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Enhanced reactivity of Lys182 explains the limited efficacy of biogenic amines in preventing the inactivation of glucose-6-phosphate dehydrogenase by methylglyoxal

Patricio Flores-Morales [a], Claudio Diema [b], Marta Vilaseca [b], Joan Estelrich [a], F. Javier Luque [a], Soledad Gutiérrez-Oliva [c], Alejandro Toro-Labbé [c], Eduardo Silva [d,*]

[a] Departament de Fisicoquímica and Institut de Biomedicina (IBUB), Facultat de Farmàcia, Universitat de Barcelona, Avda. Diagonal 643, 08028 Barcelona, Spain
[b] Unitat de Espectrometría de Masses, Institut de Recerca Biomèdica, Baldiri i Reixac 10, 08028 Barcelona, Spain
[c] Laboratorio de Química Teórica Computacional (QTC), Facultad de Química, Pontificia Universidad Católica de Chile, Casilla 306, Santiago 9840436, Chile
[d] Laboratorio de Química Biológica (QBUC), Facultad de Química, Pontificia Universidad Católica de Chile, Casilla 306, Santiago 9840436, Chile

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ABSTRACT

This study examines the inactivation of the enzyme glucose 6-phosphate dehydrogenase (G6PD) by methylglyoxal (MG) and the eventual protection exerted by endogenous amines. To determine the protective effect of amines, the rate constant of the reaction of MG with the amino group of N-α-acetyl-lysine, carnosine, spermine and spermidine was measured at pH 7.4, and the behavior of endogenous amines was analyzed on the basis of quantum chemical reactivity descriptors. A 63% reduction in the enzyme activity was found upon incubation of G6PD with MG at pH 7.4. The inactivation of G6PD was even larger when the pH was increased to 9.4, revealing a weak protective effect by the amines. The results suggest that some basic residues of G6PD exhibit an anomalous reactivity, which likely reflects a shift in the standard pK_a value due to the local environment in the enzyme. Under the experimental conditions used in the assays, this hypothesis was corroborated by mass spectrometry analysis, which points out that modification of Lys182 in the binding site is responsible for the inactivation of G6PD by MG. These results emphasize the need to search for more effective antiglycating agents, which can compete with basic amino acid residues possessing enhanced reactivity in proteins.

1. Introduction

The Maillard reaction is a non-enzymatic glycation process that involves the condensation of a carbonyl-containing molecule and a free amine. In the early steps of glycation, carbonyl groups of sugars react with amino groups of proteins through the formation of Schiff bases, which can then lead to Amadori rearrangements[1–4] (Fig. 1a). The efficiency of the imine generation depends on the amount of amino groups in neutral form capable of nucleophilic attack toward the carbonyl group. Although the neutral species should be marginally populated at physiological pH, embedding of amino groups in apolar internal sites can alter the pK_a and increase the population of the neutral form,[5–7] thus making those groups more reactive against glycating reagents.

Besides sugars, attention has also been directed toward α-dicarbonyl compounds such as glyoxal and methylglyoxal (MG), which can react with amino, guanidinium and thiol groups of proteins. They are active crosslinkers. In lens MG is formed from the degradation of triose-phosphates,[8] glycated proteins[9] and lipid peroxidation,[10] reaching a relatively high content (~1–2 μM)[11] compared to blood samples of healthy human subjects (ca. 80 nM).[12] The later stages of the complex reactive processes triggered by α-dicarbonyl compounds give rise to a variety of products, which are denoted as advanced glycation endproducts (AGEs; Fig. 1a). Examples of non-crosslinking AGEs are hydroimidazolone adducts with arginine residues, N-ε-carboxymethyl or N-ε-carboxyethyl lysines, and argpyrimidine. Crosslinking AGEs involves heterocyclic structures such as MOLD (MG-derived lysine dimmer; Fig. 1a) and GOLD (glyoxal-derived lysine dimmer; Fig. 1a).

Several studies have examined the therapeutic implications of Maillard chemistry to prevent the formation of AGEs.[13] Biogenic

Abbreviations: MG, methylglyoxal; NLys, *N*-α-acetyl-Lys; Car, L-carnosine; Sp, spermine; Spe, spermidine; εNH_2-group, ε-amino group; AGEs, advanced glycation end-products; G6PD, glucose 6-phosphate dehydrogenase; G6P, glucose 6-phosphate; NAD(P)+/NAD(P)H, oxidized/reduced nicotinamide adenine dinucleotide (phosphate); FA, formic acid; LC–MS, liquid chromatography–mass spectrometry analysis; PDB, protein data bank; MEAD, macroscopic electrostatics with atomic detail.

* Corresponding author. Address: Departamento de Química Física, Facultad de Química (536), Pontificia Universidad Católica de Chile, Casilla 306, Correo 22, Santiago 9840436, Chile. Tel.: +56 2 3544395; fax: +56 2 3544740.
E-mail address: esilva@uc.cl (E. Silva).

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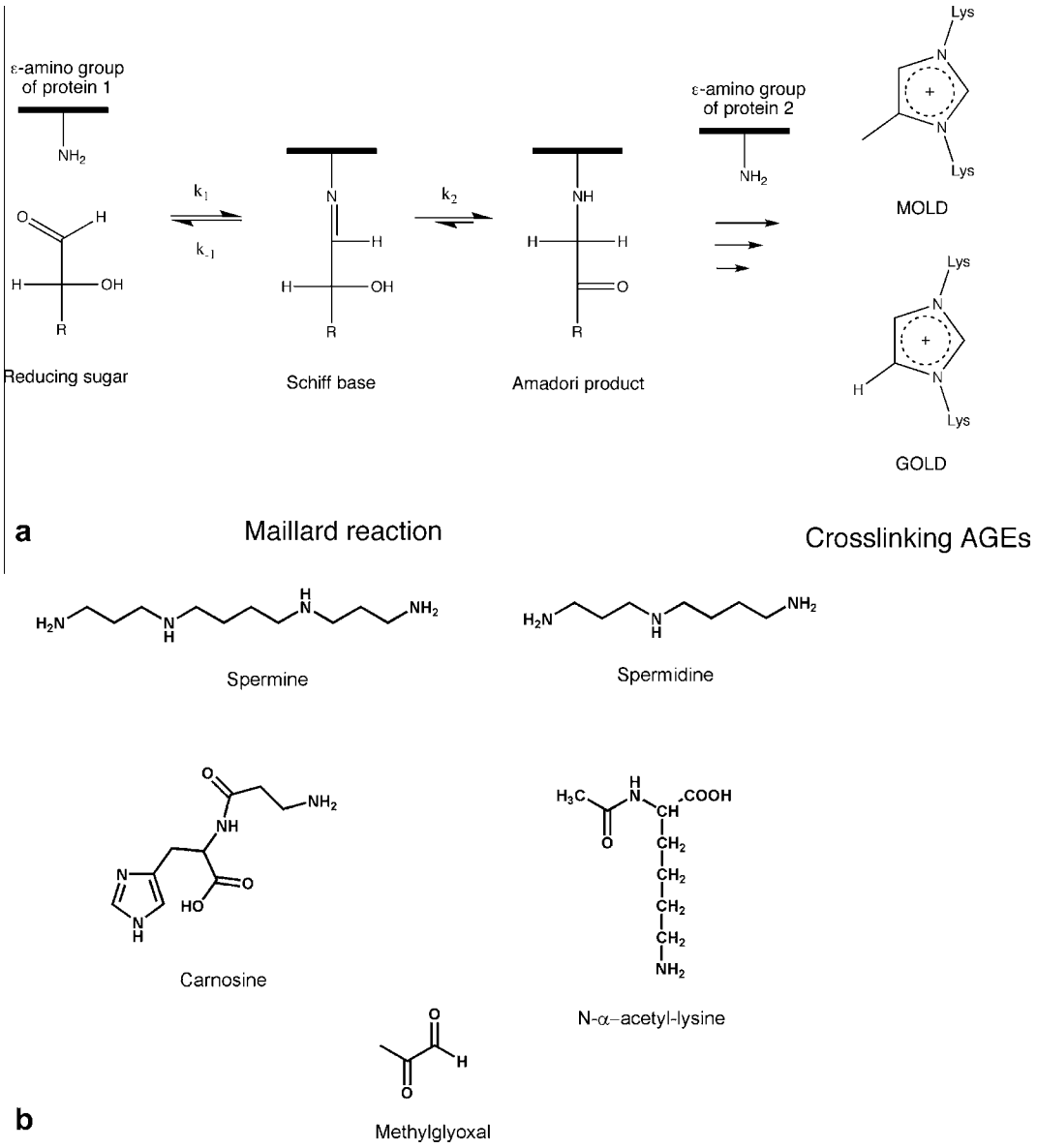

