Protein glycation and methylglyoxal metabolism in yeast: finding peptide needles in protein haystacks

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Abstract
Metabolism, the set of all chemical transformations inside a living cell, comprises nonenzymatic processes that generate toxic products such as reactive oxygen species and 2-oxoaldehydes. Methylglyoxal, a highly reactive 2-oxoaldehyde by-product of glycolysis, is able to react irreversibly and nonenzymatically with proteins, forming methylglyoxal advanced glycation end-products, which alter protein structure, stability and function. Therefore, protein glycation may influence cell metabolism and its physiology in a way beyond what can be predicted based on the implicit codification used in systems biology. Genome-wide approaches and transcriptomics, two mainstays of systems biology, are powerless to tackle the problems caused by nonenzymatic reactions that are part of cell metabolism and biochemistry. The effects of methylglyoxal-derived protein glycation and the cell’s response to this unspecific posttranslational modification were investigated in Saccharomyces cerevisiae as a model organism. Specific protein glycation phenotypes were identified using yeast null-mutants for methylglyoxal catabolism and the existence of specific protein glycation targets by peptide mass fingerprint was discovered. Enolase, the major target, endures a glycation-dependent activity loss caused by dissociation of the active dimer upon glycation at a specific arginine residue, identified using the hidden information of peptide mass fingerprint. Once glycation occurs, a cellular response involving heat shock proteins from the refolding chaperone pathway is elicited and Hsp26p is activated by glycation.

Introduction
Systems biology aims to predict and reproduce the operation of living cells, from gene regulation networks to cell metabolism and physiology. Therefore, ‘omics’ begin to fulfill their promise of an integrated approach of all knowledge regarding a living system. First we know the genome (genomics), the transcripts (transcriptomics), the proteins and their functions (proteomics) and finally the metabolite’s dynamics (metabolomics). However, in all living cells, important nonenzymatic reactions may influence the cell’s metabolism and physiology in a way beyond a simple prediction based on the implicit codification that lies at the core of systems biology. Complex nonenzymatic reactions lurking beneath the surface of glycolysis. One of the most thoroughly investigated metabolic pathways and an inescapable example in systems biology is the response of yeast cells to a glucose pulse (Wu et al., 2006). The triose phosphates dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate are chemically unstable and undergo an irreversible β-elimination reaction of the phosphate group, leading to the formation of methylglyoxal (Richard, 1993). This highly reactive compound is catabolized by the glutathione-dependent glyoxalase pathway (Thornalley, 1990), comprising the enzymes glyoxalase I (lactoylglutathione lyase, EC 4.4.1.5, coded by the glo1 gene) and glyoxalase II (hydroxycycliglutathione hydrodrolase, EC 3.1.2.6, coded by the glo2 gene) and the NADPH-dependent aldose reductase (Vander Jagt &
Hunsaker, 2003) (alditol: NADP+ oxidoreductase, EC 1.1.1.21, coded by the gre3 gene in yeast). 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). Nε-(carboxyethyl)lysine (CEL) and methylglyoxal-lysine dimers (MOLD) are the main products of the reaction of methylglyoxal with lysine residues, while it forms hydromimidazolones, tetrahydropyrimidine and argpyrimidine by reaction with arginine residues (Westwood & Thornalley, 1997) (Fig. 1). Glycation is equivalent to a point mutation, exerting profound effects on protein structure, stability and function. Glycated proteins are present in several relevant human diseases like Alzheimer, Parkinson and familial amyloidotic polyneuropathy (Vitek et al., 1994; Yan et al., 1994; Castellani et al., 1996; Chen et al., 2004; Gomes et al., 2005a). In all of these amyloidopathies, β-sheet fibrils and the presence of AGE are common features, suggesting a role of glycation in neurodegenerative diseases of amyloid type (Colaco & Harrington, 1994; Colaco, 1995).

For a problem of this nature, instead of a blind systems biology approach, we propose an integrative study, at different systems’ levels, directly related to the problem under research. Following the discovery of the genes responsible for the yeast glycation phenotypes, we begin a quest for understanding their role in glycation and protein function, integrating cellular responses, was developed.

**Materials and methods**

**Yeast strains and culture conditions**

*Saccharomyces cerevisiae* strains from the Euroscarf collection (Frankfurt, Germany) were BY4741 (genotype BY4741 MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and Δglo1, a glyoxalase I-deficient strain (isogenic to BY4741 with YML004c::KanMX4). Yeast strains were kept in YPGlu (0.5% w/v yeast extract, 1% w/v peptone and 2% w/v D-glucose) agar slopes (2% w/v agar) at 4°C and cultured in liquid YPGlu medium at 30°C, 160 r.p.m., in an orbital shaker (Infors HG).

