appear to be more relevant considering the existence of specific receptors for hydroimidazolones (Westwood *et al.*, 1997). Methylglyoxal also forms argpyrimidine [N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine], a fluorescent product (Shipanova *et al.*, 1997). Recently, argpyrimidine was found in spinal cord of familial sporadic amyotrophic lateral sclerosis (ALS) patients and mutant SOD-1 mice (Shibata *et al.*, 2002). Methylglyoxal modification of arginine may contribute to the pathophysiologies associated with aging and other diseases (Oya *et al.*, 1999; Shibata *et al.*, 2002).

In the present study, we unequivocally identified argpyrimidine, by chromatographic methods, in amyloid fibrils from Portuguese-type FAP patients. The formation of AGE in FAP may play an important role in the molecular mechanisms of amyloidogenesis either by promoting the pathway of amyloid fibril formation or increasing its toxicity to nerve cells.

3. MATERIAL AND METHODS

Reagents and materials

L-Amino acids, N^{α}-acetyl-arginine, thymol, pepsin, Pronase E (protease type XIV), aminopeptidase and dabsyl chloride were purchased from Sigma. Solid phase extraction columns (LiChrolut RP-18, 500mg) were obtained from Merck. All solvents were HPLC grade. HPLC analysis was performed in a Beckman Coulter with a high-pressure binary gradient pump 126, a Beckman-Coulter 168-diode-array detector (1 nm resolution) and a fluorescence detector FP-2020 Plus (Jasco). In all assays, a Merck LichroCART 250-4 (250 x 4 mm) with stationary phase Lichrospher 100 RP-18, 5 μm, was used at a flow rate of 1 $ml.min^{-1}$. Mass spectrometry analysis of N^{α}-acetyl-argpyrimidine was performed in an ESI-MS (electrospray mass spectrometry) Thermo-Finnigan LQC Duo and NMR analysis was performed in a Bruker Advance 400 [using a DQF (double-quantum-filtered)-COSY, HMQC (heteronuclear spin quantum correlation) and a HMBC (heteronuclear multiple bond correlation) NMR sequences].

Human samples

Adipose tissue samples from FAP patients (two males and one female; mean age 30, range 26-33 years) were collected during the initial phase of liver transplantation. Non-FAP control (three males and two females; mean age 51, range 28-69 years) comprised patients receiving transplants following liver cirrhosis or autoimmune liver disease. One of the control subjects, suffering from pancreatic tumour, did not undergo liver transplantation. Adipose tissue samples were obtained during surgery. For all FAP and non-FAP subjects, blood D-glucose concentration was determined, in fasting and post-prandial conditions. Neither FAP patients nor control subjects were diabetic or had any carbohydrate-related disorders. There were no other exclusion criteria. Moreover, blood methylglyoxal concentration was determined in the same conditions and no significant differences between FAP and control subjects were found (data not shown).

All patients gave informed written consent, and the protocol was approved by the Curry Cabral Ethical Commission according to EEC approved protocols.

Extraction of amyloid proteins from adipose tissue

Amyloid proteins were extracted from samples of FAP patients and non-FAP subjects following a modified procedure based on the method developed by Kaplan *et al.* (Kaplan *et al.*, 1994). Briefly, adipose tissue was dispersed in 5 ml of 0.154 M KCl and incubated for 1 h at room temperature. Samples were centrifuged for 10 min at 19000 g and the sediment was washed twice in the same solution. Lipid extraction was then performed with the addition of 5 ml of chloroform/methanol solution [2:1, (v/v)] and incubated for 5 min at room temperature. Samples were centrifuged for 10 min at 19000 g and the sediment was suspended with aqueous 20% (v/v) acetonitrile containing 0.1% (v/v) TFA. Samples were incubated for 1 h at room temperature and the amyloid proteins extracted were collected in the supernatant after centrifugation for 10 min at 19000 g. Amyloid protein extraction was repeated once more. The extracted material was evaporated to dryness under a stream of N_2 at 40 ºC and suspended in 500 µl of aqueous 20% (v/v) acetonitrile with 0.1% (v/v) TFA.

Protein concentration was determined using Coomassie Brilliant Blue dye as described (Cordeiro & Freire, 1994). Extracted proteins were analysed directly by HPLC using a binary gradient made of solvent A [water with 0.1% (v/v) TFA] and solvent B [acetonitrile with 0.1% (v/v) TFA]. The gradient program was: 0-20 min, 20-90% solvent B; 5 min isocratic at 90% solvent B; 25-30 min, 90-20% solvent B. The eluate was monitored at 220 and 260 nm (diode array detector) and by the fluorescence signal at $\lambda_{emission,max.}/\lambda_{excitation,max.}$ of 320/385 nm (double-monochromator fluorescence detector).

Preparation of argpyrimidine standard

Argpyrimidine was prepared by the reaction of N^{α}-acetyl-L-arginine with methylglyoxal. N^{α}-Acetyl-L-arginine (100 mM) was incubated with 100 mM methylgyloxal in 100 mM sodium phosphate buffer (pH 7.4) at 70 ºC for 72 hours.

Methylglyoxal was prepared by acid hydrolysis of 1,1-dimethoxypropanone, as described previously (Kellum *et al.*, 1978). The products obtained were analysed by HPLC using a binary gradient, solvent A [water with 0.1% (v/v) TFA] and solvent B [acetonitrile with 0.1% (v/v) TFA]. The gradient program was: 0-8 min, 2-10% solvent B; 8-13 min, 10% solvent B isocratic; 13-23 min, 10-20% solvent B; 20% solvent B isocratic for 2 min; 25-28 min, 20-2% solvent B. N^{α}-Acetyl-argpyrimidine was identified by its characteristic absorption spectra and characteristic fluorescence at excitation wavelength of 320 nm and emission wavelength of 385 nm. The resulting N^{α}-acetyl-argpyrimidine was purified in a RP-18 column (LiChrolut 500 mg). Purification was followed by HPLC analysis as described above; the fractions containing pure N^{α}-acetyl-argpyrimidine were combined and freeze-dried. The identity of N^{α}-acetyl-argpyrimidine was confirmed by mass spectrometry (molecular mass of 298.11 Da) and NMR spectroscopy analysis. To prepare the chromatographic argpyrimidine standard, the acetyl group was removed by enzymatic hydrolysis using leucine aminopeptidase for 2 days at 37 ºC.

Argpyrimidine was quantified by HPLC using a calibration curve relating argpyrimidine concentration to the area of the corresponding peak (fluorescence detection).

Amino acid analysis: chromatographic detection of argpyrimidine

Before amino acids analysis, samples containing amyloid proteins extracted from FAP patients and samples containing proteins extracted from non-FAP patients were washed by ultrafiltration (5 kDa cut-off membrane) and subjected to enzymatic hydrolysis as described previously (Ahmed *et al.*, 2002). Briefly, to 20 µl of sample (approximately 10-20 µg) 25 µl of 40 mM HCl, 5 µl of 2 $mg.ml^{-1}$ thymol and 5 µl of 2 $mg.ml^{-1}$ of pepsin (both prepared in 20 mM HCl) were added and the samples were incubated at 37 ºC for 24 h. Samples were neutralized and buffered at pH 7.4 with the addition of 25 µl of 0.5 M potassium phosphate buffer, pH 7.4, and 5 µl of 260 mM KOH. Then 5 µl of Pronase E solution (2 $mg.ml^{-1}$ in 10 mM phosphate buffer, pH 7.4) were added and the samples were incubated for 24 h at 37 ºC. Finally, 5 µl of leucine aminopeptidase and protease solutions (both prepared in 10 mM phosphate buffer, pH

7.4) were added and the mixture was incubated for 48 h at 37 ºC. This enzymatic hydrolysate (100 µl) was used for the argpyrimidine assay using dabsyl chloride as the derivatization reagent. An aliquot of the enzymatic hydrolysate (50 µl) was mixed with the same volume of 2 mM dabsyl chloride prepared in acetonitrile and incubated at 60 ºC for 10 min. The resulting sample was filtered and analysed by HPLC. A binary system of 25 mM sodium acetate buffer, pH 6.5 (solvent A) and acetonitrile (solvent B) was used. The gradient program was: 0-30 min, 20-40% solvent B; 30-55 min, 40-90% solvent B; 5 min isocratic with 90% solvent B; 60-65 min, 90-20% solvent B. The eluate was monitored at 452 nm and by the fluorescence signal at $\lambda_{emission,max.}/\lambda_{excitation,max.}$ of 320/385 nm. The argpyrimidine standard solution was submitted to the same treatment.

4. RESULTS AND DISCUSSION

Extraction and characterization of amyloid deposits from FAP patients

Amyloid proteins were extracted using a modified procedure based on the method developed by Kaplan *et al.* (Kaplan *et al.*, 1994). In the present study, an additional step was added to extract lipids and phospholipids with a chloroform/methanol mixture before amyloid protein extraction. Since proteins are the main components of amyloid fibrils, protein concentration is a measure of the efficiency of both methods. With the modified procedure we obtained a higher ratio of protein amount per initial amount of tissue [$(1.32 \pm 0.198) \times 10^{-03}$; n=3] in comparison with the Kaplan method [$(6.9 \pm 0.613) \times 10^{-04}$; n=4]. Therefore the new lipid extraction step improves the efficiency of protein extraction from adipose tissue samples. Comparing the chromatograms obtained after an HPLC analysis of the extracted material with both methods for a representative FAP patient (Figure V.1), it is clear that more protein material is obtained with the modified method. This improvement in the extraction procedure is of extreme importance, since AGE are present in vestigial quantities. Therefore, it is of utmost importance to extract the highest amount of amyloid fibrils with the least amount of contaminants. Amyloid fibrils in FAP are associated with lipids, collagen and amyloid P, all of which interfere in X-ray diffraction studies. The removal of this contaminants does not affect the chemical and structural integrity of the amyloid fibrils (Damas *et al.*, 1995).

AGE in amyloid deposits from FAP patients

One of the major consequences of protein glycation is the formation of fluorescent products with characteristic excitation and emission wavelengths (Dyer *et al.*, 1991; Monnier & Cerami, 1981). The appearance of new fluorescence properties is a strong evidence for protein glycation. Methylglyoxal, a potent and ubiquitous glycation agent, forms stable adducts with arginine residues leading to non-tryptophan fluorescence (excitation, 320 nm; emission, 340-500 nm) (Riley & Harding, 1995). Shipanova and co-workers identified a MAGE (argpyrimidine) as the major fluorescent product

($\lambda_{emission,max.}/\lambda_{excitation,max}$, 320/385 nm) present *in vivo* (Shipanova *et al.*, 1997). Recently, argpyrimidine was found in spinal cord of familial sporadic ALS patients and mutant SOD-1 mice (Shibata *et al.*, 2002).

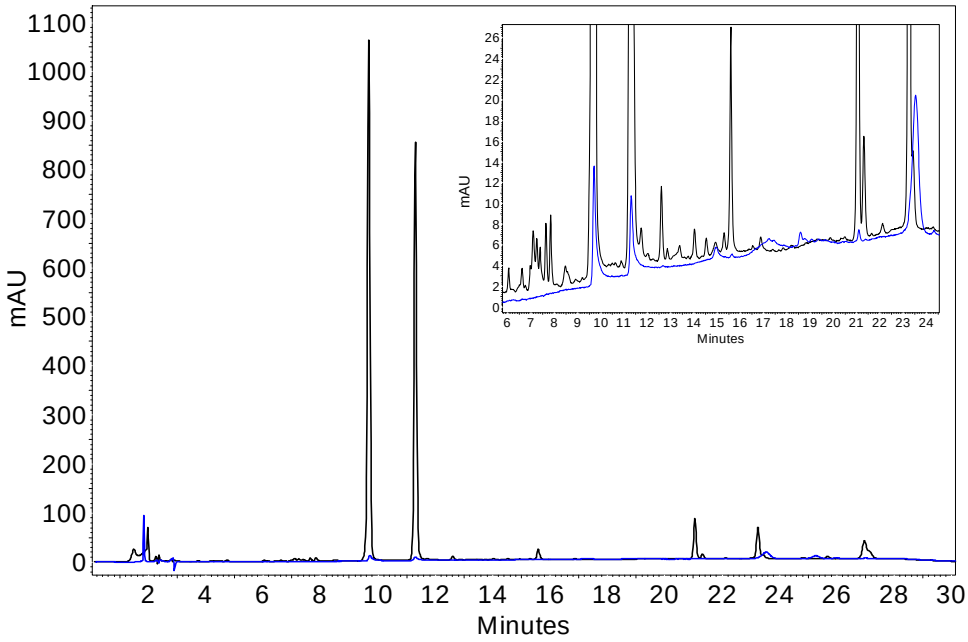

Figure V.1. Direct HPLC analysis of proteins extracted from a FAP patient with two different methods. Absorbance was monitored at 220 nm. Samples underwent ultrafiltration before analysis (5 kDa cut-off). The inset shows a magnification of the chromatogram between 5 and 25 min. Black, extraction by the modified procedure; blue, extraction by the method of Kaplan *et al* (Kaplan *et al.*, 1994). mAU, milli-absorbance units.