Figure 1. (a) Schematic representation of the glycation reaction. An ε-amino group of protein 1 reacts with a carbonyl group of a reducing sugar forming a Schiff base, which can evolve to an Amadori product containing a new carbonyl unit able to react with another ε-amino group from the same or a distinct protein. In this latter case glycation leads to advanced glycation end-products (AGEs) and cross-linked proteins (MOLD, methylglyoxal-derived lysine dimer; GOLD, glyoxal-derived lysine dimer). (b) Chemical structure of methylglyoxal, spermine, spermidine, carnosine and *N*-α-acetyl-lysine.

amines, such as spermine and spermidine (Fig. 1b), have received much interest due to their ability to inhibit protein alteration due to glycation in vitro.[14] Carnosine (Fig. 1b) also seems to attenuate the formation of AGEs[15] presumably by sequestering glyoxal and MG,[16,17] which can promote protein cross-linking between lysines.[18–20] Intense efforts have been dedicated to the discovery of novel and safer antiglycating agents, such as aminoguanidine, urea derivatives and diaminopropionic acids.[14,21,22]

Protein glycation is important in the pathophysiology of many processes related with aging, especially in diabetics, where sugar concentration in the body is unusually high. A well known example is the accumulation of modified proteins triggered by age- and environmental-related damage in eye lens, which is associated with lens opacification and senile cataract.[23] There is no removal of lens proteins with age and they remain in an environment containing reactive small molecules that can react with proteins in vivo.[24–27] Thus, it is known that glycosylation of lens proteins is increased in human cataract lenses from diabetic patients.[26–28] The glycated proteins become yellow fluorescent and aggregated, as generally found in human cataracts.[27,28]

The biochemical characteristics of the eye lens include a relatively high content of glutathione (GSH) in its reduced form (10 mM),[29] which protects against oxidative damage. Oxidation transforms GSH to the disulfide form, which is reconverted via glutathione reductase reaction. The NADPH required by this enzyme is produced in the phosphate pentose pathway, where glucose 6-phosphate dehydrogenase (G6PD) plays a key regulatory role. Decreased levels of GSH found in human cataract lenses[30,31] could reflect a low content of NADPH due to a diminished activity of G6PD. In fact, a decreased activity of G6PD has been reported in cataract[32] and in aged lens.[33]

To the best of our knowledge, the effect of MG on G6PD has not been previously studied. The aim of this work is to identify the molecular basis of the loss of G6PD activity promoted upon incuba-

tion by MG. To this end, we examine the protective effect of the amino groups present in carnosine (Car), spermine (Sp) and spermidine (Spe) against the inactivation of G6PD by MG. Both experimental and theoretical studies are performed to gain insight into the residues implicated in the chemical modification promoted by MG. In turn, this information is discussed in light of the catalytic mechanism of G6PD.

2. Results and Discussion

2.1. Determination of rate constants

The rate constants (k) for the reaction of MG with NLys, Car, Sp and Spe at pH 7.4 were determined experimentally by the fluorescamine assay.[34] The rate constants range from 1.37×10^4 ($\pm 3 \times 10^2$) to 1.76×10^5 ($\pm 5 \times 10^2$) $M^{-1}\,h^{-1}$ (Table 1). Sp and Spe have very similar values, both being slightly more reactive than Car, whose rate constant is around 10-fold smaller than that derived for NLys. The differences between the rate constants can be explained by the dual reactivity descriptor ($\Delta f(r)$; Eq. (3), see Section 4) determined for representative structures of the amines (See Fig. S1 in Supplementary data). Thus, whereas the NH_3^+-group of Car has a large electrophilic lobule and deprotonation does not increase its nucleophilic power, which instead concentrates in the imidazole ring, deprotonation of Sp and Spe gives rise to a notable increase in the nucleophilic power, which mimics the trends found for NLys. In fact, the latter compound exhibits the largest nucleophilicity. Thus, the distinct features of the dual descriptor extension correlate well with the ordering of k values for the amines ($k_{Car} < k_{Sp} < k_{Spe} < k_{NLys}$; see Table 1).

2.2. G6PD inactivation mediated by MG

The effect of MG on the activity of G6PD was examined by incubating the enzyme with MG in the absence of light for 16 h (pH 7.4). When the concentration of MG was 10-fold larger than that of the lysine residues in the enzyme, a 63% reduction in the G6PD activity was found (**ez/MG**; Fig. 2). Nevertheless, addition of Car, Sp and Spe to the incubation mixture (**ez/MG/Car**, **ez/MG/Sp** and **ez/MG/Spe**; Fig. 2) led to a moderate protection against G6PD inactivation. The magnitude of the protective effect roughly reflects the differences reported in Table 1.

Lys and Arg residues are sites susceptible to chemical modification by MG.[35,36] The fraction of NH_2-groups of the protective amines that might react with MG in the presence of G6PD can then be estimated as:

$$F_{NH_2amine} = \frac{k_{NH_2amine}\,[NH_2\text{-amine}]}{k_{NH_2\ amine}[NH_2\text{-amine}] + k_{\varepsilon NH_2}[\varepsilon NH_2\text{-G6PD}] + k_{Guanidinium}\,[Guanidinium\text{-G6PD}]} \quad (1)$$

where $k_{NH_2-amine}$ stand for the reaction rate of MG with the NH_2-groups of neutral amines ([NH_2-amine]), while $k_{\varepsilon NH_2}$ and $k_{Guanidinum}$ denote the reaction rate with neutral Lys ([εNH_2-G6PD]) and Arg ([Guanidinium-G6PD]) residues of G6PD, respectively.

