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ORIGINAL ARTICLE

A comparative study of the chemical reactivity of pyridoxamine, Ac-Phe-Lys and Ac-Cys with various glycating carbonyl compounds

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Abstract Pyridoxamine (PM) has long been known to inhibit protein glycation via various mechanisms of action. One such mechanism involves the scavenging of carbonyl compounds with glycating ability. Despite the abundant literature on this topic, few quantitative kinetic studies on the processes involved have been reported. In this work, we conducted a comparative kinetic study under physiological pH and temperature conditions of the reactions of PM, Ac-Phe-Lys and Ac-Cys with various glycating carbonyl compounds (viz. aldehydes, α-oxoaldehydes and ketones). The microscopic formation rate constants for Schiff bases of PM and various carbonyl compounds, k_1, are of the same order of magnitude as those for the Schiff bases of Ac-Phe-Lys. However, because PM exhibits a higher proportion of reactive form at physiological pH, its observed second-order rate constant is ca. five times greater than that for Ac-Phe-Lys. That could explain PM ability to compete with amino residues in protein glycation. On the other hand, the observed formation rate constant for thiohemiacetals is four orders of magnitude greater than the formation constants for the Schiff bases of PM, which excludes PM as a competitive inhibitor of Cys residues in protein glycation.

Keywords Pyridoxamine (PM) · Carbonyl compounds · Kinetics · Shiff base · Protein Glycation

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Introduction

The non-enzymatic protein glycation and the formation of its end-products, so-called Advanced Glycation End Products (AGEs), are extremely important in hyperglycemic people; as such, they have been the subject of much research over the past 30 years (Ulrich and Cerami 2001; Thorpe and Baynes 2003; Horvat and Jakas 2004; Aldini et al. 2007). Protein glycation has been deemed responsible for a wide range of diabetes-associated pathologies including some eye diseases (Stitt 2005), renal dysfunctions (Bohlender et al. 2005), arteriosclerosis (Kume et al. 1995) and Alzheimer's disease (Reddy et al. 2002).

As shown in Fig. 1 for D-glucose, protein glycation begins with the condensation of a terminal amino group in a side chain of a Lys or Arg residue with a carbohydrate or some other carbonyl compound. This initially gives a Schiff base which undergoes reversible rearrangement to Amadori compounds that ultimately produces AGEs via a complex body of reactions (Ulrich and Cerami 2001). Other reactive carbonyl species can be produced from oxidative degradation of sugars, Schiff bases, and Amadori compounds (Thornalley et al. 1999) and can also contribute to protein glycation.

Propagation of protein damage may also be mediated by reactive oxygen species which can be formed from oxidative degradation of sugars (Wolff and Dean 1987), Schiff bases and Amadori compound (Mullarkey et al. 1990). These radical species can react with protein backbone and amino acid side chains (Pro, Thr, Trp, His and Cys) (Stadtman and Levine 2003), and contribute to the development of pathology in diabetes (Wolff 1993).

Although the high D-glucose levels present in hyperglycemic people is the main culprit of protein glycation, the presence of other carbohydrates such as the aldehydes