Direct HPLC analysis with fluorescence detection at $\lambda_{emission,max.}/\lambda_{excitation,max.}$ 320/385 nm of the extracted material derived from Portuguese-type FAP patients and control subjects (Figure V.2) revealed that only the extracted amyloid fibrils from the FAP patients presented a significant fluorescence peak. Despite intensity differences of the fluorescence peak among different patients, it should be noted that protein concentration after extraction differs among samples, so fluorescence intensities are not directly comparable. In contrast, proteins extracted from non-FAP subjects have none or are barely modified. Comparing the chromatographic analysis with fluorescence detection of the controls, there were no significant differences between them. Only one

control sample showed a minor fluorescence peak in the same region of the chromatogram. All samples were purified by ultrafiltration (5 kDa cut-off membranes) prior to HPLC analysis, indicating that these samples are free from low molecular mass contaminants that could interfere with fluorescence analysis.

The differences between fluorescent properties of the extracted material from the control subjects and FAP patients hint that amyloid proteins from FAP patients are glycated. Considering the excitation and emission wavelengths that produce a measurable signal in the fluorescence detector, argpyrimidine is likely to be present in the proteins extracted from FAP patients.

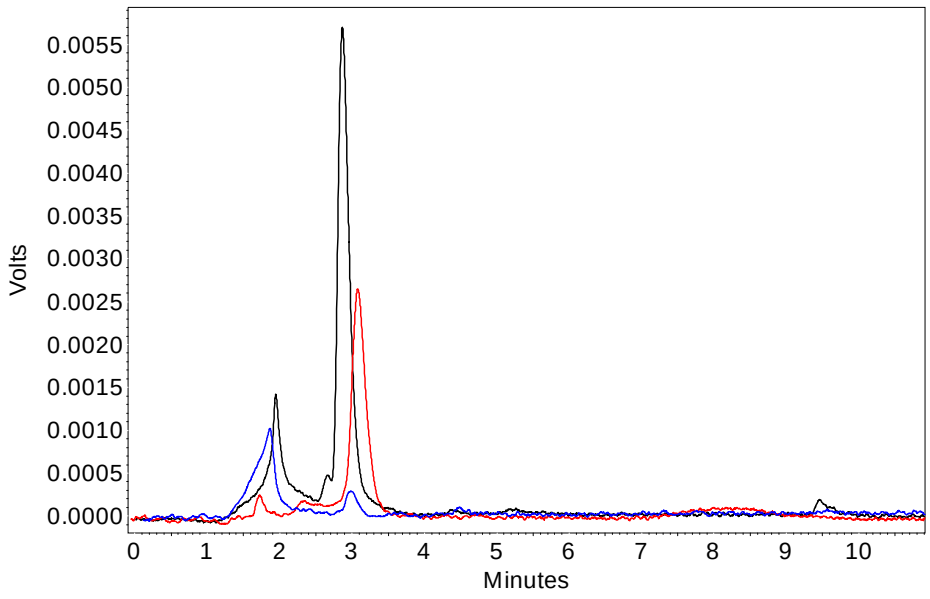

Figure V.2. Comparison of HPLC chromatograms with fluorescence detection $\lambda_{emission,max.}/\lambda_{excitation,max.}$ 320/385 nm, of the proteins extracted from FAP patients (black, FAP patient 1; red, FAP patient 2) and non-FAP subject (blue). All samples were submitted to the modified method of extraction. To simplify, just one non-FAP and two FAP samples were shown.

Amino acid analysis may be used to identify AGE since these compounds are actually modified amino acids and therefore additional peaks will appear in the chromatogram (Ahmed *et al.*, 2002). Dabsyl chloride was chosen as derivatizing reagent since it does not interfere with the fluorescence detection. Enzymatic hydrolysis of proteins is also essential, instead of the commonly used acid hydrolysis with 6 M HCl,

since argpyrimidine is acid-labile. A chromatogram of a standard amino acid mixture and a chromatogram from a representative FAP patient are presented in Figure V.3. The chromatogram of the amino acid analysis of the extracted material is substantially more complex with several new peaks appearing. These new peaks could be due to several AGE or caused by peptides resulting from incomplete protein hydrolysis.

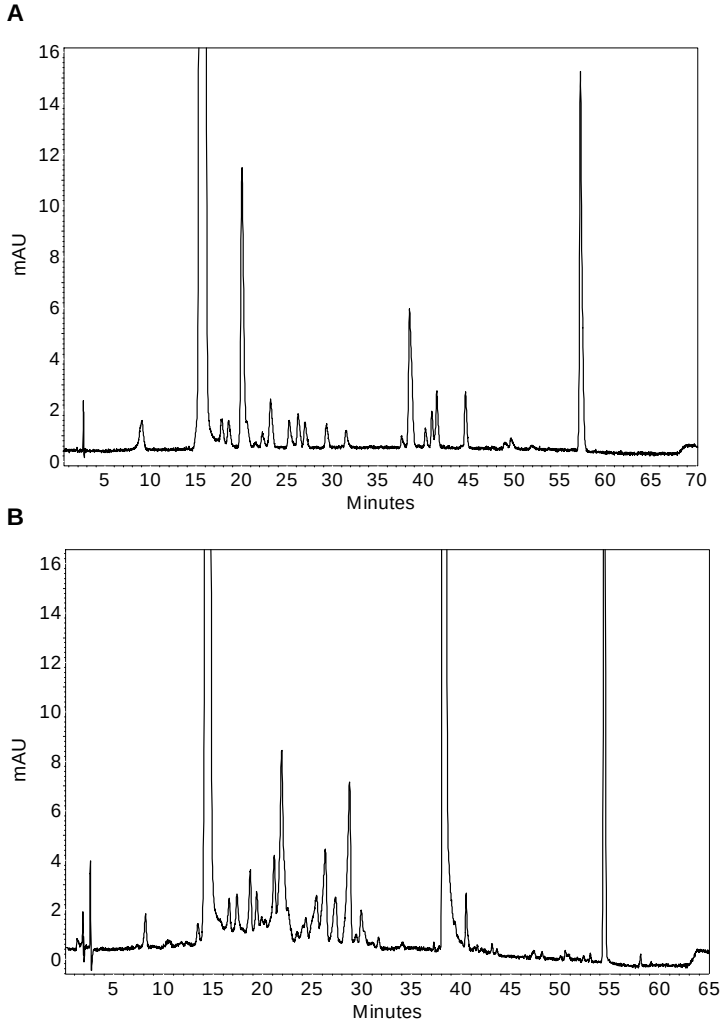

Figure V.3. HPLC amino acid analysis using dabsyl chloride of a standard amino acid mixture (A) and hydrolysed amyloid proteins extracted from a FAP patient by the modified extraction method (B). Absorvance was monitored at 452 nm. mAU, milli-absorbance units

Using fluorescence detection, two major peaks were detected with retention times of 10 and 49 min (Figure V.4). The 10 min retention time peak, found in all samples, is due to the derivatization reagent, also observed in a control derivatization assay without the addition of enzymatic hydrolysates (Figure V.5A). In contrast, the fluorescence peak with a retention time of 49 min only appears in the amino acid analysis of the extracted

amyloid fibrils from FAP patients. A comparison between this chromatogram and the argpyrimidine chromatographic standard (Figure V.5B) clearly indicates that the fluorescence peak with a retention time of 49 min is argpyrimidine. Retention time and spectroscopic properties are identical. Moreover, possible interferents like pentosidine or oxidation products of aromatic amino acids have distinct retention times (Ahmed *et al.*, 2002). Chromatographic peaks were also analysed for symmetry and purity with spectral data with 1 nm resolution using the Beckman-Coulter 32Karat software (version 5.0). No shoulders are apparent and the peaks were judged to be pure.

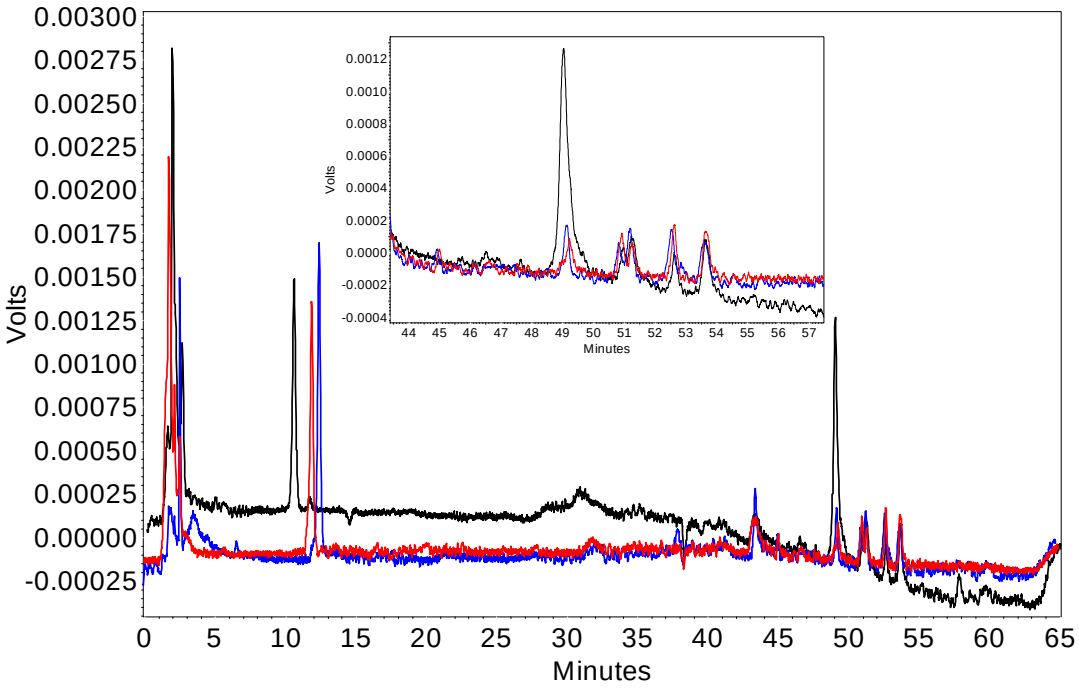

Figure V.4. HPLC amino acid analysis with fluorescence detection at $\lambda_{emission,max.}/\lambda_{excitation,max.}$ 320/385 nm of the material extracted from FAP and non-FAP subjects. Black, FAP patient; red and blue, non-FAP individuals. The inset represents a magnification of the chromatogram between 44 and 58 min, showing marked differences between the FAP and non-FAP samples. A significant fluorescence peak with the retention time of 49 min appeared only on the FAP sample. A minor fluorescence peak, later quantified as less than 1% percent of the FAP patient's values, was noted in a sample from just one control subject, who was undergoing surgery for a pancreatic tumor.