If we assume that (i) Lys and Arg residues in the enzyme retain the standard pK_a's of the free residues, (ii) the rate constant of lysines is given by the value determined for *N*-α-acetyl-lysine ($1.76 \times 10^5\ M^{-1}\,h^{-1}$), and (iii) the rate constant of arginines can be estimated from the ratio between the reaction rates of MG with hippuryllysine[35] and *N*-α-acetyl-L-arginine[36] ($1.21 \times 10^6\ M^{-1}\,h^{-1}$), then the F_{NH_2amine} values calculated for Car, Sp and Spe at pH 7.4 are 0.92, 0.94 and 0.94, respectively. This indicates that around 7% of MG reacts with basic groups present in G6PD. Accordingly, the amount of Arg and Lys residues altered by MG should be extremely low and could not explain the observed G6PD inactivation. In turn, these findings suggest an augmented reactivity of some Arg and/or Lys residues.

In order to increase the nucleophilicity of Car, Sp and Spe in the reaction medium, the inactivation of G6PD was examined at higher pH values. Remarkably, control assays performed in the absence of MG showed that the enzyme activity remained unaltered after 16 h incubation (**ez**; Fig. 2), which rules out pH-induced structural alterations in the G6PD activity. In the presence of MG the enzyme activity is especially affected by the increase in pH from 7.4 to 8.4 (**ez/MG**; Fig. 2), which can be attributed to a more extensive modification of reactive basic residues. An improvement in the relative protection by amines was also observed when the pH was increased from 7.4 to 9.4 (Fig. 2). However, this effect is relatively weak considering the high concentration of the amines.

The preceding findings allow us to hypothesize the existence of basic residues in G6PD that are especially reactive toward MG (Fig. S2 in Supplementary data). Among arginines, four potential candidates for modification by MG are Arg46, Arg121, Arg223 and Arg395. Arg46 assists coenzyme binding through interaction with the 2′-phosphate of $NADP^+$. Arg121 and Arg223 are close to the glucose and adenine moieties of G6P and $NADP^+$, respectively. Finally, Arg395 forms a salt bridge that stabilizes the dimeric state of the enzyme. Since MG incubations were made in the absence of both substrate and coenzyme, Arg46, Arg121 and Arg223 are expected to be fully exposed to the solvent. Moreover, since the enzyme is in its monomeric state during incubation, Arg395 must also be solvent-exposed. Under these conditions, the pK_a of these latter residues should be close to the standard one.

In order to explore the ionization state of these residues, we first carried out a series of PROPKA[37,38] calculations for different G6PD structures available in the PDB (see Section 4). PROPKA is an empirical method for the structure-based prediction of the pK_a values of ionizable residues in proteins, which takes into account both intra-protein interactions and desolvation effects through an empirically adjusted function related to the chemical nature and spatial location of the residues. With regard to lysines, there are five residues (Lys21, Lys148, Lys182, Lys338 and Lys343) in the substrate binding site. According to PROPKA computations, notable deviations from the standard pK_a value (10.5) are found for certain residues depending on the specific X-ray structure considered in the calculations. In particular, Lys148 and Lys182 are predicted to have pK_a values diminished by 1–2 units. This pK_a reduction is not unexpected taking into account an extreme example of pK_a value of 5.6 recently reported for a buried Lys in the hydrophobic core of staphylococcal nuclease.[7] To further check these findings, additional computations were performed with

Table 1
Rate constants (k; $M^{-1}\,h^{-1}$) for the reaction between methylglyoxal and several amines determined at physiological pH

Amine	k
Carnosine	$1.37 \times 10^4 \pm 3 \times 10^2$
Spermine	$3.91 \times 10^4 \pm 2 \times 10^2$
Spermidine	$4.28 \times 10^4 \pm 3 \times 10^2$
N-α-Acetyl-lysine	$1.76 \times 10^5 \pm 5 \times 10^2$

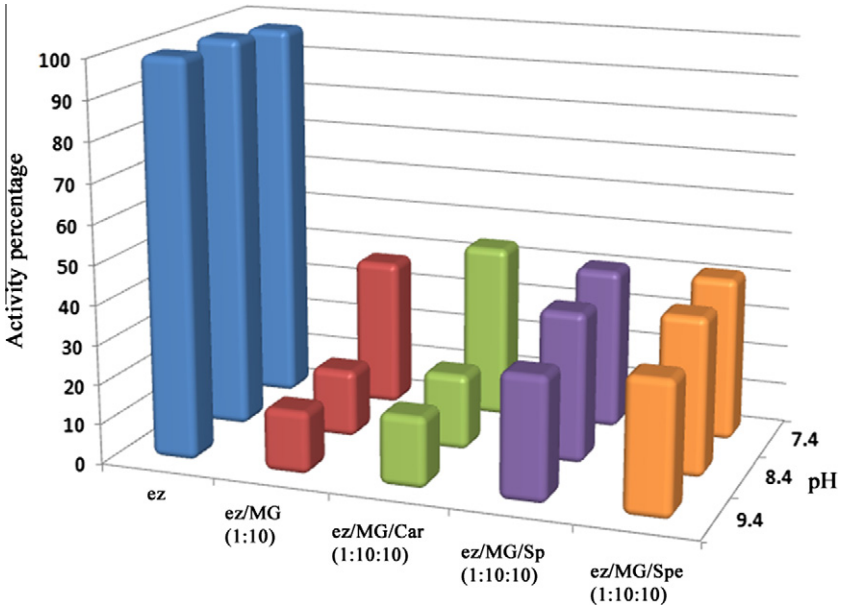

Figure 2. Inactivation of G6PD by MG and protective effect of amines. The reaction was carried out at 37 °C, pH 7.4–9.4 and dark conditions (**ez**: lysine residues + amino-terminal group ratio into the enzyme; **MG**: methylglyoxal ratio; **Car**, **Sp** or **Spe**: carnosine, spermine or spermidine NH_2-group ratio).

MEAD (Macroscopic Electrostatics with Atomic Detail).[39] MEAD is a method that relies on the detailed structural information of atoms in a protein and applies a macroscopic dielectric model to evaluate the solvent-screened electrostatic interactions in a solvated macromolecular system. To this end, the protein is treated as a low dielectric medium, characterized by the distribution of charges located at atomic positions, immersed in a high dielectric medium for the surrounding solvent, and the electric potential is determined by solving the Poisson–Boltzmann equation. MEAD calculations also showed a large dependence on the predicted decrease in pK_a with the dielectric constant for the more buried Lys residues (Fig. 3). This behavior is especially notable for Lys182 and Lys343, which are localized at the inner part of the binding site, in comparison with Lys19 (totally exposed to the aqueous solvent). Experimental evidence from the reaction rate of NLys with MG at low water content and high proportion of dioxane, confirms this fact (Fig. 3, insert).