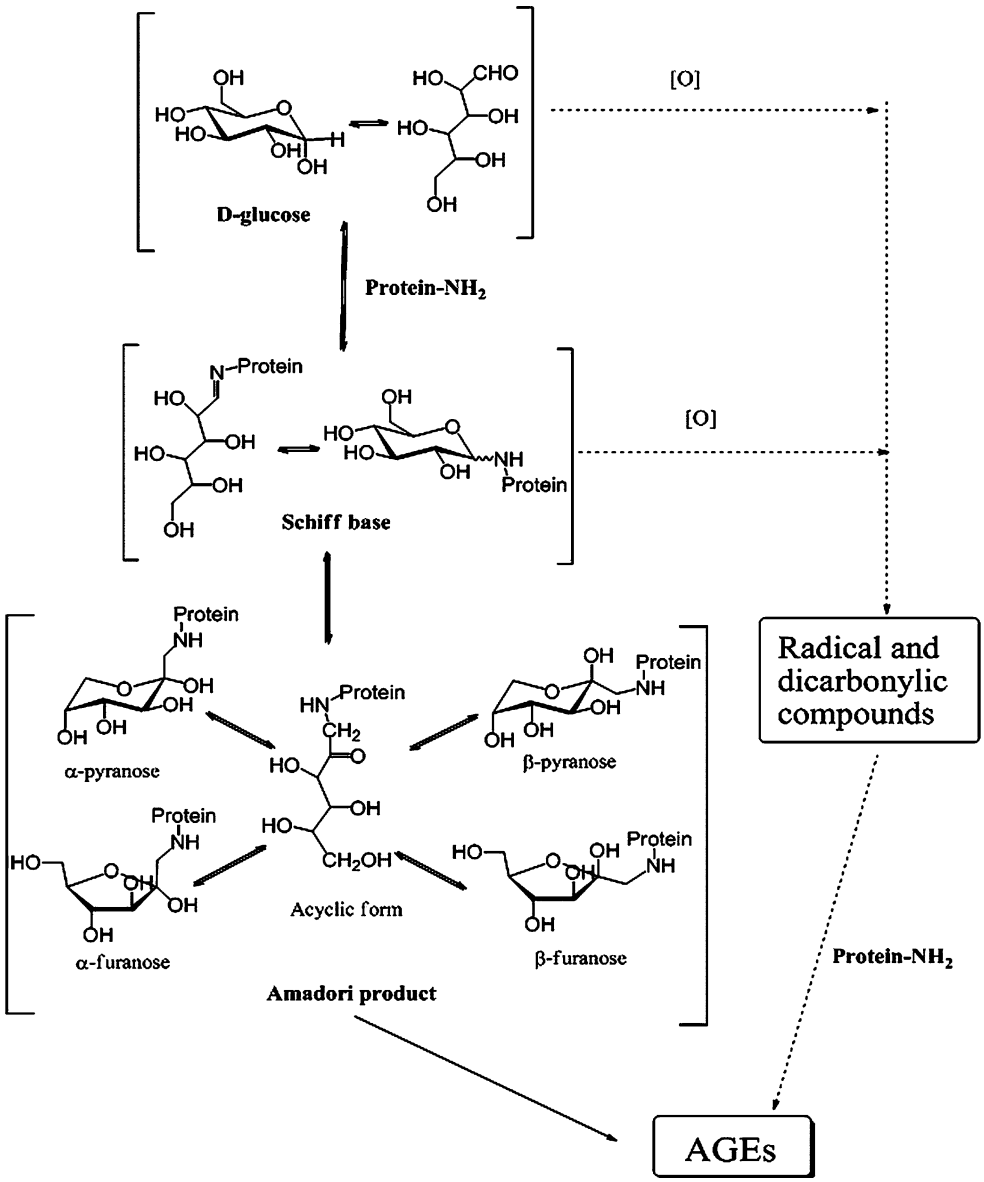

Fig. 1 Protein glycation pathways

D-ribose, D-arabinose and 3-deoxyglucosone, has also been found to induce the formation of AGEs (Dyer et al. 1991). Some small molecules such as glycolaldehyde, glyoxal and methylglyoxal, which result from the peroxidation of lipids and autoxidation of carbohydrates, are also highly reactive towards proteins (Nagai et al. 2000). Also, acetaldehyde, which is the main oxidation product of ethanol in vivo, is behind the protein damage leading to the development of alcoholism-related diseases (Braun et al. 1995). In addition, malondialdehyde, hydroxynonenal and isolevuglandins (γ-ketoaldehydes) formed during the oxidation of LDL cholesterol and fatty acids clearly play some role in protein lipoxidation (Aldini et al. 2007).

Ketonic compounds can also act in various steps in protein damage. Thus, dehydroascorbic acid is regarded as a powerful glycating agent (Reihl et al. 2004) and acetone, which occurs at increased concentrations in individuals with diabetic ketosis, is one of the principal protein modification agent for aminophospholipids in vivo (Kuksis et al. 2005). Also, acetone can be converted into methylglyoxal by the action of cytochrome P450, the intermediate acetol formed being an effective inhibitor for the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Morgan et al. 2002). Amadori compounds, which are key intermediates in the non-enzymatic protein glycation, and diketones formed in their autoxidation, provide effective electrophilic sites for protein cross-linking (Ulrich and Cerami 2001).