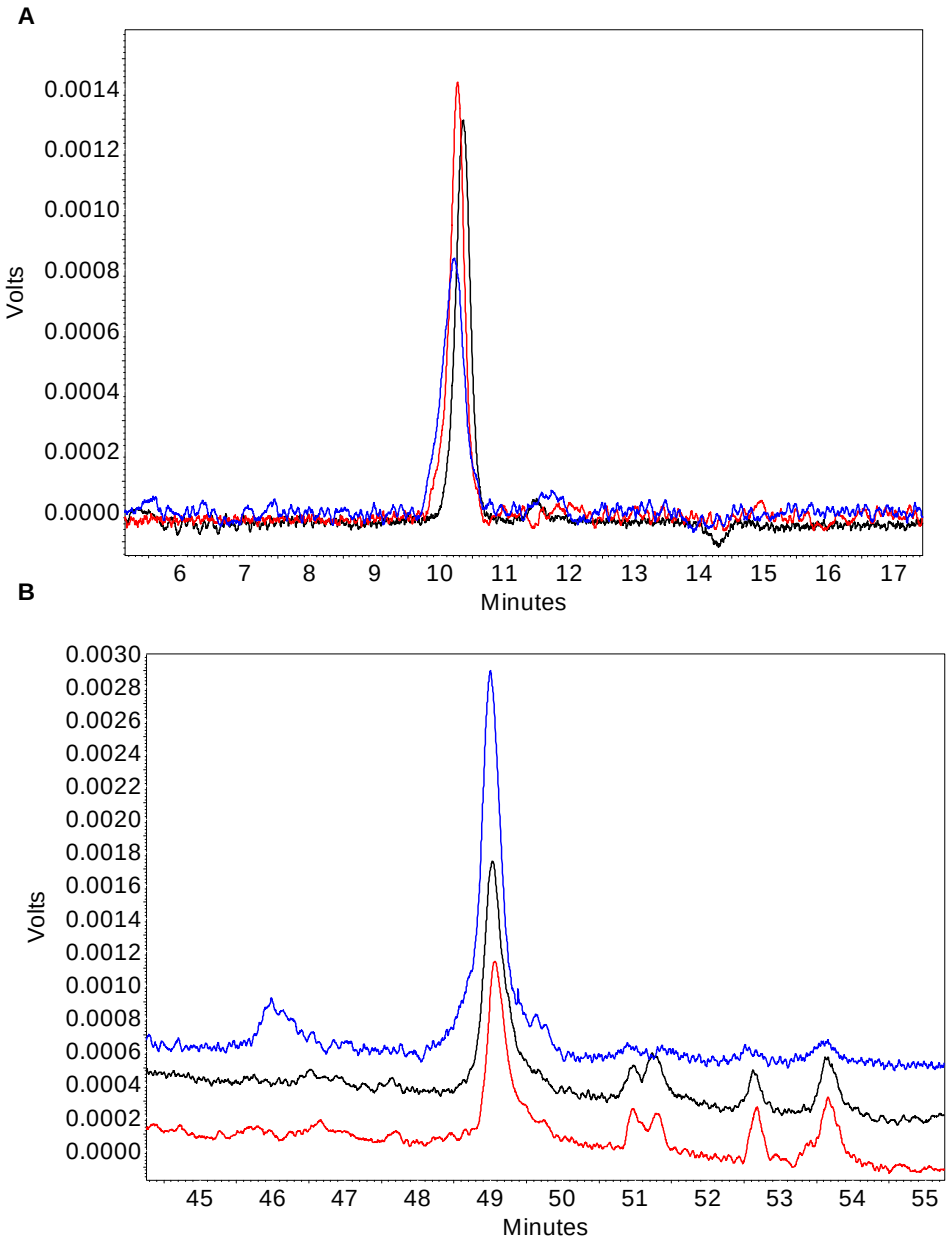

Figure V.5. Argpyrimidine in amyloid proteins extracted from adipose tissue samples of FAP patients. (A) HPLC analysis of a control derivatization assay, with no sample added (red), amino acid analysis of a FAP sample (black) and non-FAP sample (blue). A fluorescence peak with a retention time of 10 min appears in all three assays. (B) Argpyrimidine was detected by HPLC analysis with fluorescence detection at $\lambda_{emission,max.}/\lambda_{excitation,max.}$ 320/385 nm. The argpyrimidine chromatographic standard was treated as the samples from FAP and non-FAP subjects. The amino acid analysis of the FAP sample reveals a fluorescence peak at 49 min (black and red) coincident with the argpyrimidine standard (blue). To simplify, just two of the three FAP samples are shown.

Our data clearly demonstrate the presence of AGE in amyloid deposits characteristic of FAP patients and the chromatographic methods allowed us to identify argpyrimidine in these deposits. In FAP patients, 152 pmol, 166.7 pmol and 168.5 pmol of argpyrimidine per milligram of protein were detected (162.40 ± 9.05 pmol/mg protein, n=3). By contrast, only 1 pmol of argpyrimidine per milligram of protein in just one of the five non-FAP patients analysed was measured. This subject, undergoing surgery for a pancreas tumour, was the oldest individual analysed. The amount of argpyrimidine quantified in the present study is similar to recent results obtained by others: using a LC-MS based method, Ahmed and co-workers (Ahmed *et al.*, 2003) obtained 205 ± 19 pmol of argpyrimidine/mg of protein in human lens proteins, which are known to accumulate high levels of AGE.

The Maillard reaction in FAP

Increased AGE formation has been linked to the development of cataracts (Lyons *et al.*, 1991), clinical complications of diabetes *mellitus* (Tanaka *et al.*, 2000), uraemia (Miyata *et al.*, 1999), atherosclerosis (Kume *et al.*, 1995) and age-related disorders (Brownlee, 1995). In amyloid diseases, the formation of AGE is relevant in Alzheimer's disease (Du Yan *et al.*, 1997a; Vitek *et al.*, 1994; Yan *et al.*, 1994a), Parkinson's disease (Castellani *et al.*, 1996) and dialysis-related amyloidosis (Miyata *et al.*, 1994a). In Alzheimer's disease, glycation of β-amyloid peptide promotes the nucleation and precipitation of this peptide, suggesting an additional mechanism by which the Maillard reaction may accelerate the progression of Alzheimer's disease (Vitek *et al.*, 1994).

In FAP, the mechanisms involved in amyloid deposit formation and toxicity are largely unknown. Concerning amyloidogenesis, it has been shown that TTR tetramer dissociates to non-native monomeric species at physiological conditions (Quintas *et al.*, 1999). These non-native monomeric species lead to the formation of partially unfolded monomeric species and high molecular mass soluble aggregates (the so-called proto-fibrils). These soluble aggregates will lead to amyloid fibrils by an unknown mechanism. Based on our data and the FAP amyloidogenic model discussed below, we propose that protein glycation could be involved in amyloid fibril formation from these soluble

aggregates. Indeed, the formation of high molecular mass soluble aggregates precedes amyloid fibril formation by several years in FAP patients. Transthyretin can be detected by immunohistochemical methods in different tissues, years before amyloid fibrils are formed (Sousa *et al.*, 2001a). Similar results, on a different time scale, were obtained with a transgenic rat model overexpressing a particularly aggressive transthyretin mutation (Sousa *et al.*, 2002). The missing link in amyloidosis models lies in the mechanisms that cause the structural transition from soluble aggregates of partially unfolded proteins to insoluble fibrils. Recent research suggests that glycation of albumin is involved in conformational transitions, inducing partially unfold intermediary formation which in turn leads to the aggregation and fibril formation of albumin. A native all-α-motif protein turns into a cross-β structure characteristic of amyloid fibrils, as shown by Congo red, thioflavin T assays and by transmission electron microscopy (Bouma *et al.*, 2003).

Moreover, glycation of soluble protein aggregates can be seen as a fixative process since, after glycation, it is impossible to reverse the process of amyloid fibril formation. In contrast, the formation of soluble aggregates can be reversed (Quintas *et al.*, 2001). Therefore extensive glycation could mark the difference between soluble aggregates and toxic amyloid deposits. The current understanding of amyloid diseases indicates that all forms of amyloid share biophysical and biochemical features (most important is the similar structure with an extensive β-sheet), despite the lack of any relevant homology between the different amyloid precursor proteins. This observation suggests that the same process may be involved in amyloidogenesis of different types of amyloid and that this process is common to all forms of amyloidosis. The involvement of protein glycation also explains the formation of amyloid deposits derived from non-mutated TTR, as in SSA.

Amyloid deposits are thought to be the direct cause of cell toxicity in different forms of amyloidosis, as they are present in areas of neurodegeneration. An important observation is the presence of oxidative stress markers co-localizing with amyloid deposits (Beckmann *et al.*, 1994; Kume *et al.*, 1995; Sousa *et al.*, 2001c). In Alzheimer's diseases and in FAP, lesions have been correlated with lipid membrane peroxidation indicative of oxidative stress. Moreover, toxicity could be blocked *in vitro* by catalase indicating a free-radical-dependent mechanism (Andersson *et al.*, 2002). Studies

concerning Alzheimer's diseases reveal an apoptotic induction by the amyloid deposits *in vivo* and *in vitro* through a mechanism involving the generation of free radicals (Dickson, 2004). The formation of AGE in FAP amyloid deposits can also contribute to cell toxicity and neurodegeneration via oxidative stress. In agreement to this hypothesis, glycated proteins may generate oxidative stress themselves (Yim *et al.*, 1995) or by the interaction with RAGE (Huttunen *et al.*, 1999; Yan *et al.*, 1994a). Moreover, AGE-modified proteins are toxic to animal cells (Iversen *et al.*, 1995). So, protein glycation induces cell toxicity due to the production of local oxidative stress. This local oxidative stress formed upon interaction between AGE and RAGE is responsible for the activation of important transcriptional factors like NFκB and TNF-α that could trigger a neuronal inflammatory and apoptotic pathway that is important in FAP neurodegeneration (Huttunen *et al.*, 1999; Sousa *et al.*, 2001c). Several studies demonstrated that the tissue targeting of amyloid-induced toxicity is not cell-type-specific and it was proposed that local factors might facilitate conformational change of mutated TTR, leading to toxic aggregates (Andersson *et al.*, 2002). We propose that these "local factors" might modulate the glycation of the proteins in amyloid fibrils or proto-fibrils.

The present work provides new insights into the role of glycation in amyloid deposits formation since argpyrimidine was identified unequivocally and quantified for the first time in extracted fibrils from FAP patients, using chromatographic methods. Since argpyrimidine is not one of the major AGE found *in vivo*, we expect that other AGE might be present in higher quantities in these deposits. Nyhlin and co-workers (Nyhlin *et al.*, 2000) found AGE immunoreactivity in tissue samples rich in amyloid deposits from FAP patients. No particular AGE were identified or quantified, but the results present here show that AGE are indeed present in amyloid deposits in FAP.

So far, the major research in this area was directed to *in vitro* studies, with the production of the fibrils *in vitro*, and mainly concerned structural changes in TTR induced by amyloidogenic mutations (Hamilton *et al.*, 1993; Redondo *et al.*, 2000; Sebastiao *et al.*, 1998). In contrast, in the present study we used samples from FAP patients and studied directly amyloid deposits found *in vivo*.

Despite the genetic origin of the disease, glycation should be seriously considered as an additional factor in FAP.