2.3. Intact G6PD MS analysis

Intact proteins (native, MG-treated and $NaBH_4$-reduced MG-treated) eluted at a retention time of 31.18–32.05 min by

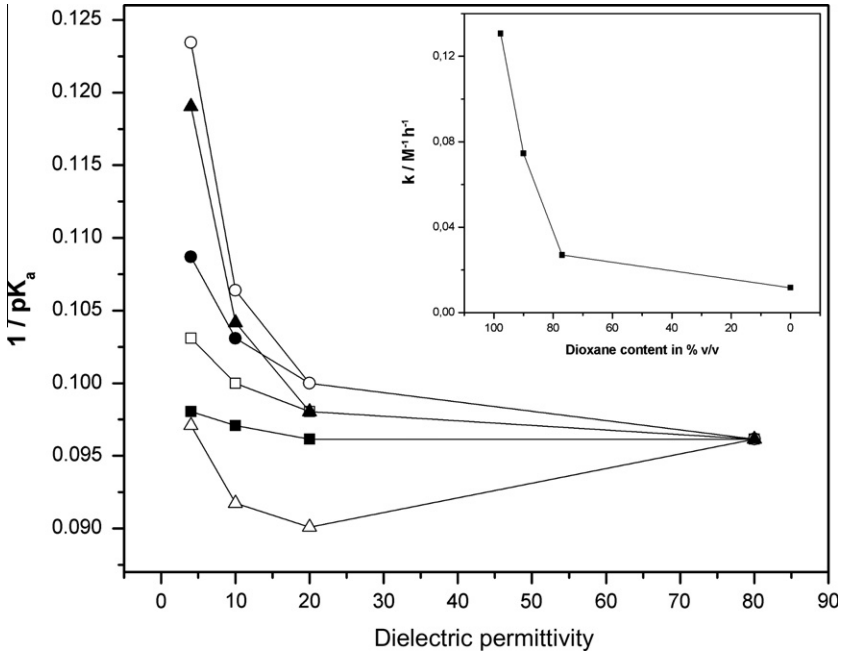

Figure 3. Reciprocal of calculated pK_a for lysine residues (-■- Lys19, -●- Lys21, -□- Lys148, -○- Lys182, -△- Lys338, -▲- Lys343) in the G6P binding site of a mutant of G6PD (from *Leuconostoc mesenteroides*) as a function of the dielectric permittivity. Lys-19 is totally exposed to the solvent. [Insert: Variation of log k_{rel} (in M^{-1} h^{-1}) as a function of 1,4-dioxane content (by volume), for the reaction between NLys and MG].

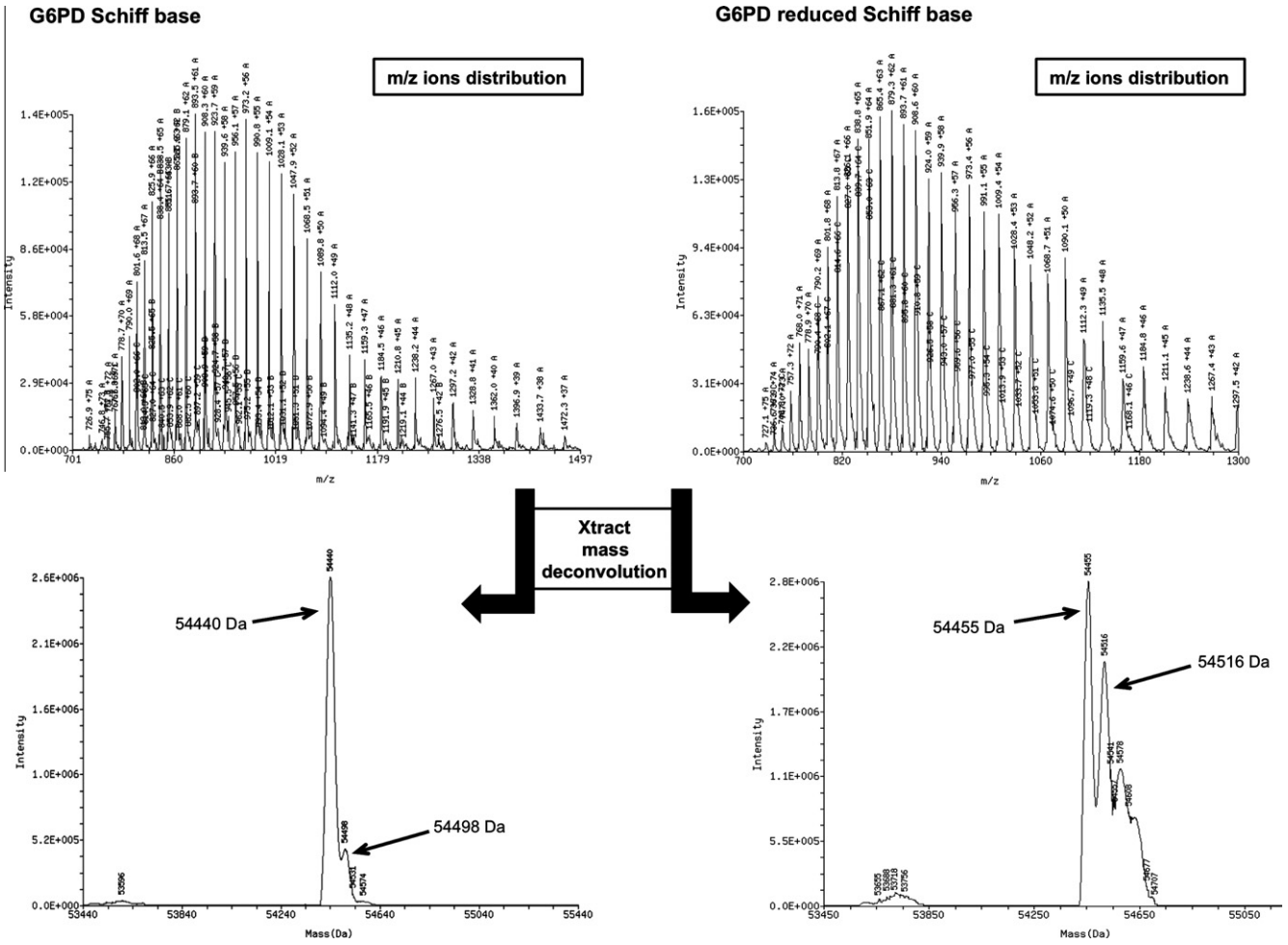

Figure 4. Intact MS analysis of MG-treated and $NaBH_4$-reduced MG-treated G6PD samples: (top) *m*/*z* ion distribution spectra and (bottom) Mass deconvolution spectra.

LC-nanoESI-MS coupling. An average mass of 54,440 Da, matching the expected mass for G6PD, was observed as the main peak after deconvolution of the MS spectra for the native and MG-treated protein samples (Fig. 4; note that the mass includes also a methionine residue at the N-terminal side in the protein used in MS analysis). Deconvolution of the MS spectra recorded for the reduced sample affords a main peak with an average mass of 54,455 Da. Both in the non-reduced MG-treated sample and in the $NaBH_4$-reduced one, an additional mass, shifting +54–69 amu, was also observed, which would agree with the formation of a Schiff base on a basic residue. The higher proportion of this adjacent mass in the reduced sample versus the non-reduced one can be explained by the higher stability of the hypothetical modification after its selective reduction with $NaBH_4$. Nevertheless, the MS analysis of the intact protein is still not resolutely enough to determine accurately the mass of the modified protein due to the limitations of the deconvolution algorithm at high molecular masses and in the presence of mixtures.