Pyridoxamine (PM), a naturally occurring derivative of vitamin B_6, has proved to be an effective inhibitor for protein glycation and lipoxidation both in vivo and in vitro (Voziyan and Hudson 2005). The inhibitory action of PM

involves chelation of metal ions with catalytic oxidation capacity (Price et al. 2001); neutralization of radical species by releasing the phenol proton at 3′ (Voziyan and Hudson 2005); and scavenging of carbonyl species with a high glycation ability—e.g. D-glucose and other carbohydrates (Adrover et al. 2007), glyoxal and glycolaldehyde (Voziyan et al. 2002), methylglyoxal (Nagaraj et al. 2002) and as recently shown 3-deoxyglucosone (Chetyrkin et al. 2008a)—or lipoxidation ability—e.g. γ-ketoaldehydes (Davies et al. 2006), malondialdehyde (Kang et al. 2006), and arachidonate and other peroxidation products of linoleic acid (Onorato et al. 2000).

One of the mechanisms by which PM exerts its inhibitory action is by scavenging carbonyl compounds, exhibiting competitive inhibition with nucleophilic groups in protein side chains. However, the significance of this process has scarcely been examined in quantitative and kinetic terms (Voziyan et al. 2002; Amarnath et al. 2004). In this work, we studied the reactions between PM and various glycating carbonyl compounds including aldehydes, ketones, and α-oxoaldehydes. In addition, we examined the reactivity of Ac-Phe-Lys as a protein side-chain model towards the same carbonylic compounds. For comparison, we also studied the reactions of Ac-Cys with glyoxal, glycolaldehyde and methylglyoxal.

Based on the results obtained in this work we can conclude that the thiol group in Cys exhibits the largest kinetic rate constants. Furthermore, the observed formation rate constants for the Schiff bases of PM exceed those for Ac-Phe-Lys.

Materials and methods

Materials

Pyridoxamine, D-glucose, D-ribose, D-arabinose, acetaldehyde, glycolaldehyde, glyoxal, methylglyoxal, 1-deoxy-1-morpholino-D-fructose, and D_2O (99.9% D) were purchased from Sigma-Aldrich; acetol was obtained from Fluka; Ac-Phe-Lys was supplied by Bachem Inc.; sodium cyanoborohydride, Ac-Cys, potassium hydrogen phthalate and acetone were obtained from Acros Organics; *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan was synthesized as described in a previous work (Adrover et al. 2008). All reagents were used as received. The buffering material was reagent-grade and Mili-Q water was used throughout.

High performance liquid chromatography (HPLC)

HPLC analyses were conducted on a *Shimadzu-LC 10AT* chromatograph equipped with a *Rheodyne 7725i* universal injector and a *Shimadzu SPD-M20A* UV/Vis photodiode array detector. The column was a *Tracer Excel 120 ODSB* model (25 × 0.46 cm, 5 μm). The target compounds in the reaction mixtures were separated by using MeCN/water-50 mM potassium phosphate (pH 6.0) in various isocratic and gradient modes.

NMR spectroscopy

NMR spectra were recorded on a *Bruker AMX-300* spectrometer, using sample tubes 5 mm in diameter and 3-(trimethylsilyl)-1-propanesulphonic acid (DSS) as internal reference. The solutions in D_2O were stabilized at p*D* 7.4 (p$D = -\log [D^+]$) with 0.5 M phosphate buffer. ^{13}C multiplicities were determined by using the distortionless enhancement bipolarization transfer method (DEPT-135).

LC–MS

Mass analyses were performed on an *Agilent 1110 Series LC–MS* instrument. The mass spectra for the target compounds were obtained by injecting the reaction mixtures through a *Tracer Excel 120 ODSB* column (25 × 0.46 cm, 5 μm). By exception, those for the thiohemiacetals were obtained by using a columnless flow injection analysis (FIA) system. Mass spectral detection of the compounds was done by using an electrospray ionization interface in conjunction with a quadrupole mass analyser. The mobile phase was 5 mM ammonium acetate at pH 6.0—NH_4AcO initiates the ionization for MS detection and circulated at a flow-rate of 0.3 ml/min for the FIA analyses. Various isocratic and gradient modes were used in conjunction with MeCN/water−5 mM ammonium acetate (pH 6.0) to separate the target compounds in the column analyses. The mobile phase was nebulized into an electrospray mass analyser by using gaseous nitrogen at 350°C at a flow-rate of 10 ml/min. The detector was used in the scan mode to count positive ions spanning the *m*/*z* range 100–800; with thiohemiacetals, however, it was used to count negative ions over the same range. A nebulization pressure of 415.6 kPa, a fragmentor voltage of 70 V and a capillary voltage of 3,000 V were used.