5. Acknowledgments

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CHAPTER VI

PROTEIN GLYCATION INDUCES TRANSTHYRETIN AMYLOID FIBRIL FORMATION *IN VIVO*

1. SUMMARY

The hallmark of familial amyloidotic polyneuropathy (FAP), a neurodegenerative disorder that primarily affects the peripheral nervous system, is the deposition of transthyretin (TTR), which assumes a β-sheet fibril structure forming amyloid deposits. This is a fatal disease for which no therapy is currently available, except liver transplantation, a stressing and debilitating procedure. The processes that lead to the transition from a functional innocuous protein into toxic structures are not fully understood. Although more than eighty TTR point mutations were associated with FAP, the existence of sporadic cases like senile systemic amyloidosis (SSA), derived from non-mutated TTR, and the wide variation of disease onset for patients bearing the same mutation, point to the involvement of non-genetic factors. Protein glycation might just be one of those factors, given its occurrence in all amyloid deposits in neurodegenerative diseases. To address this issue, we investigated TTR amyloid fibril formation in *S. cerevisiae*. Yeast is an ideal model since it is highly susceptible to protein glycation and the occurrence of glycation can be controlled. For this purpose, TTR variants with different amyloidogenic potentials were expressed in yeast and the formation of amyloid deposits was monitored by fluorescence microscopy after thioflavin-S staining. In non-glycation conditions, TTR-amyloid fibrils were detected only in cells expressing the highly amyloidogenic synthetic TTRd-D variant. No amyloid deposits were observed with TTR-wt and TTR-L55P. In glycation conditions (non-growing yeast cells challenged with 250 mM of D-glucose), TTR amyloid aggregates are observed in cells expressing the amyloidogenic TTR-L55P variant. Moreover, TTR glycation *in vivo* by methylglyoxal was observed by western blot analysis. These results provide the first experimental evidence that protein glycation promotes the formation of TTR-amyloid deposits *in vivo*.

2. INTRODUCTION

Transthyretin (TTR), also referred in older scientific literature as pre-albumin, is the main component of amyloid deposits preferentially found associated to the peripheral nervous system, characteristic of familial amyloidotic polyneurophaty (FAP) (Andrade, 1952; Costa *et al.*, 1978). FAP was first described in a group of Portuguese patients who had a fatal hereditary amyloidosis, inherited in an autossomal dominant pattern. This disease is characterized by a sensorimotor peripheral polyneurophaty and autonomic dysfunction (Andrade, 1952).

Like all amyloidogenic proteins, TTR is normally an innocuous protein found in the plasma and cerebrospinal fluid (Soprano *et al.*, 1985). Its physiological role is the transport of thyroxine hormone and retinol, the latter in association with the complex formed between retinol and retinol binding protein (RBP) (Kanai *et al.*, 1968; Peterson, 1971; Robbins & Rall, 1960). Native TTR is a homotetramer with two identical thyroxine-binding sites located in a channel at the core of the molecule (Blake *et al.*, 1978) and four RBP binding sites at the surface (Monaco *et al.*, 1995). The monomer has a high β-sheet content with eight β-strands (A to H) and a short helix between strands E and F (Blake *et al.*, 1978). Two TTR monomers join edge-to-edge to form a dimer and the tetramer results from the association of two dimers (Blake *et al.*, 1978; Hamilton *et al.*, 1993).

Under certain conditions, TTR undergoes massive structural changes to produce β-amyloid fibrils, by mechanisms far from being fully understood. TTR variants with single amino acid replacements as a consequence of single point mutations were associated with several clinical forms of FAP (Saraiva *et al.*, 1984). Over eighty different amyloidogenic point mutations have been reported (Saraiva, 2001). Among them, TTR-V30M (substitution of methionine for a valine at position 30) is the most common, being detected in many kindred around the world, including Portugal, Sweden and Japan (Connors *et al.*, 2003). In an aggressive form of FAP the amyloid fibrils are composed predominantly by a new TTR variant, L55P (substitution of a proline for a leucine at position 55) (Jacobson *et al.*, 1992). Age at disease onset for TTR-L55P carriers was reported in the second decade of life, much earlier than for TTR-V30M variant carriers

(normally between the fourth to fifth decades of life) (Ando *et al.*, 1993; Araki, 1995; Nakazato *et al.*, 1992). Since TTR-L55P is highly amyloidogenic, it is likely that amyloid fibril formation is directly related to disease onset and progression. Besides these naturally occurring mutations, the deletion or multiple substitutions in the D β-strand increases the amyloidogenic behaviour of TTR (Goldsteins *et al.*, 1997; Goldsteins *et al.*, 1999). With the discovery of TTR point mutations associated with the disease, it was believed that as a result of amino acid substitutions, structural and stability changes occur, ultimately leading to tetramer dissociation and amyloid fibril formation. Considerable research efforts were directed towards comparative structural studies of TTR mutants. For most cases, only small structure differences were observed and a clear association between the structural changes induced by point mutations and amyloidogenesis is not perceived (Lei *et al.*, 2004).

TTR amyloid formation and its amyloidogenic properties were extensively investigated *in vitro*, leading to one of the best established models for amyloid TTR fibril formation (Quintas *et al.*, 1997; Quintas *et al.*, 1999; Quintas *et al.*, 2001). In physiological conditions, the TTR tetramer may dissociate into partially unfolding monomers that associate into high molecular mass soluble aggregates that will evolve into insoluble amyloid β-fibril deposits (Quintas *et al.*, 1997; Quintas *et al.*, 1999). In strong agreement with this model, a correlation between the thermodynamic stability of TTR variants and their potential to form partially unfolded monomers and soluble aggregates was found. The TTR-L55P shows the lowest thermodynamic stability, being the less stable TTR variant (Quintas *et al.*, 2001). However, this model does not explain the transition from soluble, partially unfolded protein aggregates to highly insoluble, protease resistant and structurally well-defined β-amyloid fibrils. More importantly, it fails to explain the wide differences on disease onset between patients carrying the same amyloidogenic mutation. These doubts hint at the existence of others factors that must be considered in the pathogenesis and progression of this neurodegenerative disorder. Consistent with this idea, non-mutated TTR also form amyloid fibrils in patients with senile systemic amyloidosis (SSA), a widespread geriatric disease that affects approximately 25% of the population aged above 80 years (Westermark *et al.*, 1990). Thus, TTR has an intrinsic amyloidogenic behavior that is enhanced by specific point

mutations. Moreover, different amyloidogenic proteins without any homology form amyloid fibrils with virtually identical structures, suggesting that similar processes might be involved in amyloid fibril formation in different amyloidosis.

One likely non-genetic factor towards amyloidogenic behaviour is protein glycation. This non-enzymatic post-translational modification occurs in most amyloid disorders like Alzheimer's (Vitek *et al.*, 1994; Yan *et al.*, 1994a) and Parkinson's diseases (Castellani *et al.*, 1996; Munch *et al.*, 2000) and DRA (Miyata *et al.*, 1993). FAP is no exception, with argpyrimidine being detected in amyloid deposits extracted from FAP patients (Gomes *et al.*, 2005a). To address the involvement of protein glycation in TTR amyloid fibril formation, we used yeast cells as a eukaryotic cell model to host human recombinant TTR variants. In yeast, glycation phenotypes can be controlled through growth conditions and by deletion of genes coding for methylglyoxal catabolic enzymes (Gomes *et al.*, 2005b). Thus, different TTR variants with distinct amyloidogenicity (TTR-wt, TTR-L55P and TTRd-D) were expressed in yeast and the effects of protein glycation on TTR amyloid formation *in vivo* were investigated.

3. MATERIAL AND METHODS

Reagents and Materials

Peptone, yeast extract, agar and yeast nitrogen base (YNB) were obtained from Difco while D-glucose (microbiology grade) was obtained from Merck. Tryptone, 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, PMSF, glass beads (452-600 microns), sorbitol, thioflavin-S, formaldehyde, β-mercaptoethanol, agarose, lithium acetate, lyticase, gelatine G-9382, tween-20 and DAPI (4',6-diamidino-2-phenylindole) 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. VectaShield® was from Vector Labs.

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.

Bacteria, yeast strains and culture conditions

Escherichia coli strain used (DH5α, F-; *rec*A1; *end*A1; *thi*-1; *gyr*A96; hsdR17; *sup*E44; *rel*A1;ϕ89d; *lac*Z; DM15 λ-) was cultured in LB medium [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract] at 37 ºC. Solid LB medium contained 2% (w/v) agar. Transformed strains, carrying the plasmids, grew in LB medium supplemented with 0.1 $mg.ml^{-1}$ ampicillin.

Saccharomyces cerevisiae strain used was the BY4741 (genotype BY4741 *MATa*; *his3*Δ1; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) 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 slopes 2% (w/v) agar] at 4 ºC and cultured in liquid YPGlu medium at 30 ºC. BY4741 strain expressing the plasmids containing TTR variants was cultured in YNB

minimal medium without uracil [0.67% (w/v) yeast nitrogen base, 2% (w/v) D-glucose and 0.025% (w/v) L-methionine, L-histidine, L-leucine].

Plasmids and yeast transformation

Plasmids carrying the different TTR genes (TTR-wt, TTR-L55P and TTRd-D) were constructed by Dr. Tiago Outeiro (Massachusetts General Hospital, Harvard Medical School, USA) who kindly provided the transformed DH5α *E. coli* strains. Human TTR genes were cloned using *Bam*HI and *Xho*I sites 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).

E. coli cells were grown in liquid LB medium supplemented with ampicillin (as referred), and plasmid DNA extraction was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega), following manufacturer's instructions. DNA concentration was evaluated spectrophotometrically at 260 nm and purity was assessed by the absorbance ratio 260/280 nm and by electrophoretic analysis in 0.8% (w/v) agarose gel in 1x TAE (Tris/acetate/EDTA buffer), according to standard procedures (Ausubel *et al.*, 1990).

Plasmids carrying different TTR genes were used to transform BY4741 yeast strain using the lithium acetate method and transformants were selected on minimal agar plates deficient in uracil, following the procedure described in the Yeast Protocols Handbook ("Small-scale LiAc Yeast Transformation Procedure", from Clontech). The same yeast strain was also transformed with the p426GPD vector without the gene, as a control. For protein expression, transformants were grown in YNB minimal medium without uracil.

Yeast growth curves

Yeast growth assays were performed using a method described by Sainhas and co-workers (Sainhas *et al.*, 1998). The cultures were diluted into fresh medium to an initial absorbance at 640 nm of 0.25. Cells were then grown at 30 ºC in a water bath (with

magnetic stirring in the flask) and the growth was followed at 640 nm, for 24 hours in a Beckman DU-7400 diode array spectrophotometer, with temperature control and magnetic stirring in the cuvette, essential to maintain isotropic conditions. A continuous circulation system using a multichannel peristaltic pump from the cuvette to the culture flask was designed for continuous monitoring of the cell concentration.

Glycation experiments

Cells were harvested at the end of the exponential phase of growth, washed twice in type II water, suspended at a concentration of $5.2x10^8$ in 0.1 M MES/NaOH pH 6.5 with 250 mM D-glucose and incubated at 160 r.p.m, 30 ºC, in a orbital shaker (Infors HT). Samples were collected at defined times for protein glycation, TTR analysis and detection of TTR-amyloid deposits.

Immunoblot analysis of transthyretin expression and protein glycation

Total yeast protein extraction was performed by glass bead lysis as described (Ausubel *et al.*, 1990; Gomes *et al.*, 2005b). Proteins (30 μg per lane) were separated by SDS-PAGE in a Mini-protean 3 (Bio-Rad) using a 12% polyacrilamide 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) with transfer buffer 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. 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 ºC in 1% (v/v) blocking solution in TBS (50 mM Tris and 150 mM NaCl, pH 7.5). For argpyrimidine detection, 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), diluted 1:2000 in blocking solution. An anti-human-TTR antibody (a gift from Dr. Fiona Campbell, University of Glasgow UK), diluted 1:350 in blocking solution, was used to detect this protein in membranes.

Washes, secondary antibody and detection procedures were performed using the BM Chemiluminescence Western Blotting Kit (Roche) following manufacturer's instructions. Each immunoblot was repeated at least two times from independent experiments.

Transthyretin analysis by mass spectrometry

Proteins (30 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 kDa TTR) 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 manufacturer's instructions and eluted directly to the MALDI plate (Anchorchip MALDI target from Brucker) with 0.8 μl of DHB matrix (10 $mg.ml^{-1}$) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) TFA. The samples were air dried. Peptide mixture was analysed by MALDI-FTMS in a Bruker Apex Ultra with a 7 Tesla magnet. Monoisotopic peptide masses, determined by the Snap 2 algorithm in Data analysis 3.4 software, were used to search for homologies with theoretical TTR tryptic digestion using Brucker Daltonics BioTools 3.1 software. A mass accuracy of at least 5 ppm was considered; Cys carbamidomethylation and Met oxidation were taken into account as fixed and variable amino acid modifications, respectively.