In order to make a more accurate mass determination of the modified protein, the intact MG-treated G6PD sample was reanalyzed on a 12 Tesla LTQ-FT mass spectrometer and data were processed using 'in-house' deconvolution software (On-line automation CRAWLER software). A mass of 54463.16 Da was obtained for the modified protein, differing only by two units from the expected monoisotopic mass for a Schiff base modification (54461.14 Da). Nozzle-skimmer dissociation of the infused sample resulted in the characterization of the C- and N-terminal ends of the protein. However, no information about the core of the protein could be obtained using this strategy (Fig. S3 in Supplementary data). Therefore, further protease digestion of the samples was performed in order to localize precisely the main modification detected on the protein sequence.

Peptides obtained from the protein digestion with the Glu-C endoproteinase covered 83% of the protein sequence (Fig. S4 in Supplementary data). For the MG-treated protein, two peptides with monoisotopic masses of 2194.04443 and 2248.05932 were found matching the monoisotopic masses of the peptide corresponding to the sequence 166–183 (NAFDDNQLFRIDHYLGKE) with no chemical modification and with a Schiff base modification of Lys182, respectively (Fig. 5; see also Fig. S4). The isotopic distributions of the corresponding charged precursor ions perfectly matched the theoretical isotopic distributions expected for both peptides (see Fig. S5 in Supplementary data). The collected LC fraction containing the eluted modified peptide was infused for high resolution MS/MS analysis. Precursor ion (*m*/*z* 750.36080; Fig. 5, top) was isolated and 37% energy was applied for CID fragmentation. The resulting fragments were deconvoluted to charge zero and further analyzed with PROSIGHT PC software. Detection of one MS/MS fragment ion of peptide 166–183 with less than 10 ppm accuracy is indicative of the presence of a modification (+54.01001, Schiff base) in the C-terminal end of the peptide, which can be attributed to the only Lys present in the peptide (Lys182).

The LC-nanoESI-MS analysis of the Glu-C digested $NaBH_4$-reduced MG-treated protein also showed two peptides with monoisotopic experimental masses of 1746.86893 and 1802.90990

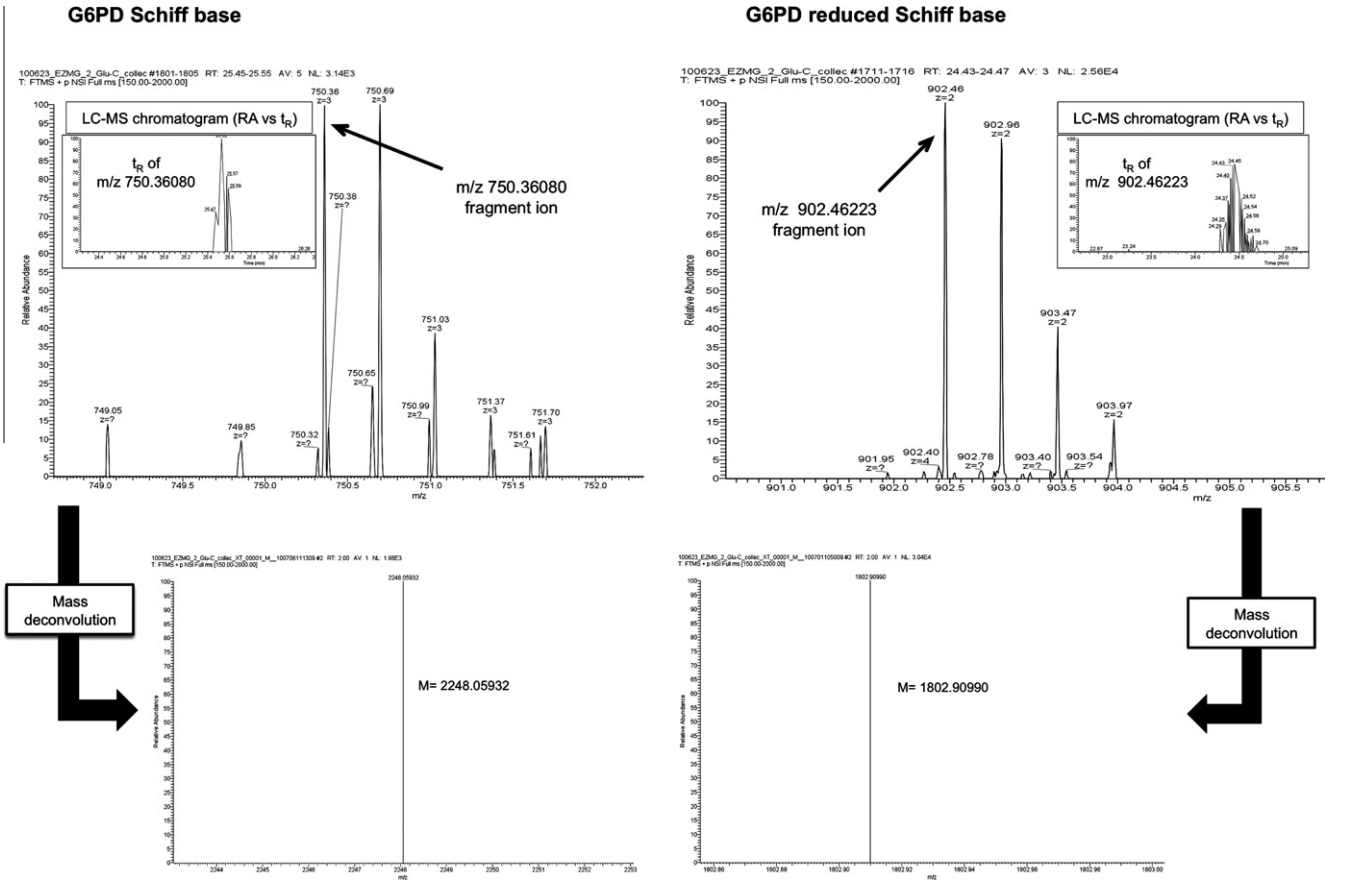

Figure 5. LC-nanoESI-MS analysis of the Glu-C crude reaction of MG-treated and $NaBH_4$-reduced MG-treated G6PD samples. Top: zoom spectrum at ion m/z 750.36080 ($z = 3$) corresponding to peptide 166–183 from MG-treated G6PD and 902.46223 ($z = 2$) corresponding to peptide 170-183 from $NaBH_4$-reduced MG-treated G6PD. Top Insert: Xtracted chromatogram showing experimental retention time (25.5 and 24.4 min for m/z ions 750.36080 and 902.46223, respectively), bottom: Xtract deconvolution spectrum of ions to the zero charged monoisotopic mass (Legend; RA, relative abundance; tr, retention time).