Stopped-flow spectroscopy

The kinetics of the reactions of Ac-Cys with glyoxal, methylglyoxal and glycolaldehyde were monitored with a *Biologic SFM-20* mixer furnished with a *TC-100/10T* quartz cell of 1cm path length and coupled to a *J&M Tidas16 256* diode array detector for multi-wavelength data collection at 37°C. The mixer-cell ensemble has a dead time of 3.7 ms for a flow velocity of 10 ml/s. A 75 W Xe lamp and a coupled monochromator were used to irradiate samples with a selected wavelength in order to avoid

photoinduced processes in aldehydes. The diode array detector had a spectral acquisition time of 0.8 ms over the wavelength range used.

Reaction mixtures for Schiff base kinetic analysis and products identification

The kinetic study of the reactions of PM and Ac-Phe-Lys with D-glucose, D-ribose, D-arabinose and acetone was performed with solutions that were 5 mM in the nucleophile, 200 mM in the carbonyl compound and 10 mM in $NaCNBH_3$. The reactions of the previous two nucleophiles with acetaldehyde and glycolaldehyde were studied in solutions containing 0.1 mM nucleophile, 3 mM in carbonyl compound and 1 mM in $NaCNBH_3$. Finally, those of PM and Ac-Phe-Lys with glyoxal and methylglyoxal were studied in solutions containing 0.5 mM nucleophile, 10 mM in carbonyl compound and 1 mM in $NaCNBH_3$. The kinetic study of the reactions of Ac-Phe-Lys with acetol, 1-deoxy-1-morpholino-D-fructose and *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan was performed with solutions that were 10 mM in the nucleophile, 200 mM in the carbonyl compound and 100 mM in $NaCNBH_3$. The addition of sodium cyanoborohydride ($NaCNBH_3$) was performed in order to selectively reduce imino groups in physiological conditions and facilitate the scavenging of the Schiff bases (Borch et al. 1971; Bunn and Higgins 1981). All reaction mixtures were prepared in 0.5 M phosphate buffer at pH 7.4 and thermostated at 37°C. Their kinetics were monitored by HPLC, using a wavelength of 321 and 230 nm for the reactions involving PM and Ac-Phe-Lys, respectively, as the nucleophile. All reactions were performed in duplicate. The reaction product formed by reduction of each Schiff base was identified by using ^{1}H, ^{13}C NMR and HPLC–MS techniques.

In order to demonstrate the Schiff base formation and subsequent selectively reduction by $NaCNBH_3$, the major reaction product formed between Ac-Phe-Lys and acetol was isolated by HPLC, freeze-dried, and characterized by NMR in 0.5 M phosphate buffer at p*D* 7.4. ^{1}H-NMR: δ 7.35 (dd, 1H, $^{3}J_{H3'-H4'} = 7.34$, $^{3}J_{H4'-H5'} = 7.45$ Hz, H–C(4′)); 7.32 (d, 1H, H–C(5′)); 7.29 (d, 1H, Hz, H–C(3′)); 4.59 (dd, 1H, $^{3}J_{H4-H1'A} = 5.36$ Hz, $^{3}J_{H4-H1'B} = 8.21$ Hz, H–C(4)); 4.08 (t, 1H, $^{3}J_{H7-H1''} = 8.22$ Hz, H–C(7)); 3.89 (m, 1H, $^{3}J_{H6''-H7''A} = 3.84$ Hz, $^{3}J_{H6''-H7''B} = 7.13$ Hz, $^{3}J_{H6''-H8''} = 6.47$ Hz, H–C(6'')); 3.57 (dd, 1H, $^{2}J_{H7''A-H7''B} = 11.62$ Hz, H–C(7″A); 3.40 (dd, 1H, H–C(7″B); 3.17 (dd, 1H, $^{2}J_{H1'A-H1'B} = 14.14$ Hz, H–C(1′A)); 2.99 (t, 2H, $^{3}J_{H3''-H4''} = 7.62$ Hz, H–C(4″)); 2.93 (dd, 1H, H–C(1′B)); 1.95 (s, 3H, H–C(1)); 1.76 (m, 2H, $^{3}J_{H1''-H2''} = 7.89$ Hz, H–C(1″)); 1.67 (m, 2H, $^{3}J_{H2''-H3''} = 7.13$ Hz, H–C(3″)); 1.32 (m, 2H, H–C(2″)); 1.13 (d, 3H, H–C(8″)). ^{13}C-NMR, DEPT-135: δ 180.82 C(8); 176.35 C(2); 174.7 C(5); 138.92 C(2′); 131.61 C(4′); 131.14 C(3′); 129.52 C(5′); 70.41 C(6″); 69.42 C(7″); 57.43 C(4); 57.37 C(7); 41.82 C(4″); 39.87 C(1′); 33.47 C(3″); 28.81 C(1″); 24.52 C(2″); 21.38 C(1); 20.68 C(8″). The molecular weight of isolated compound was determined by injecting the solution into the LC–MS system, which provided a spectrum with a major signal at *m/z* 416. The NMR data and mass spectra (according to the $[M + Na]^{+}$ peak of 2-(2-acetamido-3-phenylpropanamido)-6-(1-hydroxypropan-2-ylamino)hexanoic acid) confirms the reduced Schiff base formation between Ac-Phe-Lys and acetol (Fig. 2).