Detection of amyloid deposits *in vivo* by thioflavin-S staining

Thioflavin-S staining was performed as described (Kimura *et al.*, 2002; Zabrocki *et al.*, 2005), with some modifications. Cells from late-exponential phase (absorbance at 640 nm between 1.5 and 2.0) were collected, diluted to $3x10^7$ cells and harvested by centrifugation (1700 g for 4 min at 4 ºC). Cells were then washed twice with potassium phosphate buffer 0.1 M pH 6.5, before fixation with 4% (w/v) formaldehyde in PBS (0.02 M sodium phosphate buffer with 0.15 M NaCl, pH 7.4) for 20 min at room temperature. Cells were washed twice with 0.1 M phosphate buffer pH 6.5 containing 1.2 M sorbitol

(S-buffer), suspended in 1 ml S-buffer and pre-treated with 0.002% (v/v) β-mercaptoethanol for 45 min at 30 ºC and 160 r.p.m. in an orbital shaker. Spheroblasts were then prepared by incubation with 50 μg lyticase *per* ml of cell suspension for 60 min at 160 r.p.m. and 30 ºC. Spheroblasts were washed once more in S-buffer (centrifuging at 700 g, 4ºC for 5 min) and permeabilized with 10% (v/v) triton X-100 (20 μl / ml cells) for 10 min at room temperature. Spheroblasts were stained with thioflavin-S. A 1% (w/v) thioflavin-S solution was freshly prepared in water and centrifuged before use to remove any undissolved material. Spheroblasts were then incubated for 15 min with 0.001% (v/v) thioflavin-S and washed three times with 0.1 M phosphate buffer pH 6.5 containing 1% (w/v) gelatin, 0.12 M NaCl and 0.1% (v/v) Tween 20. Finally, cells were suspended in VectaShield® mounting medium and DAPI (2 $mg.ml^{-1}$) was added for fluorescence DNA labelling. Cells were placed on microscope slides for observation using fluorescence microscopy.

Fluorescence microscopy

The imaging setup consists of an Olympus IX-50 inverted microscope, Ludl BioPoint filter wheels and a 12-bit PCO Sensicam cool CCD. Integrated control of filter wheel and image acquisition is achieved by Image-Pro Plus 5.0 and Scope-Pro 3.1 (Media Cybernetics). The settings for image acquisition (camera exposure time, filters, time interval, and storing modes) were determined by custom-made macros. The images were collected with Olympus 40 or 100 plan apo objectives (numerical apertures 0.95 and 1.4, respectively). Once defined, the settings were kept constant throughout the data collection. Fluorescence analysis was performed with the software package Image-Pro Plus 5.0 (Media Cybernetics). The following filter configurations were used: thioflavin S, excitation 436 nm, emission > 455 nm; DAPI, excitation 358 nm, emission 463 nm.

4. RESULTS

TTR expression in yeast cells

To study the molecular mechanisms underlying TTR-amyloid formation and its relationship to protein glycation, yeast cells were used as a well-defined cellular model system for which different glycation phenotypes exist (Gomes *et al.*, 2006; Gomes *et al.*, 2005b). Three TTR variants with different amyloidogenic behaviour were expressed in the reference yeast strain BY4741: the wild-type TTR (TTR-wt), the highly aggressive variant TTR-L55P and the synthetic extremely amyloidogenic TTR variant with deletion of the β-strand D (TTRd-D).

BY4741 cells at the end of the exponential phase of growth show similar expression levels of the different TTR variants, as shown by western blot analysis with anti-human TTR antibody (Figure VI.1A). A protein band with an apparent molecular weight of 14 kDa, corresponding to monomeric TTR, is clearly observed. Furthermore, another immunoreactive protein was also detected at 32 kDa indicating the presence of TTR dimers. Similar observations, showing the presence of monomeric and dimeric TTR in a western blot analysis, were reported (Purkey *et al.*, 2001; Wilce *et al.*, 2001). In dimeric TTR, the strong interaction between monomers may prevent the dissociation of all TTR molecules in the denaturating conditions of SDS-PAGE electrophoresis (Chang *et al.*, 1999). Since TTR is a homotetrameric protein produced by the interaction between two dimers, the observation of dimeric TTR hints that, when expressed in yeast, TTR acquires its native tetrameric folded structure. No positive signal was observed for yeast cells carrying the plasmid without insert, confirming the specificity of the TTR immunoreactive signal (Figure VI.1A, control). To further confirm the presence of TTR, the protein at 14 kDa was excised from the gel and subjected to in gel trypsin hydrolysis with the resulting peptide mixture analysed by MALDI-FTMS. Four peptides derived from human TTR sequence, without the first 20 amino acids that correspond to the signal peptide, were identified providing a sequence coverage of 21.3% (Table VI.1). The low number of TTR peptides observed is due to the intrinsic sequence of the protein. With trypsin digestion, the vast majority of the resulting peptides show an m/z greater than

2000, hampering their detection by MALDI-FTMS. Nevertheless, the four TTR peptides were detected with a very high mass accuracy, below 3 ppm, and covered more than 20% of the protein sequence, rendering the identification unambiguous (Table VI.1).

Even though highly aggressive amyloidogenic TTR variants (such as the synthetic mutant lacking the β-strand D) were expressed in yeast, no effects on cell growth were observed (Figure VI.1B). Likewise, no growth changes were observed in yeast expressing a single copy of another amyloidogenic protein, the α-synuclein (wild-type and amyloidogenic variants) (Outeiro & Lindquist, 2003). However, doubling α-synuclein expression leads to inclusion body formation and strongly inhibits growth (Outeiro & Lindquist, 2003). This indicates that the toxic effects are directly related to the expression levels of the amyloidogenic protein, an interesting observation since aggregation and amyloid fibril formation are two concentration-dependent phenomena.

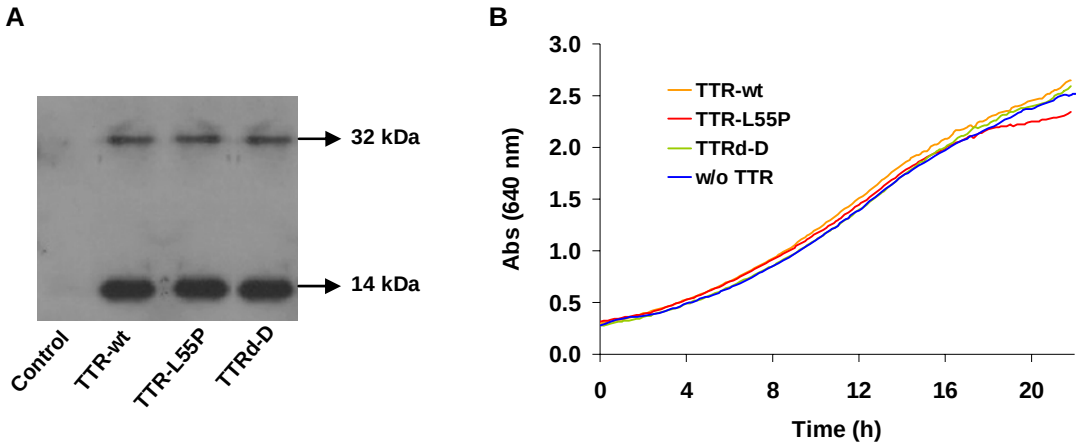

Figure VI.1. Expression of wild-type transthyretin and amyloidogenic variants in yeast. (A) Western blot analysis with anti-human TTR antibody of total protein extracts from BY4741 yeast strain carrying the empty plasmid (control, lane 1) and expressing wild-type TTR (TTR-wt) and variants TTR-L55P and TTRd-D. TTR monomer and dimer are observed with a molecular weight of approximately 14 and 32 kDa, only in cells expressing transthyretin. Similar expression levels were obtained for each TTR variant. (B) Growth curves of BY4741 strain expressing different TTR variants. Although some highly amyloidogenic TTR variants were used, no noticeable change in BY4741 strain growth was observed.

Table VI.1. MALDI-FTMS analysis of in gel tryptic digests of TTR monomer.

Observed mass (Da)	Theoretical mass (Da)	Peptide sequence*	Deviation (ppm)
1366.7592	1366.7589	GSPAINVAVHVFR (42-54)	0.220
1394.6216	1394.6222	AADDTWEPFASGK (56-68)	0.712
1494.8544	1494.8539	GSPAINVAVHVFRK (42-55)	0.041
1522.7218	1522.7172	KAADDTWEPFASGK (55-68)	2.617

* numbering considers the first 20 amino acids of the signal peptide

Formation of TTR amyloid aggregates in yeast

The formation of TTR-amyloid aggregates in yeast was investigated in cells expressing different TTR variants by thioflavin-S staining. Thioflavin-S is a methylated, sulfonated polymerized primulin mixture that specifically binds to amyloid fibrils like amyloid-β (Westermark *et al.*, 1999).

In cells expressing TTR-wt and the aggressive amyloidogenic TTR-L55P, no thioflavin-S staining was observed, indicating the absence of TTR-amyloid aggregates (Figure VI.2B and C). As expected, in cells expressing the empty expression vector no thioflavin-S staining was detected (Figure VI.2A). On the other hand, *foci* stained with thioflavin-S were detected in cells expressing the synthetic amyloidogenic variant TTRd-D, indicating the presence of TTR amyloid deposits (Figure VI.2D1 and D2). These β-sheeted aggregates do not co-localize with the nucleus, observed by DAPI staining (Figure VI.2D2 and D3). The thioflavin-S staining morphology is similar to the observed positive staining in yeast prions aggregation *in vivo* (Kimura *et al.*, 2003; Kimura *et al.*, 2002). It was already observed that the deletion of β-strand D yields TTR molecules that rapidly form aggregates, with a typical crossed β-pattern in X-ray diffraction studies and a positive signal after staining with Congo red or thioflavin-T (Goldsteins *et al.*, 1997; Goldsteins *et al.*, 1999). These results validate yeast as a model to study TTR dynamics and aggregation in living cells and to investigate the role of protein glycation in the process.

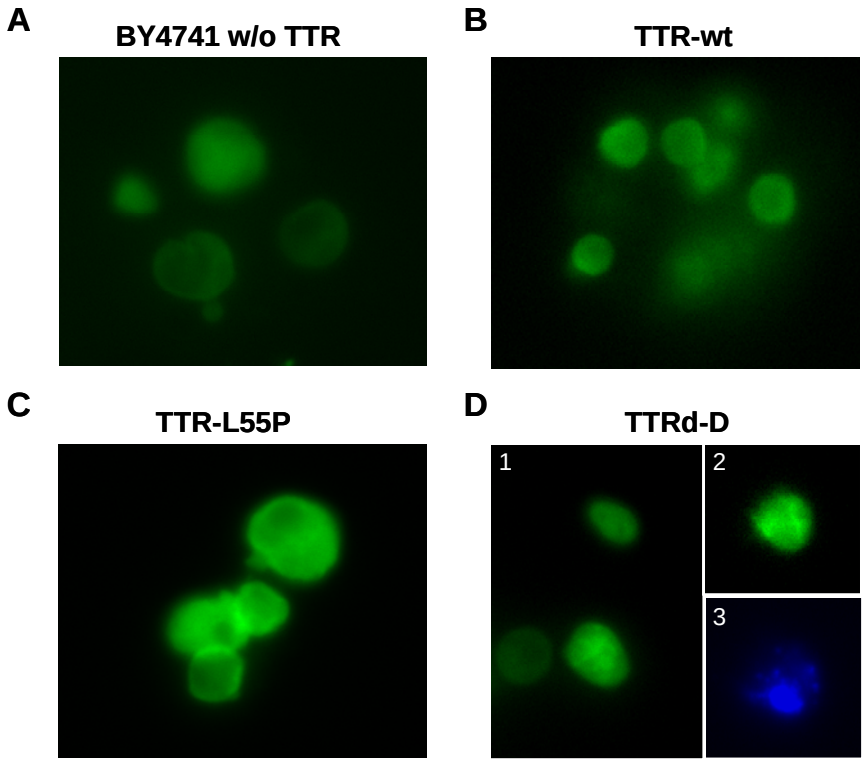

Figure VI.2. Formation of TTR amyloid aggregates *in vivo* probed by thioflavin-S staining. BY4741 yeast strain with the empty plasmid (A) and expressing TTR-wt (B), TTR-L55P (C) and TTRd-D (D) were used. In these experimental conditions, intracellular amyloid aggregates were detected only in yeast cells expressing the synthetic amyloidogenic TTR variant TTRd-D. The β-sheeted aggregates, observed in 2 do not co-localize with the nucleus, observed by DAPI staining, shown in 1. No amyloid deposits derived from the highly amyloidogenic TTR-L55P variant and wild-type TTR were observed. X1000.