(Fig. 5; see also Fig. S4), which match the monoisotopic masses of the expected G6PD Glu-C digested peptides corresponding to the sequence 170–183 (DNQLFRHIDHYLGKE) in both non-modified and reduced Schiff base forms, respectively. Further identification was performed by comparison of the isotopic distribution of the double charged experimental ion detected (m/z 902.46223; Fig. 5, top) with the theoretical one considering the presence of a reduced Schiff base in the peptide (see Fig. S6 in Supplementary data). On the other hand, no matching was observed with the theoretical isotopic distribution of the same peptide considering a modification by bi-formylation. After LC fraction collection, this peptide was infused for MS/MS analysis. Following precursor ion (m/z 902.46223) isolation, 40% CID energy was applied for fragmentation. Data analysis with Prosight PC software showed the presence of two MS/MS fragment ions matching accurately (<10 ppm) a modification (+56.02567, reduced Schiff base) in the C-terminal end of the peptide, which can be attributed to chemical modification of Lys182.

Overall, these results indicate that, under the experimental conditions used for the inactivation of G6PD with MG, the reduction in the enzymatic activity can be attributed to the formation of a Schiff base with Lys182. The enhanced reactivity of this residue in the binding site would also explain the limited protection observed upon addition of amines at physiological pH.

2.4. Ionization of Lys182 and its enhanced reactivity toward MG

The chemical modification of Lys182 by carbonyl groups reveals an enhanced reactivity, which might arise from an anomalous low pK_a. This suggestion is supported by distinct experimental evidences.

(i) The plot of log k_{cat}/K_m versus pH reveals the presence of four ionizable groups in *Leuconostoc mesenteroides* G6PD.[40,41] In particular, two pK_a values at 8.9 and 10.0 were assigned to amino acids that affect the binding of G6P. Cosgrove et al. pointed out that residues Lys182 and Lys343, which are located in the binding site that accommodates the phosphate moiety of G6P, could be probable candidates for those ionizations.[41] Our results support the assignment of the lowest pK_a (8.9) to Lys182, whose enhanced reactivity is reflected in the chemical modification by MG. In contrast, the highest pK_a (10.0) would correspond to Lys343.

(ii) These assignments are reinforced from the change in the apparent K_m for G6P (114 μM for the wild type enzyme in presence of NADP at pH 7.6) induced by mutations of those basic residues.[42] Mutation of Lys182 to either Gln or Arg gives rise to similar increases in the K_m value (Lys182→Gln: 13,100 μM; Lys182→Arg: 16,100 μM). In contrast, whereas the mutation of Lys343 to Arg promotes a smaller change in the apparent K_m (3080 μM), the mutation to Gln increases dramatically the K_m value (106,000 μM). Therefore, the presence of a positive charge in position 343 is necessary for the binding of G6P, whereas there is greater flexibility regarding position 182. Accordingly, the highest pK_a value should be assigned to Lys343, enabling an Arg residue to play a similar role. In contrast, the lowest pK_a value would correspond to Lys182, where mutation to either polar (Gln) or charged (Arg) residues triggers similar shifts the K_m value.

Taken together, the preceding discussion allows us to conclude that the enhanced reactivity of Lys182 is due to an unusual low pK_a

value of 8.9, which facilitates the chemical modification by carbonyl groups of MG.

From a biochemical point of view an important question is then why Lys182 exhibits such a significant reduction in its pK_a. We hypothesize that the two Lys residues play distinct roles in mediating the binding of G6P. Lys343 is directly implicated in the binding of the substrate through electrostatic stabilization due to the formation of a salt bridge with the phosphate moiety of G6P. Nevertheless, Lys182 might play an indirect role, as this residue could participate in the protonation of His178 upon binding of G6P to the enzyme. Inspection of the X-ray structures available for substrate-bound forms of the enzyme (PDB entries 1E77 and 1E7Y; crystallized at pH ~7.6) indicates that the phosphate moiety of G6P is tightly bound to both Lys343 and His178 (interatomic distances of 2.6–2.8 Å). In fact, the relevance of His178 in binding G6P has been confirmed by site-directed mutagenesis, as the His178→Asn mutant showed a 400-fold increase in the K_m for G6P (42,900 μM).[43] We propose here that the interaction of His178 with G6P is facilitated by the proton transfer from Lys182, thus enabling His178 to reorient its side chain to stabilize electrostatically, in conjunction with Lys343, the binding of the phosphate moiety of G6P.

From a therapeutic point of view the unusual reactivity of Lys182 toward carbonyl groups explains the extremely low protection exerted by biogenic amines localized in the bulk of the solution, notwithstanding the fact that they were used at concentrations 10-fold higher than that of the basic residues in the protein. This finding is in agreement with previous studies focused on the structural requirements of α-dicarbonyl compounds implicated in protein crosslinking,[44] which have challenged the use of compounds containing amino groups as a suitable strategy to intervene against the Maillard reaction. Similarly, the local environment of basic residues located in the interface of multimeric aggregates, such as those found in the eye lens proteins, could also explain the low protective effect of biogenic amines toward glycating agents.[45,46]

Taking into account that trapping of reactive dicarbonyl species by drugs containing amine groups in their structures is one of the strategies proposed to inhibit AGE formation, our results suggest that the development of more effective therapeutic solutions to the problem of glycation requires biogenic amines with increased nucleophilicity, whose reactivity could then make an effective competition with basic residues with anomalously low pK_a values, and therefore prone to chemical modification by carbonyl-containing compounds. In fact, the enhanced reactivity of those basic residues likely contributes to the limited success of current Maillard inhibitors, which justifies the intense research effort devoted to the development of novel glycation inhibitors.[47]

A plausible strategy to make more protective agents consists of the introduction of suitable chemical modifications that enhance the reactivity properties of amines. The effective tuning of the reactivity of amines via chemical modification is supported by the changes of pK_a's of aliphatic amines upon fluorination of the methylenic chain (see Table S1 in Supplementary data). The pK_a of aliphatic amines is insensitive to the length of the chain, as the pK_a is close to 10.6 for methyl-, ethyl-, propyl- and butylamine. In contrast, the inclusion of two fluorine atoms in beta position reduces the pK_a to 7.5. The addition of a third fluorine atom (2,2,2-trifluoroethylamine) further reduces the pK_a to 5.7. Clearly, this effect can be attributed to the electron withdrawing effect of fluorine, which facilitates deprotonation of the protonated amine.

Similar trends can also be observed in the pK_a values predicted for fluorinated analogs of alanine, as replacement of the methyl group in the side chain of this residue by a trifluoromethyl unit reduces the estimated pK_a from 9.7 to 7.0 (see Table S2 in Supplementary data). Other functional groups can trigger similar changes. For instance, the addition of a second amine unit in gamma position reduces the experimental pK_a of propylamine from 10.6 to 8.6 (see Table S1 in Supplementary data). This factor likely contributes to the enhanced protective effect recently reported for N-terminal 2,3-diaminopropionic acid peptides by Sasaki et al. who have considered the use of 1,2-vicinal diaminoalkyl compounds as potential scavengers of α-dicarbonyl compounds.[48]

The preceding considerations suffice to demonstrate that a proper exploration of chemical substituents could be effective to open new avenues for developing anti-glycating amines. Those modifications must be properly chosen to yield an optimal pK_a that ensures a balance between ionization properties of the amine and its intrinsic nucleophilicity. Thus, the reduction in the pK_a must be large enough in order to ensure the presence of significant populations of the neutral species at physiological pH. However, as the intrinsic nucleophilicity due to the lone pair of the deprotonated amine is also affected by the presence of the electron withdrawing atoms, those chemical substituents should be carefully chosen as to retain the intrinsic nucleophilicity of the neutral amine against carbonyl compounds. Thus, the precise nature and location of the chemical modifications made in the amine should be properly chosen in order to keep the balance between those factors.