**Glycation conditions**

In all experiments, nongrowing yeast cells were used. Before assays, cells were harvested at the end of the exponential phase, washed twice in type II water (produced by reversed osmosis in Wasselab equipment and of quality above that of double-distilled water) and suspended at a concentration of 5 × 10⁶ cells mL⁻¹ in 0.1 M MES/NaOH, pH 6.5.

To study the dependence of glycation on the glycolytic flux, BY4741 cells were challenged with different concentrations of D-glucose (up to 250 mM). Samples were taken after 5 h and analysed by Western blot for the presence of argpyrimidine-modified proteins and the amount of enolase. To investigate the effects of glycation on enolase amount, BY4741 and Δglo1 cells were incubated with 250 mM D-glucose, and samples were taken at defined times (0, 1 and 5 h) and probed with antienolase antibody.

**Western blot analysis: detection of argpyrimidine-modified proteins, yeast enolase and Hsp26p**

Soluble cytosolic 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 sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis in a Mini-protean 3 system (Bio-Rad) using a 12% polyacrylamide separation gel and a 6% polyacrylamide stacking gel. Proteins were transferred to polyvinylidene fluoride 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. Total proteins were stained with Ponceau S solution (0.5% (w/v)
Ponceau S in 1% v/v glacial acetic acid) to confirm protein transfer. The membrane was blocked overnight at 4 °C in 1% (v/v) blocking solution in TBS (50 mM Tris and 150 mM NaCl, pH 7.5). For argpyrimidine detection, blots were probed for 2.5 h with antiargpyrimidine monoclonal antibody, a kind gift from Dr K. Uchida (Laboratory of Food and Biodynamics, Nagoya University Graduate School of Bioagricultural Sciences, Japan) diluted 1:2500 in TBS. To identify enolase, an antienolase antibody, a kind gift from Dr H. Park (Department of Microbiology, Chungnam National University, Korea), was used diluted 1:5000 in TBS (2.5 h). The small heat shock protein Hsp26 was identified by an anti-Hsp26p antibody, a kind gift from Professor Johannes Buchner (Institut für Organische und Biochemie, Technische Universität München, Germany) diluted 1:6000 in TBS by incubation for 3 h. 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.

Modelling and computer simulation

The kinetic models of methylglyoxal metabolism used to study the dependence of methylglyoxal formation rate on glycolytic flux, described in Gomes et al. (2005b), comprises the glyoxalase pathway, aldose reductase and methylglyoxal formation from the triose phosphates. The model can be found in the Online Cellular Systems Modelling database at http://jjj.biochem.sun.ac.za/database/gomes/index.html. Simulations were performed with the software package PLAS, Power-Law Analysis and Simulation (A.E.N. Ferreira, Faculdade de Ciências, Universidade de Lisboa, Portugal; http://www.dqb.fc.ul.pt/docentes/aferreira/plas.html).

MALDI-TOF analysis of tryptic digests of glycated proteins

MALDI-TOF-MS spectra of the tryptic digests were acquired as described by Pandey et al. (2000). Protein bands were excised and subjected to reduction, alkylation and digestion in gel with sequencing-grade modified trypsin (Promega). 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 reconstituted matrix α-cyano-4-hydroxycinnamic acid (CHCA) (10 mg mL⁻¹) prepared in 70% (v/v) acetonitrile with 0.1% (v/v) Trichloroacetic acid (TFA). Mass spectra were acquired in a Voyager-DE STR MALDI-TOF-MS (Applied Biosystems). Proteins were identified by peptide mass fingerprint using MASCOT (http://www.matrixscience.com). The location and chemical nature glycated aminoacid residues were identified using MALDI-TOF data. A theoretical digestion of the major protein glycation target identified, enolase2 (Gomes et al., 2006), considering up to two trypsin miscleavages was made (Peptidemass, Exasy, http://www.exasy.ch/tools/peptide-mass.html), and the mass increment due to a specific MAGE was added to the resulting peptide masses. Peptides with m/z values that appear solely in the mass spectrum of glycated enolase that do not correspond to theoretical enolase peptides were compared with the mass list of theoretical peptides plus the mass increment due to MAGE. Positive results have a mass match and a miscleavage in the correct residue (arginine residues for MAGE hydroimidazolones, argpyrimidine and tetrahydropyrimidine while lysine residues were analysed for CEL).