Glycation conditions induces TTR glycation and TTR amyloid-aggregation in yeast

When non-growing yeast cells are exposed to 250 mM of D-glucose, the increased intracellular methylglyoxal concentration leads to the formation of MAGE-modified intracellular proteins (Gomes *et al.*, 2006; Gomes *et al.*, 2005b). Taking advantage of this finding, we first analysed intracellular protein glycation in yeast cells expressing different TTR variants challenged by glycation conditions. Consistent with our earlier findings (Gomes *et al.*, 2006; Gomes *et al.*, 2005b), four main argpyrimidine-modified proteins

were observed (Figure VI.3A), previously identified as enolase2, aldolase, phosphoglycerate mutase/Hsp26 and Hsp71/72 (Gomes *et al.*, 2006). After 5 hours incubation, a new argpyrimidine-modified protein was also detected with a molecular weight of approximately 14 kDa only in cells expressing TTR variants. This immunoreactive signal was not previously observed and is also absent from the control BY4741 immunoblot (Figure VI.3A). These observations show that monomeric TTR expressed in yeast is glycated *in vivo* by methylglyoxal. In fact, when the same blot was probed with anti-human-TTR antibody, monomeric TTR detection coincided with the novel MAGE-modified protein (Figure VI.3B). Interestingly, TTR glycation is only observed after 5 hours while naturally-occurring protein glycation targets in yeast are present after just 1 hour. TTR glycation levels of the variants used, TTR-wt, TTR-L55P and TTRd-D, are similar (data not shown).

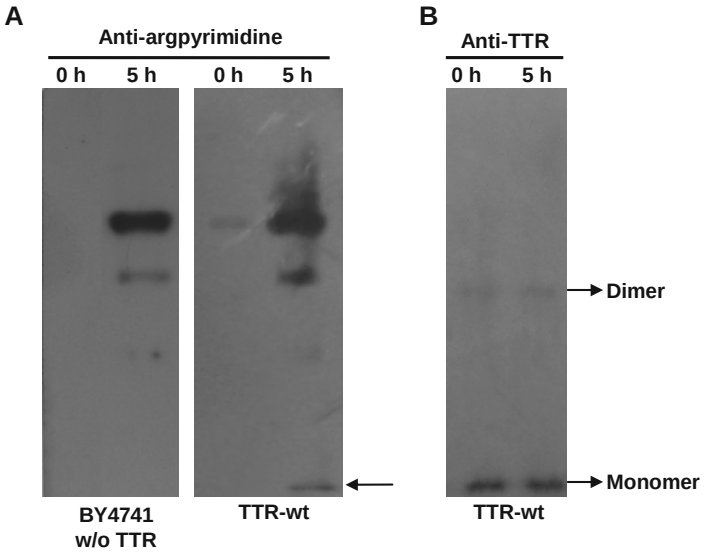

Figure VI.3. *In vivo* glycation of TTR expressed in yeast. (A) Western blot analysis of argpyrimidine-modified intracellular proteins in glycation conditions of BY4741 yeast strain carrying the empty plasmid and TTR-wt. Besides the detection of the yeast glycation targets (Gomes *et al.*, 2006), an additional imunoreactive signal at approximately 14 kDa emerges in cells expressing TTR after 5 hours of D-glucose incubation (indicated by the arrow). (B) This additional protein corresponds to the TTR monomer, as probed by western blot with anti-TTR antibody.

This observation led us to investigate the hypothesis that increased protein glycation (including in recombinant TTR) caused by carbonyl stress could trigger TTR-amyloid formation *in vivo*. Therefore, TTR-amyloid aggregates were analysed by thioflavin-S staining in cells exposed to glycation conditions. Like our previous observations, thioflavin-S stained citoplasmatic inclusions derived from TTRd-D variant at time zero and after 5 hours incubation (data not shown). With TTR-wt no differences were observed compared to non-glycating conditions, indicating that no amyloid deposits derived from TTR-wt were produced in either experimental condition (Figure VI.4A). On the contrary, glycation seems to induce a noticeable difference in the highly amyloidogenic TTR-L55P-aggregation. Albeit no thioflavin-S staining was observed in cells at the end of exponential phase of growth, after 5 hours incubation with D-glucose, cells expressing TTR-L55P variant show thioflavin-S stained citoplasmatic inclusions (Figure VI.4B). This result indicates that protein glycation directly causes the formation of TTR-amyloid deposits *in vivo*. Noteworthy, no changes on TTR-L55P and TTR-wt expression were detected by western blot (Figure VI.4C).

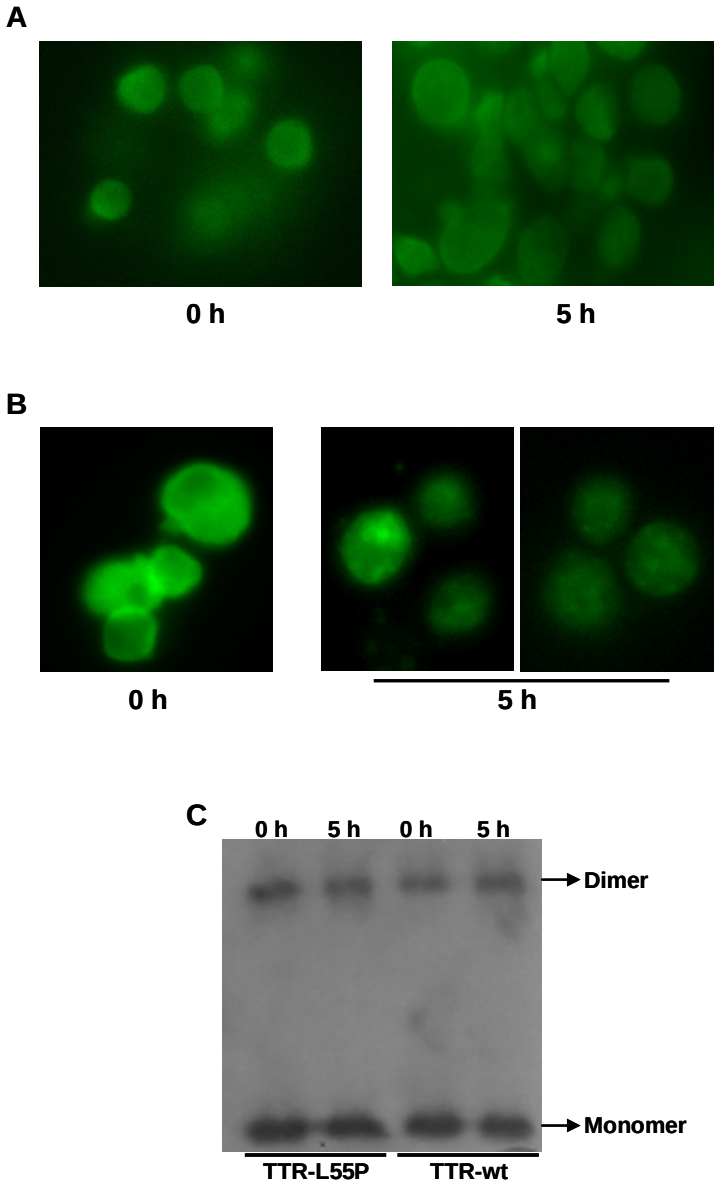

Figure VI.4. Protein glycation induces amyloid fibril formation in yeast expressing TTR-L55P. Yeast cells expressing TTR-wt (A) and TTR-L55P (B) variant were challenged with glycation conditions and analysed before (time zero) and after 5 hours incubation. After exposure to glycation conditions, intracellular amyloid aggregates become visible in cells expressing the amyloidogenic TTR-L55P. No amyloid aggregates become noticeable in cells expressing TTR-wt. X1000. (C) Western blot analysis of TTR expression. Similar TTR levels are obtained after five hours D-glucose incubation suggesting that the formation of TTR-L55P-amyloid deposits is not due to an increase on TTR expression.

5. DISCUSSION

The aberrant TTR aggregation and amyloid β-sheets structures are the pathological hallmark of amyloid disorders such as FAP and SSA (Costa *et al.*, 1978; Westermark *et al.*, 1990). Point mutations are believed to trigger the process of amyloid formation (Saraiva, 2001). Despite the high amount of research work in this area, the mechanism(s) by which an innocuous protein like TTR forms amyloid deposits with the consequent cell toxicity is not understood. The existence of sporadic cases like SSA in which non-mutated TTR forms amyloid fibrils (Westermark *et al.*, 1990) and the variation of disease onset time for patients bearing the same mutation (Ando *et al.*, 1993; Araki, 1995; Nakazato *et al.*, 1992) suggest that non-genetic factors like post-translational modifications are involved in protein misfolding *in vivo*. Protein glycation has been implied in amyloid disorders based on the detection of AGE-modified amyloidogenic protein components of amyloid deposits (Castellani *et al.*, 1996; Gomes *et al.*, 2005a; Miyata *et al.*, 1993; Vitek *et al.*, 1994; Yan *et al.*, 1994a). *In vitro*, protein glycation causes aggregation and amyloid fibril formation (Bouma *et al.*, 2003). However, it is still unclear whether glycation is directly involved in amyloid formation *in vivo* or is merely a result of the accumulation of amyloid fibrils which, due to their extreme insolubility and protease resistance, persist for a long period of time.

This study provides direct evidence that protein glycation by methylglyoxal increases TTR-amyloid fibril formation *in vivo*. Glycation conditions induce the formation of argpyrimidine-modified TTR *in vivo* and promote TTR-amyloid fibril formation in yeast cells expressing the amyloidogenic TTR-L55P variant. By contrast, although TTR glycation was also observed in TTR-wt, no amyloid fibrils were observed after exposure to glycation conditions. Interestingly, TTR glycation extent is quite similar between different TTR variants indicating that the presence of these mutations does not change TTR glycation susceptibility *in vivo*. These results show that glycation acts synergistically with amyloidogenic mutations, accelerating fibril formation. Glycation may cause an additional conformational change that, together with the destabilizing effects of the amyloidogenic mutations, could enhance β-amyloid deposit formation. In this context, the conformational changes imposed by glycation on TTR variants with

amyloidogenic potential that have a lower tetramer and non-native monomer stability might be sufficient to promote tetramer dissociation and amyloid fibril formation. Indeed, it was shown that conformational perturbation of the β-strands C and D give rise to an intermediate prone to self-assembly (Serag *et al.*, 2002). Non-specific post-translational modifications like glutathionylation and cysteinylation on Cys10 destabilize the tetrameric form of TTR and promote amyloidogenesis (Takaoka *et al.*, 2004; Zhang & Kelly, 2003).