Besides the direct chemical modification of the amine, an alternative approach for tuning the reactivity of amines might rely on the use of microheterogeneous systems. The inclusion of amine compounds into cyclodextrins or nanoparticles can provide a more hydrophobic environment that enhances the nucleophilicity of the amines. Thus, previous studies have shown that complexation of a tertiary amine with β-cyclodextrin decreases the pK_a (from 9.4 in aqueous solution to 8.9 in 0.01 M β-cyclodextrin solution).[49] Similarly, the enhanced chemical reactivity of amines is also supported by kinetic studies on the aminolysis of esters by primary alkylamines in the absence and presence of cyclodextrins.[50] Enhanced deacylation of esters containing *t*-butylphenyl groups has also been reported for systems comprising β-cyclodextrin linked to dendrimer poly-ethylenimines.[51]

Overall, the design of systems containing chemically modified amines enclosed in less polar environments could be a promising strategy to modulate the anti-glycating activity, thus enabling the use of lower concentrations of amines. This latter aspect is also relevant, taking into account the convenience to avoid side reactions that can occur between amines and endogenous carbonyl groups present in biological systems in vivo.[52]

3. Conclusions

The results reported here reveal that biogenic amines exert a very modest protective effect on the inactivation of G6PD by MG. This finding could be attributed to an anomalous reactivity of specific basic residues in the enzyme, which would then lead to an enhanced reactivity toward carbonyl groups. In the case of G6PD, the results point out that, under the experimental conditions examined here, Lys182 should have an enhanced nucleophilicity, as this residue is involved in the formation of a Schiff base modification by MG. In fact, this residue is suggested to be responsible for the pK_a value of 8.9 determined experimentally in previous kinetic studies.[41–43] Since Lys182 is found in the inner part of the binding site, both limited exposure to water molecules and the nature of the surrounding residues in the binding pocket, particularly His178, could account for the enhanced nucleophilicity. In this context, the design of therapeutic strategies that combine the modulation of the intrinsic reactivity of amines through chemical modification with the use of microencapsulating systems could be valuable for developing more effective anti-glycating agents.

4. Materials and methods

4.1. Chemicals

MG, Car, Sp, Spe, NLys, fluorescamine, G6PD, the oxidized form of nicotinamide adenine dinucleotide (NAD^+), glucose 6-phosphate (G6P), were purchased from Sigma–Aldrich. Sodium borohydride ($NaBH_4$) was purchased from Fluka. Magnesium chloride ($MgCl_2$) was purchased from Merck. Acetonitrile (LC–MS quality, Scharlau), water (LC–MS quality, Sigma–Aldrich), formic acid (FA) (LC–MS quality, Sigma–Aldrich) and endoproteinase Glu-C (Roche) were employed for liquid chromatography–mass spectrometry analysis (LC–MS).

4.2. Determination of rate constants

Solutions of MG and amines were prepared in phosphate buffer (pH 7.4) at a 100 mM concentration and incubated at 45 °C in the absence of light. The decrease of the concentration of primary amino group over time was followed by the fluorescamine assay.[34] To this end, assays were performed using a final concentration of 1 mM for NLys, Car, Sp and Spe, while the MG concentration was varied from 25 to 100 mM when incubated with NLys or Car, and from 50 to 200 mM when incubated with Sp or Spe. The reaction rate is given by,

$$-\frac{d[\text{R-NH}_2]}{dt} = k[\text{MG}][R\text{-NH}_2] \quad (2)$$

where $[R\text{-NH}_2]$ and [MG] stand for the concentration of the primary amino group and MG, respectively, and k is the actual rate constant.

Upon excess of MG, the concentration of the amino group is given by $[\text{R-NH}_2] = [\{\text{R-NH}_2\}]_o\, e^{-kobst}$, where $[\text{R-NH}_2]_o$ is the concentration of the primary amino groups at $t = 0$, and k_{obs} is the observed or apparent rate constant.

A plot of $\ln [\text{R-NH}_2]/[\text{R-NH}_2]_o$ versus time leads to a straight line with slope $-k_{obs}$, and the representation of k_{obs} against MG concentration affords a linear plot with slope k_{rel}. Since the concentration of non-hydrated MG, which is more reactive than the hydrated adducts, corresponds to 1% of the total MG present in the reaction medium,[53] the actual rate constant (k) is obtained from the expression $k_{rel} = k \cdot F_N$, where F_N corresponds to the fraction of amine groups in their neutral nucleophilic form.

4.3. G6PD inactivation

The activity of G6PD was measured in a Genesys 10-S UV Scanning Thermo Spectronic spectrophotometer using Langdom modified method.[54] The total reaction mixture (3 mL) consisted of 2 mL of a solution containing 1.84 U of G6PD (from *L. mesenteroides*) in 0.55 mM NAD^+ and 1 mL of 1:1 mixture of $MgCl_2$ (6.7 mM) and G6P (1 mM). The activity was estimated from the increase in the absorption band at 340 nm (corresponding to the formation of reduced nicotinamide adenine dinucleotide; NADH) for 1 min. Measurements were performed in triplicate and the results expressed as percentage of the activity measured at zero time incubation.

For the incubation of G6PD with MG and amines, a solution containing 850 μL of enzyme (1.1 μM final concentration), plus 66 μL of MG (0.41 mM final concentration) with or without amines (final concentration of 0.41 mM for Car and 0.21 mM for Sp and Spe), were incubated in buffer phosphate (pH 7.4–9.4) for 16 h at 37 °C in the absence of light. The final volume was 1500 μL. The concentration of amino groups (Lys residues + amino terminal) was 0.041 mM, so that the amino-group/MG/amine ratio was 1:10:10 (Car) or 1:10:5 (Sp, Spe). The activity of the enzyme was assayed at 0 and 16 h.

Two samples of G6PD incubated with MG at pH 8.4 under the same experimental protocol (16 h incubation, 37 °C, and absence of light) were taken for mass spectrometry analysis. After incubation, $NaBH_4$ was added to one of those samples in order to reduce the Schiff base that might be formed upon modification of amino groups by MG. Finally, a third sample of G6PD treated with the same experimental protocol but without addition of MG was used as control. The samples were then dialyzed for 6 days, and diluted in ammonium acetate (25 mM) to obtain a final 10 μM concentration of the enzyme for intact MS analysis (top-down MS)[55–58] or digestion with Glu-C previous to MS analysis (middle-down MS).[59] In the latter case, a volume of 16 μL of endoproteinase Glu-C (1 μg/μL in H_2O) was added to 100 μL of protein solutions, and incubation was performed at room temperature overnight with gentle agitation. The samples were lyophilized and reconstituted in 50 μL of a 1% formic acid (FA) aqueous solution.