Reaction mixtures for thiohemiacetal kinetic analysis and products identification

Solutions containing 8 mM Ac-Cys (solution A) and 100 mM glycolaldehyde (solution B) were prepared in 0.5 M phosphate at pH 7.4 and kept at 37°C. Variable reactant volumes were inserted in the stopped-flow mixing chamber in order to obtain a V_A:V_B ratio of 1:1, 1:2 and 1:2.5. Each test was performed in triplicate and involved monitoring the absorbance at 286 nm, where a maximum increase was observed. An identical procedure was used to study the reaction between Ac-Cys and glyoxal. The reaction between Ac-Cys and methylglyoxal was examined by preparing solutions containing 4 mM Ac-Cys (solution A) and 40 mM methylglyoxal (solution B) in 0.5 M phosphate buffer at pH 7.4 that were mixed in V_A:V_B ratios of 1:1, 1:2 and 1:2.5. Each test was performed in triplicate and involved monitoring the absorbance at 305 nm, where a maximum increase was

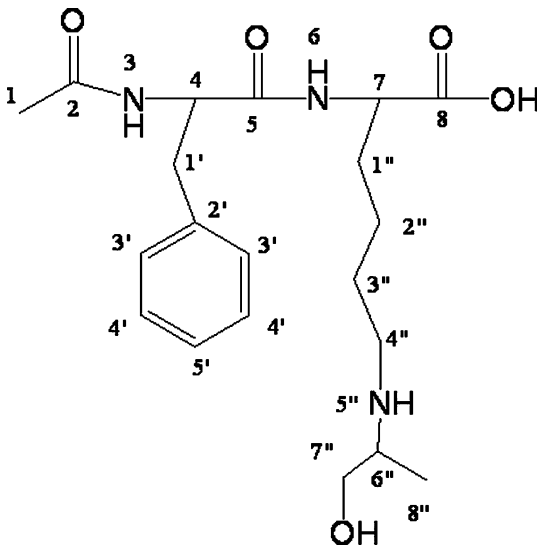

Fig. 2 Structure of the 2-(2-acetamido-3-phenylpropanamido)-6-(1-hydroxypropan-2-ylamino)hexanoic acid isolated formed from reduced Schiff base formed between Ac-Phe-Lys and acetol