Another interesting possibility is the involvement of glycation in the latter steps of amyloid formation: production of soluble aggregates or the transition to amyloid fibrils. Glycation could promote the formation of soluble aggregates and amyloid fibrils from the non-native monomers that result from TTR tetramer dissociation, which is known to occur in high extent when amyloidogenic mutations like L55P are present (Quintas *et al.*, 1999; Quintas *et al.*, 2001). Glycation may also act on soluble aggregates promoting the subsequent irreversible formation of insoluble amyloid fibrils. This is a very important issue that needs to be further investigated since it is becoming clear that the monomeric and small oligomers are the most toxic species (Reixach *et al.*, 2004) and that the formation of amyloid fibrils may be a protective mechanism to cope with those species (Ross & Poirier, 2004). Indeed, it was recently reported that 5-[4-(4-chlorobenzoyl)-1-piperazinyl]-8-nitroquinoline reduced toxicity in Huntington's and Parkinson's disease by promoting aggregation (Bodner *et al.*, 2006).

The discussed hypotheses imply that the modification of TTR by glycation induces amyloid formation, in agreement with the observation that TTR is glycated *in vivo*. However, protein glycation of other cellular proteins may lead to misfolding stress decreasing the cell quality control capacity, essential to avoid the harmful TTR misfolding pathways. Actually, one hypothesis to explain the late onset of several misfolding disorders is its occurrence with aging when the protein quality control system capacity to cope with accumulating misfolded proteins is exceeded (Outeiro *et al.*, 2006). Consistently, it was shown that cellular misfolding stress exists when yeast cells are exposed to methylglyoxal glycation conditions (Gomes *et al.*, 2006).

The mechanism(s) by which glycation induces TTR-fibril formation, either due to TTR glycation that may interfere with amyloid formation pathway and/or diminishing the

cellular quality control system due to misfolding stress, it is currently unknown. Nevertheless, we show that carbonyl stress increase, associated with methylglyoxal-protein glycation, promotes amyloid β-sheet aggregation in yeast cells expressing amyloidogenic TTR variants. This observation together with several evidences showing that glycation is involved in cellular toxicity associated with amyloid diseases, suggests that protein glycation must be accounted in the quest of effective therapeutic strategies for this multifactor disorder. The presented yeast model is certainly useful to increase our knowledge about protein misfolding, abnormal aggregation and cellular responses.

6. Acknowledgments

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CHAPTER VII

CONCLUDING REMARKS

CHAPTER VII – CONCLUDING REMARKS

Protein glycation has gained particular attention in the context of several human pathologies, such as age-related disorders and neurodegenerative diseases of amyloid type, from the earlier observations that glycated haemoglobin increases as a function of glycaemia in diabetic patients. Glycated proteins are characterized by modified biochemical features often associated with neurophatological amyloid lesions like high insolubility, protease resistance, cross-link structures and, in some cases, fluorescence and brown-colour (Colaco & Harrington, 1994; Harrington & Colaco, 1994). These modified proteins endure changes in their structure, with consequent function loss, and become toxic to cells. Therefore, extensive investigations were focused on finding novel therapeutic strategies capable to inhibit protein glycation and minimize its deleterious physiological effects. Nevertheless, the success has been quite limited. The best example of a Maillard reaction inhibitor is aminoguanidine, a hydrazine compound highly reactive towards dicarbonyl molecules (Brownlee *et al.*, 1986). However, there is still considerable controversy about its mechanism of action *in vivo*. Aminoguanidine is pharmacologically effective at low plasma concentration compared to the much higher doses typically required for inhibiting the Maillard reaction *in vitro* (Thorpe & Baynes, 1996). This hydrazine compound is a strong inhibitor of amine oxidases and nitric oxide synthase, affecting the vascular tone (Tilton *et al.*, 1993), and it also inhibits lipid peroxidation (Picard *et al.*, 1992). Therefore, this compound may be effective in the context of several human diseases, like diabetes *mellitus*, for reasons other than the inhibition of glycation reactions. This emphasizes the complexity of protein glycation and also our limited knowledge about the mechanisms, biochemical effects and cellular responses to this post-translational modification. Not surprisingly, the role of protein glycation in the development of pathological conditions is far from being understood.

Protein glycation has been mainly investigated *in vitro*, in which clinical relevant or model proteins are glycated under non-physiological conditions. Several examples may be found in the literature in which glycation agents were used in millimolar to molar concentrations for extended periods of time, from days to years (Bakhti *et al.*, 2007; Bouma *et al.*, 2003; Kang, 2003; Luthra & Balasubramanian, 1993; Raabe *et al.*, 1996).

In addition, protein interactions, which may influence glycation reactions, and the cellular responses to the detrimental effects of protein glycation, are not present *in vitro*. Importantly, cells have the ability to recover or degrade damaged proteins and synthesize new protein molecules in a continuous fashion thereby avoiding the deleterious effects of having a non-functional altered protein. Protein turnover is also absent from test tube experiments. Another important issue is the glycation agents' metabolism. Since AGE formation is a non-enzymatic process, the knowledge of the reactivity and metabolism of glycation agents *in vivo* is of utmost importance. The metabolism of methylglyoxal, the most relevant glycation agent *in vivo*, was studied in model systems but this is still an obscure area, where multiple catabolic pathways, of unknown relative importance, were described. The glutathione-dependent glyoxalase system is commonly accepted as the main catabolic pathway (Thornalley, 1990; Thornalley, 1996), but the existence of a few other enzymes capable of using methylglyoxal as substrate casts some doubts on this matter. All these observations highlight the importance of investigating protein glycation *in vivo* using cellular models.

In this work, we investigated protein glycation by methylglyoxal in *S. cerevisiae*, an outstanding cellular model for metabolic regulation studies, being easy to manipulate genetically. It is an extremely well-characterized eukaryotic cell with a wide collection of gene deletion mutants readily available, allowing global screens for induced phenotypes. Common to all eukaryotic cells, yeast has protein quality control systems, including degradation and folding pathways. It has been successfully used as a model organism to investigate protein misfolding in the context of conformational disorders (Outeiro & Lindquist, 2003; Outeiro & Muchowski, 2004). Prion transmission and the effects of α-synuclein aggregation were also investigated in yeast (Krishnan & Lindquist, 2005; Outeiro & Lindquist, 2003).

The first issue addressed was the occurrence of protein glycation in yeast. By choosing a set of gene deletion mutants in methylglyoxal catabolism and associated processes, protein glycation by methylglyoxal was found to occur when cells reached the steady-state stage of growth. This was the first observation that glycation occurs in a microrganism. Despite the association of protein glycation to complex organisms and

long-lived proteins, this post-translational modification also occurs in yeast in a short time-scale.

Contrary to the common belief that the glyoxalase system is the main methylglyoxal detoxification pathway *in vivo*, aldose reductase emerged as an equally important enzyme. This is in agreement with the observation that aldose reductase overexpression increases methylglyoxal tolerance and complements glyoxalase I deficiency in a ΔGLO1 strain (Aguilera & Prieto, 2001; Aguilera & Prieto, 2004). A sensitivity analysis of the kinetic model of methylglyoxal metabolism in yeast showed that aldose reductase and glyoxalase I are equally relevant in maintaining a low steady-state methylglyoxal concentration. The glutathione concentration also showed marked effects on methylglyoxal steady-state concentration, suggesting that oxidative stress, which leads to GSH depletion, methylglyoxal concentration and consequently MAGE formation, are linked processes (Figure VII.1). A higher methylglyoxal formation rate and/or a reduction in GSH concentration may impair methylglyoxal catabolism leading to its accumulation. Moreover, the reduction of oxidized glutathione (GSSG) requires NADPH, the cofactor for the aldose reductase-catalysed reaction. Noteworthy, methylglyoxal appears to be directly involved in the cellular response to oxidative stress in *S. cerevisiae*. Under these stressful conditions, several anti-oxidant coding genes are up-regulated upon the activation of transcriptional factors. In yeast, several genes responsible for glutathione metabolism, such as those involved in glutathione synthesis (GHS1) and glutathione-dependent antioxidant systems (GPX2, glutathione peroxidase, and GLR1, glutathione oxidoreductase), are up-regulated by YAP1, a functional homologue of the mammalian AP-1 (Grant *et al.*, 1996; Inoue *et al.*, 1999; Sugiyama *et al.*, 2000; Wu & Moye-Rowley, 1994). Importantly, the activity of YAP1 is reversibly modulated by methylglyoxal (Maeta *et al.*, 2004), indicating that YAP1 could therefore act as an intracellular sensor for methylglyoxal. In addition, YAP1 activation leads to an increase in GSH concentration, essential to methylglyoxal catabolism. In fact, we show that a yeast strain lacking YAP1 is susceptible to protein glycation. Methylglyoxal also functions as a signal initiator of the HOG-MAP kinase pathway, which induces glyoxalase I expression (Inoue *et al.*, 1998; Maeta *et al.*, 2005a).

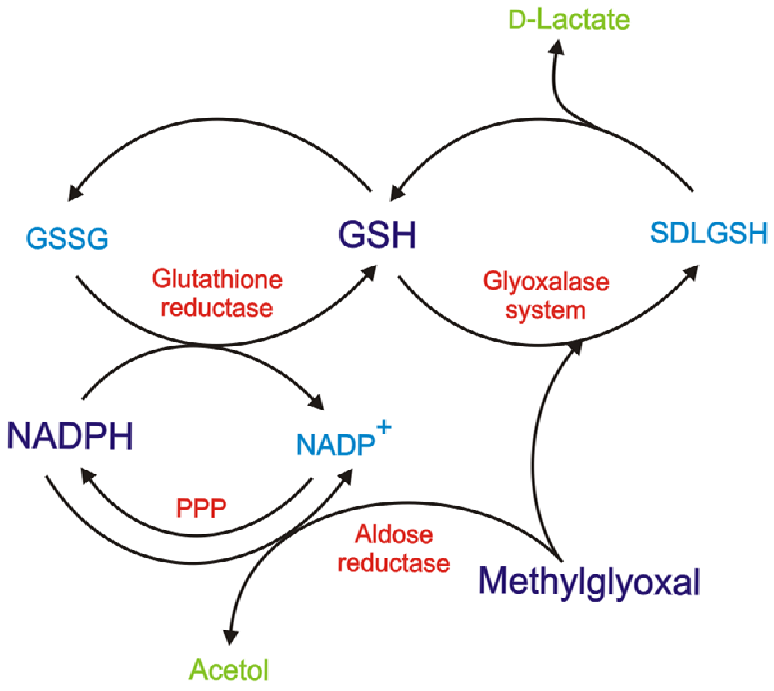

Figure VII.1. Model of the oxidative-carbonylic cycles. The model links the glutathione redox cycle, NADPH oxidation-reduction and methylglyoxal catabolism. Reduction of GSH concentration and/or an increase of methylglyoxal formation rate may impair methylglyoxal catabolism. In addition, NADPH, the cofactor of aldose reductase enzyme, is needed for the reduction of oxidized GSH (GSSG). In this conditions, methylglyoxal concentration increases and the formation of MAGE-modified proteins is enhanced. The pentose phosphate pathway (PPP) is the main route of NADPH formation. Interestingly, an increase in the methylglyoxal concentration triggers a cellular response with the activation of YAP1, responsible for the up-regulation of genes involved in GSH metabolism, and the activation of the HOG-MAP kinase pathway that induces glyoxalase I gene expression.

Parameter scanning with the model of methylglyoxal metabolism in yeast also revealed the extreme importance of its formation rate. The kinetic model predicts a linear increase of methylglyoxal concentration, suggesting that glycation would be more pronounced when methylglyoxal formation is increased. In fact, protein glycation was observed in the reference strain, despite all enzymatic defenses against methylglyoxal, after challenging non-growing yeast cells with a high D-glucose concentration. Thus, when key anti-glycation defenses are overcome, like in the ΔGLO1 and ΔGRE3 mutant

strains, or when methylglyoxal formation rate is increased, MAGE-modified proteins accumulate.

Different glycation levels can be obtained by different experimental conditions or mutant yeast strains, emphasizing the use of yeast as an excellent cellular model to investigate protein glycation *in vivo*. An important finding, again in sharp contrast with the current knowledge, is the existence of specific glycation protein targets, suggesting that glycation in yeast is a non-random process. All proteins are putative glycation targets, but only a few are modified *in vivo*. By peptide mass fingerprinting, three glycolytic enzymes (enolase2, aldolase and phosphoglycerate mutase) and the heat shock proteins Hsp71/72 and Hsp26 were identified.