Protein structure analysis

Saccharomyces cerevisiae enolase dimer structure was represented by PDB entry 1ehb, containing Mg. It has 95% identity and 4% homology with Eno2p. 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).

Results and discussion

Glycation in yeast

Because of its high glycolytic activity, yeast cells produce methylglyoxal, a highly reactive intracellular glycation agent, at a rate of c. 0.3% of the glycolytic flux (Martins et al., 2001). Yeast cells growing in Yeast extract, peptone dextrose (YPD) with 100 mM of D-glucose accumulate MAGE-modified proteins in strains with deficiencies in methylglyoxal catabolism, while glycated proteins are not observed in the reference strain BY4741 (Gomes et al., 2005b). The appearance of glycated proteins in strains with deficiencies in methylglyoxal catabolism Δglo1, Δglo2, Δgsh1 (lacking γ-glutamyl cysteiny1 synthetase gene), Δgre3 and Δglo1 Δglo2 (lacking both glyoxalase I and aldose reductase) depends on the increase of methylglyoxal concentration (Gomes et al., 2005b). In fact, even the reference strain BY4741, when challenged with a high D-glucose concentration, which increases methylglyoxal formation, presents argpyrimidine-modified proteins (Gomes et al., 2005b).

Using a previous kinetic model of the methylglyoxal metabolism, which comprises the glyoxalase pathway and aldose reductase (Gomes et al., 2005b), we observe a linear relationship between methylglyoxal formation rate (methylglyoxal input) and its steady-state concentration (Fig. 2a). Because methylglyoxal is a by-product of glycolysis, different glycolytic fluxes lead to different intracellular methylglyoxal concentrations and consequently to different glycation levels. To test this hypothesis, BY4741 yeast cells were
incubated with different D-glucose concentrations and, after 5 h incubation, soluble proteins were harvested and argpyrimidine-modified proteins were detected by Western blot using a specific antibody against argpyrimidine-modified proteins. When BY4741 cells are challenged by different D-glucose concentrations (different glycolytic rates), an increase in argpyrimidine-modified proteins is clearly observed (Fig. 2b), especially in the major glycation target that was previously identified as enolase2 (Gomes et al., 2006). This is valid only for nongrowing yeast cells, when most glucose is used by the glycolytic pathway. However, an increase in argpyrimidine content on aldolase and phosphoglycerate mutase, two other identified glycation targets (Gomes et al., 2006), is also observed. Therefore, protein glycation in yeast is directly related to methylglyoxal formation, which in turn depends on the glycolytic flux. An increase in the D-glucose metabolism via glycolysis unavoidably leads to protein glycation. So, although there are enzymatic systems for methylglyoxal detoxification, the increase of methylglyoxal concentration in certain experimental or pathological conditions is responsible for the formation of MAGE-modified proteins.

In nongrowing conditions, intracellular protein glycation is a fast process (Gomes et al., 2005b). To understand if there is any difference in enolase amount in these experiments due to protein glycation we probed the membrane with an antienolase antibody. No major differences were observed and the amount of enolase is very similar after 5 h incubation with each D-glucose concentration used (data not shown).

**Glycation effects on enolase, the main target of protein glycation in yeast**

Enolase2, identified as the major glycation target in yeast, endures a glycation-dependent activity loss (Gomes et al., 2006). This is directly related to glycation levels because
Activation of the refolding chaperone pathway by glycation

Besides the modification of specific glycolytic enzymes by methylglyoxal, the heat shock proteins Hsp71p/72p and Hsp26p also contain MAGE (Gomes et al., 2006). Contrary to Hsp71p/72p, Hsp26p was only identified in glycated samples (Gomes et al., 2006). Because we are analysing the soluble cytosolic fraction, this result indicates that in glycation conditions, Hsp26p is mainly found in the soluble fraction. Hsp26p forms 24-meri complexes and it has been shown that the dissociation of the complex, caused by heat or oxidative stress, is a prerequisite for its efficient chaperone activity (Haslbeck et al., 1999). Therefore, the presence of Hsp26p in soluble form under glycation conditions suggests activation of the refolding chaperone pathway. In fact, during 5 h incubation of BY4741 cells with 250 mM of D-glucose, the amount of soluble Hsp26p increases with time, as evaluated by Western blotting (Fig. 5a), confirming the above observation. Altogether, these results indicate that the chaperone pathway could be involved in the cell’s response to protein glycation in vivo.