4.4. Intact protein MS analysis

For the top-down MS, an aliquot of 100 μL of the MG-treated sample solution was cleaned-up and concentrated using a C4 zip-tip (Millipore). Final elution from the zip-tip was made with 5 μL of a 78% acetonitrile, 1% acetic acid aqueous solution, and the sample was further diluted in 10 μL of an electrospray solution (methanol/1% FA) for MS infusion analysis on a LTQ-FT 12 Tesla (ThermoFisher Scientific). Direct sample introduction was performed by automated nanoelectrospraying using the Advion Triversa Nanomate (Advion Biosciences) at 1.65 kV spray voltage and 0.6 psi gas pressure. The mass spectrometer was operated on the positive polarity mode and parameters were set to 200 °C capillary temperature, 42 V capillary voltage and 135 V tube lens. Full MS spectra (m/z 200–2000) were acquired at sid (source induced dissociation or nozzle-skimmer dissociation) energies of 0 and 75 V and 200,000 resolution.

A volume of 10 μL of the 10 μM ammonium acetate reconstituted enzymes (native G6PD, treated and non-treated with MG) was analyzed by on-line LC-nanoESI-MS. Samples were injected through a Finnigan Micro AS autosampler (ThermoElectron Corporation) and loaded onto a BioSuite pPhenyl 1000 column (10 μm, 2.0 × 75 mm, Waters) using a Surveyor Finnigan MS quaternary pump (ThermoElectron Corporation). A 60 min gradient of 5–80% buffer B (A = 0.1% FA in water, B = 0.1% FA in acetonitrile) at 100 μL/min flow rate was used. The column outlet was directly connected to an Advion Triversa nanomate (Advion Biosciences) fitted to an LTQ-FT Ultra mass spectrometer (ThermoFisher Scientific). The nanomate was used as a splitter (1:250) and as a nanoESI source (Spray voltage and delivery pressure were set to 1.75 kV and 0.3 psi, respectively). The mass spectrometer was operated in positive polarity mode. Capillary voltage, tube lens and capillary temperature were tuned to 35 V, 135 V and 200 °C, respectively. Full scan MS spectra (m/z 295–2000) were acquired at 100,000 resolution (after accumulation of a target value of 10^6).

4.5. Glu-C digested protein MS analysis

For the middle-down MS, a volume of 10 μL of the Glu-C crude sample digestions was loaded onto a BioBasic-18 column (5 μm, 2.1 × 150 mm, ThermoFisher) and analyzed by LC-nanoESI-MS/MS using the chromatographic system described above. Peptides were separated in a 60 min gradient of 5–80% buffer B (A = 0.1% FA in water, B = 0.1% FA in acetonitrile) at 100 μL/min flow rate. The Advion Triversa nanomate was set to collection mode allowing simultaneous online nanoESI ionization and fraction collection. Spray voltage and delivery pressure in the nanomate source were set to 1.75 kV and 0.3 psi, respectively. The LTQ-FT ultra mass spectrometer was operated in positive polarity mode. Capillary voltage,

tube lens and capillary temperature were tuned to 35 V, 135 V and 200 °C, respectively. Acquisitions were performed in data-dependent mode. Survey full-scan MS spectra (m/z 295-2000) were acquired in the FT at 100,000 resolution (after accumulation of a target value of 10^6). The three most intense ions were sequentially isolated for fragmentation and detection in the linear ion trap using collision induced dissociation (CID) at a target value of 50,000, 1 microscan averaging and a normalized collision energy (CE) of 35%. Target ions already selected for MS/MS were dynamically excluded for 30 s. Further CID analyses were also performed off-line using the nanomate source by infusing the LC-collected fractions of interest. A normalized CE of 35–42% was applied to the ion trap isolated ions with high resolution (100,000) detection in the FT and 200–1000 scan averaging.

4.6. MS data analysis

Mass spectrometers were powered by Xcalibur software 2.07. Xcalibur Xtract algorithms and ProMass software version 2.8 (ThermoFisher Scientific) were used for charged state ion deconvolution to zero charged species. MS/MS high resolution spectra of intact proteins were deconvoluted with the in-house on-line automation cRawler software (Prof. N.L. Kelleher's property). Bioworks 3.3.1 and Protein Calculator from Xcalibur software were used to calculate theoretical molecular weights of intact and digested proteins. Single protein and Sequence gazer options from Prosight PC 2.0 (ThermoFisher Scientific)[60] software were used to localize modified residues from experimental high resolution MS/MS data, after fragment ions Xtract deconvolution to zero charged monoisotopic masses. MS raw data files from digested proteins were processed with Proteome Discoverer vs. 1.1 (ThermoFisher Scientific) against G6PD sequence.

4.7. Quantum chemical calculations

The reactive properties of amines were characterized by chemical reactivity descriptors determined at the B3LYP/6-311G(d) level,[61,62] considering the aqueous solvent effect by using the Polarizable Continuum Model (PCM)[63] as implemented in GAUSSIAN-03.[64] Attention was paid to the dual descriptor of chemical reactivity and selectivity defined as:[65]

$$\Delta f(\mathbf{r}) = f^{+}(\mathbf{r}) - f^{-}(\mathbf{r}) \approx \rho_L(\mathbf{r}) - \rho_H(\mathbf{r}) \quad (3)$$

where $f^{+/-}(\mathbf{r})$ stands for Fukui functions that characterize the response of the system at point $\mathbf{r}$ when dealing with nucleophilic ($f^{+}(\mathbf{r})$) or electrophilic ($f^{-}(\mathbf{r})$) attacks, and $\rho_L(\mathbf{r})$ and $\rho_H(\mathbf{r})$ are the densities associated to the lowest unoccupied and highest occupied molecular orbitals, respectively. Positive/negative values of $\Delta f(\mathbf{r})$ identify regions susceptible to react with nucleophilic/electrophilic reagents.

4.8. pK_a predictions

The ionization properties of selected basic residues were examined by titration computations performed for a diverse set of X-ray crystallographic structures of *L. mesenteroides* G6PD (PDB entries 1DPG, 1E77, 1E7Y, 1E7M, 1H93, 1H94 and 1H9A). In all cases, any substrate and/or co-factor were deleted prior to computations. The ionization state was determined using PROPKA method,[37,38] which takes into account the desolvation cost of ionizable residues, as well as hydrogen-bonding and charge–charge interactions with neighboring residues in the protein. Additional calculations were performed with MEAD program,[39] which relies on a semi-macroscopic treatment of biomolecules where the interiors of the protein and the solvent have different dielectric constants. The boundary between the different dielectric regions depends on the detailed atomic structure of the molecule, and the electrostatic potential is determined by using the Poisson–Boltzmann equation.[66] The dielectric constant for the solvent was 80, and for the protein was ranged from 4 to 20. Partial atomic charges were taken from the AMBER (parm99)[67] force field, and atomic radii from the optimized values developed by Poisson–Boltzmann computations with AMBER charges.[68] The ionic strength was fixed at 0.1 M. The protein was initially located in the geometric center (101 Å^3 box; grid step of 1.0 Å), and then refined by focusing the grid on the active site (101 Å^3 box; grid step of 0.5 Å).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.01.044.

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