The glycolytic enzyme enolase is the main glycation target, enduring a glycation-dependent activity loss with dimer dissociation and protein unfolding. Based on the knowledge of the molecular location of modified amino acid residues, using the hidden information of peptide mass fingerprint, an explanation for the observed effects was proposed (Figure VII.2). Glycation occurs at a critical arginine residue (R414) essential for dimer stabilization through an ionic pair formed with a glutamate residue from the other monomer (E20). The irreversible modification of R414 side chain with the formation of a hydroimidazolone would disrupt the electrostatic interactions that stabilized the dimer. Dimer dissociation into inactive monomers would then occur, followed by protein unfolding. Analysis by circular dichroism of purified enolase glycated *in vivo* showed secondary structure changes, with an increase of random structures and α-helix content decrease, and the presence of unfolded, inactive protein was confirmed by size-exclusion chromatography. If purified enolase is glycated *in vitro*, under conditions often described in the literature, these effects are enhanced. However, there are critical differences between these two glycation conditions, casting some doubts about *in vitro* glycation studies. In the latter, β-sheet content is increased and there is a considerable molecular heterogeneity of glycation. While *in vivo* the same amino acid residues appears to be consistently glycated forming the same MAGE, *in vitro* different MAGE were found on the same amino acid residue in different enolase molecules.

Considering the non-enzymatic nature of glycation, we found intriguing why enolase is the main glycation target. With the identification of the molecular location of

MAGE in enolase we propose that the arginine-rich cage located at the dimer interface could provide a favourable glycation environment for MAGE formation. In fact, reactivity of arginine residues towards glycation was shown to depend on their chemical environment (Ahmed *et al.*, 2005; Speer *et al.*, 2003). This enolase arginine-rich cave could be a trap for free methylglyoxal that evaded its catabolic routes, preventing modifications of other cellular proteins. Thus, enolase could function as a methylglyoxal scavenger. Remarkably, enolase interacts with some other vital proteins related to protein degradation via ubiquitin-dependent proteasome, transcriptional regulation, protein import/export and RNA export (Gavin *et al.*, 2006; Gavin *et al.*, 2002). Being merely quoted as a glycolytic enzyme, this observation is quite unexpected. As for the other two glycolytic enzymes, their role as minor glycation targets requires further investigation.

Protein unfolding as a consequence of glycation may be connected to specific cellular responses like the refolding chaperone pathway, and possibly the ubiquitin-mediated protein degradation. These two major components of cellular protein quality control system are specialized to deal with misfolded and/or aggregated proteins (Bukau *et al.*, 2006; Outeiro & Tetzlaff, 2007). Our results showed that under glycation conditions, a cellular response involving the activation of Hsp26 is triggered. Hsp26 functions as a captor of misfolded proteins, being involved in the refolding processes (Cashikar *et al.*, 2005; Ehrnsperger *et al.*, 1997; Haslbeck, 2002). Proteins that cannot be refolded will follow a degradation pathway where the role of small Hsps, like Hsp26, is poorly understood (Cashikar *et al.*, 2005; Han *et al.*, 2005; Park *et al.*, 2007). Therefore, active Hsp26p may sequester denatured enolase to be refolded or, more likely, degraded, thereby avoiding its aggregation.

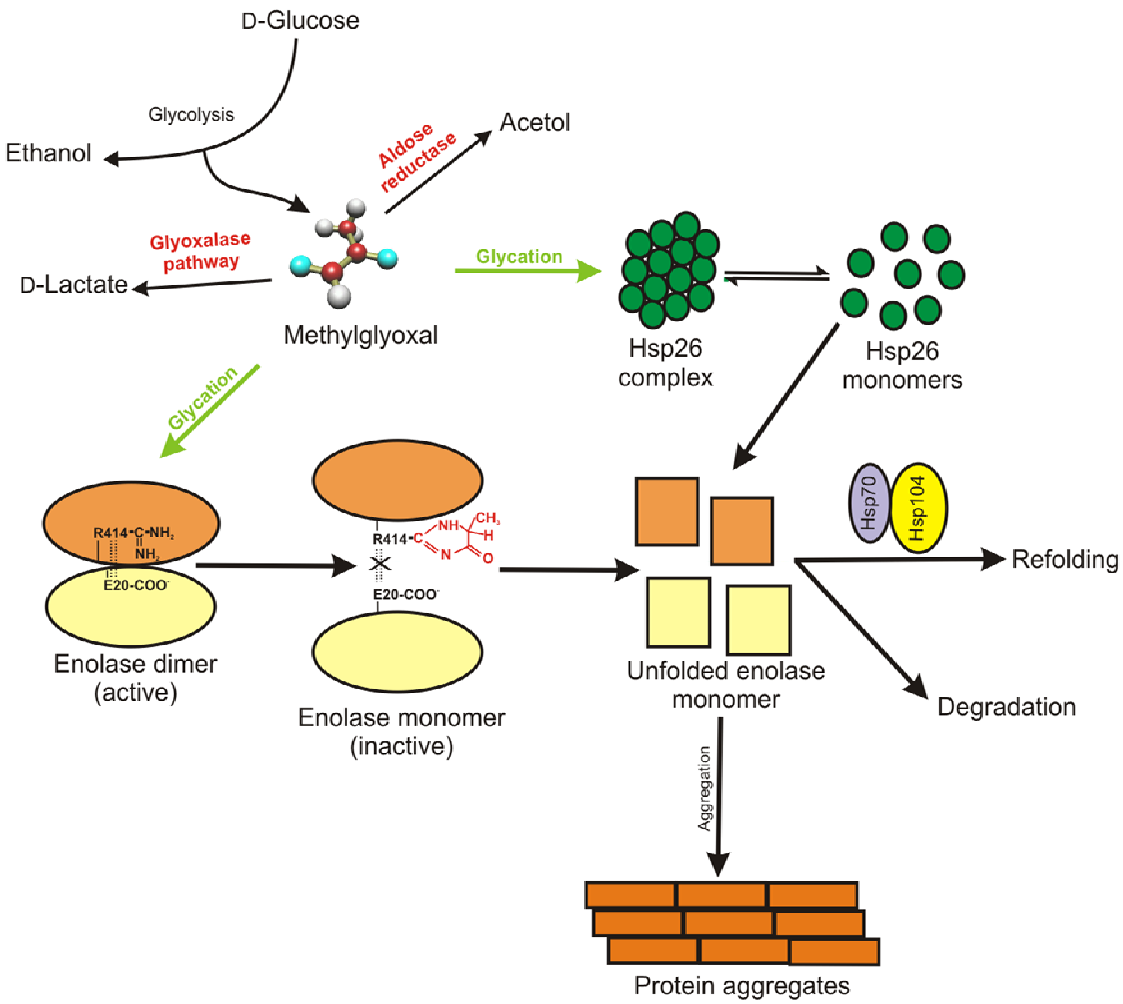

Figure VII.2. Model of protein glycation *in vivo*, unfolding stress and cellular responses. Methylglyoxal is mainly catabolised by the GSH-dependent glyoxalase system and the NADPH-dependent aldose reductase. Nevertheless, protein glycation by methylglyoxal still occurs and causes structural changes leading to protein unfolding. Extensive protein unfold could results in the formation of protein aggregates. Cellular responses include the activation of the refolding chaperone pathway, the deployment of methylglyoxal-protein scavengers and protein degradation pathways. These responses will counteract the harmful effects of protein misfolding induced by protein glycation.

The mechanisms underlying the observed Hsp26 activation are not yet perceived. Is specific methylglyoxal glycation required for the activation of heat shock proteins and other components of the cellular quality control system? If so, do cells "sense" methylglyoxal concentration through this mechanism and immediately activate cellular defenses to counteract its harmful effects? Our discoveries hint that specific methylglyoxal modifications through the Maillard reaction may regulate the activity of Hsp26. In this context, post-translational modifications by methylglyoxal may play an important role in the regulation of several cellular proteins. In fact, argpyrimidine-modified proteins were constitutively detected in several human cancer cell lines and the main glycation target was identified as Hsp27 (Padival *et al.*, 2003; Sakamoto *et al.*, 2002; Schalkwijk *et al.*, 2006). Argpyrimidine modification of Hsp27 specifically occurs on R188 at the C-terminal and is essential to the anti-apoptotic activity of this protein (Sakamoto *et al.*, 2002). Hsp27 and α-crystallins enhance their chaperone activity upon glycation by methylglyoxal (Nagaraj *et al.*, 2003; Oya-Ito *et al.*, 2006). This may link methylglyoxal to protein unfolding stress, associated with several conformational pathologies. Moreover, it raises the possibility that methylglyoxal-derived glycation of Hsp71/72 and Hsp26 lead to their activation and/or improved chaperone properties, being involved in the cellular response to glycation. This is a fundamental point that needs to be addressed in the future.

The described biochemical effects and cellular responses to methylglyoxal-derived protein glycation may also be associated with the role of this post-translational modification in amyloid fibril formation. The mechanisms of protein misfolding and aggregation have been investigated in yeast cells, which display most properties of eukaryotic cells regarding protein synthesis and quality control systems (Outeiro & Muchowski, 2004). The link between protein glycation and amyloid diseases was addressed in yeast, taking advantage of the known biochemical effects of protein glycation *in vivo*. Transthyretin, involved in familial amyloidotic polyneuropathy, is one of the most extensively investigated and characterized amyloidogenic proteins, and different point mutations with distinct amyloidogenic potential are known (Goldsteins *et al.*, 1997; Jacobson *et al.*, 1992; Saraiva, 2001; Saraiva *et al.*, 1984). We showed that amyloid deposits from FAP patients also contain MAGE modifications, suggesting that

glycation may be involved in this neurodegenerative disorder. However, little is known about the role of protein glycation regarding the genesis, progression or toxicity in this disorder. Thus, different TTR variants were expressed in yeast and the effects of glycation on the formation of TTR-amyloid aggregates *in vivo* was investigated. This is a relevant breakthrough on TTR misfolding and aggregation research since most previous efforts were directed towards the investigation of the structural and stability effects of point mutations on amyloidogenesis *in vitro*.

With these studies, we observed that TTR is glycated by methylglyoxal *in vivo* and that under glycation conditions TTR amyloid fibril formation is enhanced. This provides an opportunity to investigate the role of TTR glycation *in vivo* in amyloid fibril formation. Are the conformational changes imposed by glycation, together with amyloidogenic mutations, the trigger for the initial unfolding and aggregation? Or, are the soluble aggregates susceptible to glycation leading to the irreversible formation of amyloid structures? These issues remain in discussion. On the other hand, protein glycation appears to induce misfolding stress with the activation of the refolding chaperone pathway. It is still unknown whether, in these conditions, the cell quality control system is able to cope with the TTR misfolding and subsequent aggregation. Under certain conditions, yet unknown, cellular defenses including molecular chaperones and degrading pathways are exceeded and extensive protein misfolding and aggregation occurs leading to severe pathological conditions. In fact, a less effective protein quality control system has been implicated in neurodegenerative disorders (Outeiro & Tetzlaff, 2007; Ross & Poirier, 2004).

Since transthyretin is an extracellular protein, another possibility is that protein glycation interferes with its synthesis, translocation and secretion. This can also be investigated in yeast since it displays most of the properties of eukaryotic cells concerning protein synthesis, folding and maturation of extracellular proteins.

The investigation of protein glycation *in vivo* with this model organism and its relationship with amyloid fibril formation represents a step-forward in our understanding of the role of glycation in the development of several human disorders such as FAP. It is quite noticeable however, that many open questions need be addressed. Fortunately, the

model described here will be useful and further research work will provide new essential information and more detailed insights into the mechanisms of protein glycation *in vivo*, as well as its role on several human pathologies such as misfolding disorders. This increased knowledge will certainly be useful to develop new or improved therapeutic strategies to inhibit protein glycation and neutralize its harmful effects, thus improving the prognosis for a broad range of human disorders.

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