Methylglyoxal, unfolding stress and heat shock proteins

Methylglyoxal formation and protein glycation escape the reasoning underlying systems biology. These noncoded processes are nonenzymatic in nature and result from the inescapable chemical nature of some metabolites. Its occurrence generated a selective pressure that resulted in the evolution of cellular responses against methylglyoxal stress. In consequence, cells developed enzymatic defences like the glutathione-dependent glyoxalase pathway (Thornalley, 1990) and the NADPH-dependent aldose reductase (Vander Jagt & Hunsaker, 2003) that catabolize methylglyoxal formed from triose phosphates during glycolysis (Richard, 1993) (Fig. 1). However, some methylglyoxal still evades these catabolic routes and generates MAGE in proteins. As expected, the irreversible modification of lysine or arginine side chains in proteins exerts a profound impact on their structure and function. Enolase glycation in yeast materializes in dimer dissociation and activity loss. Despite these effects, glycolytic flux and cell viability remain unchanged (Gomes et al., 2005b). Although a Δglo1 strain, with
deficiencies in methylglyoxal catabolism, is viable, it appears to produce a larger amount of enolase2 than the reference strain under glycation conditions (incubation with 250 mM D-glucose for 5 h) (Fig. 5b). This hints at a possible function of enolase2 as a methylglyoxal scavenger. In this context, spontaneous protein glycation of specific targets could diminish the concentration of free methylglyoxal, preventing changes in the biochemical functionalities of other proteins. Enolase is an essential protein that remarkably interacts with some other vital proteins. Most are related to critical cellular processes like protein degradation via ubiquitin-dependent proteasome, transcriptional regulation, protein import/export and RNA export (Gavin et al., 2002; Gavin et al., 2006). Therefore, the arginine-rich cage in enolase structure (Fig. 4) could provide a highly favourable glycation environment for the formation of MAGE, thereby sequestering free methylglyoxal.

Glycation of heat shock proteins and, above all, the appearance of glycated Hsp26p, a small heat shock protein, in the soluble cytosolic protein extracts hint at a physiological role of methylglyoxal in the activation of chaperone proteins. Small heat shock proteins (sHsp), including Hsp26p, have been shown to exhibit chaperone activity and protect proteins from irreversible aggregation in vitro (Jakob...
et al., 1993). Environmental stress leads to aberrant protein conformations and aggregation. Cells antagonize the detrimental consequences of protein misfolding by activating refolding and degradation. In yeast, Hsp26p functions as a captor of misfolded proteins and modulates their disaggregation by rendering aggregates more accessible to Hsp104p/Hsp71p/Hsp40p action (Cashikar et al., 2005). Besides the disaggregation of misfolded proteins, Hsp26p and other sHsp are involved in the refolding of denatured proteins by holding them in a reactivation-competent state, creating a reservoir of nonnative refoldable proteins (Ehrenspgerger et al., 1997; Haslbeck, 2002). If proteins cannot be refolded, they will follow a degradation pathway where the role of sHsp is still poorly understood (Cashikar et al., 2005; Han et al., 2005; Park et al., 2007). Our molecular model of the glycation-dependent enolase activity loss involves the dissociation of enolase dimer to inactive monomers that will unfold. Thus, as a consequence of glycation, enolase will unfold in vivo. Active Hsp26p may sequester denatured enolase to be refolded or, more likely, degraded, thereby preventing its aggregation.

Besides the identification of Hsp26p and Hsp71p/72p in glycation conditions, these proteins are glycated in vivo, meaning that their role may be regulated by glycation (Gomes et al., 2006). Indeed, in mammalian cells, Hsp27p is the major glycation target (Padival et al., 2003; Schalkwijk et al., 2006) and recent work has shown that Hsp27p requires glycation by methylglyoxal to perform its physiological functions (Sakamoto et al., 2002). Other proteins, such as crystallins, enhance their chaperone activity upon glycation by methylglyoxal (Nagaraj et al., 2003). These observations raise the question of whether Hsp26p and Hsp71p/72p glycation causes their activation and/or enhances their chaperone activity. This may link methylglyoxal to protein unfolding stress, associated with several conformational pathologies (Fig. 6). Although these hypotheses need further research, we established a model for the molecular mechanism of cellular responses to protein glycation by methylglyoxal.

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