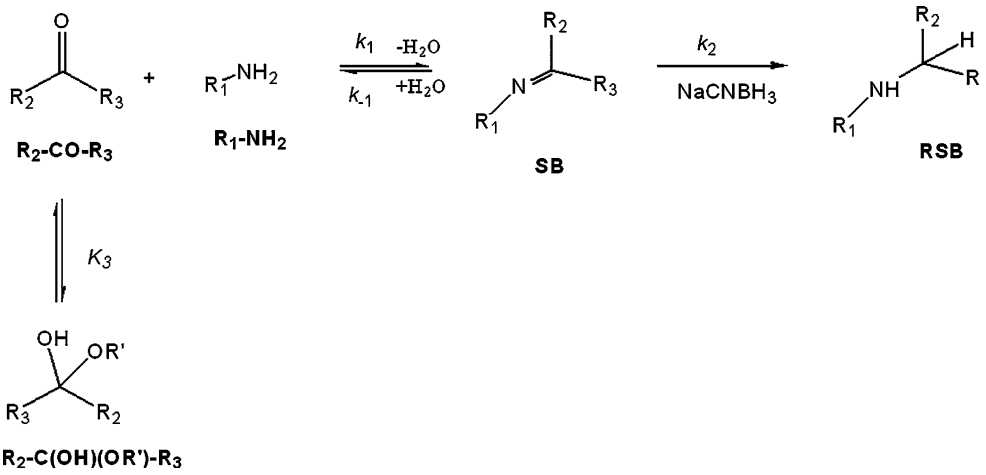

Fig. 3 Mechanism of formation of the secondary amine by reduction of the Schiff base

observed. The kinetic study of the reaction between Ac-Cys and methylglyoxal was repeated under identical conditions at pH 6.7 and 8.5 in order to assess the influence of this variable on the reaction rate. The resulting thiohemiacetals were identified in the reaction mixtures by HPLC–MS and ^{1}H-NMR spectroscopy. The ^{1}H-NMR results revealed that the thiohemiacetal formation equilibrium was completely displaced to the product, consistent with previous results of Lo et al. 1994.

Determination of Schiff base formation rate constants

Figure 3 shows the kinetic mechanism for the formation of a Schiff base (SB) between an amino group and a carbonyl group, and its subsequent reduction with $NaCNBH_3$. The kinetic equation defining the rate of disappearance of the terminal amino compounds is

$$\frac{-\mathrm{d}[\mathrm{R_1}-\mathrm{NH_2}]}{\mathrm{d}t} = k_1[\mathrm{R_2}-\mathrm{CO}-\mathrm{R_3}][\mathrm{R_1}-\mathrm{NH_2}] - k_{-1}[\mathrm{SB}]. \quad (1)$$

Application of the steady-state approximation for the Schiff base to this equation under the assumption that $k_2 >>> k_{-1}$ (Bunn and Higgins 1981) yields

$$\frac{-\mathrm{d}[\mathrm{R_1}-\mathrm{NH_2}]}{\mathrm{d}t} = k_1[\mathrm{R_2}-\mathrm{CO}-\mathrm{R_3}][\mathrm{R_1}-\mathrm{NH_2}]. \quad (2)$$

The carbonyl compounds studied in this work can be as reactive forms (R_2–CO–R_3) or unreactive forms (R_2–C(OH)(OR′)–R_3) in solution. This last form can be in hydrated (R′ = H) or cyclic form (R′ = hidrocarbon chain) as in carbohydrates. The equilibrium constant is given by

$$K_3 = \frac{[\mathrm{R_2}-\mathrm{CO}-\mathrm{R_3}]}{[\mathrm{R_2}-\mathrm{C(OH)(OR'\ )}-\mathrm{R_3}]}. \quad (3)$$

Under the experimental conditions, PM occurs mainly as single ionic specie with three different tautomers in equilibrium (see Fig. 4a) (Metzler et al. 1973), PM_1a being the reactive one, which concentration is related by the apparent constant K_4:

$$K_4 = \frac{[\mathrm{PM\_1a}]}{[\mathrm{PM\_1b}]+[\mathrm{PM\_1c}]}. \quad (4)$$

Tacking into account the mass balance for carbonyl compounds $[R_2–CO–R_3]_T = [R_2–CO–R_3] + [R_2–C(OH)(OR')–R_3]$, and for PM $[R_1–NH_2]_T = [PM\_1a] + [PM\_1b] + [PM\_1c]$ it can be demonstrated (Adrover et al. 2008) that Eq. 2 yields,

$$\frac{-\mathrm{d}[\mathrm{R_1}-\mathrm{NH_2}]_T}{\mathrm{d}t} = \frac{k_1K_3K_4[\mathrm{R_2}-\mathrm{CO}-\mathrm{R_3}]_T[\mathrm{R_1}-\mathrm{NH_2}]_T}{1+K_3+K_4+K_3K_4} \quad (5)$$

where all constants terms can be grouped as:

$$k_{\mathrm{obs}} = \frac{k_1K_3K_4[\mathrm{R_2}-\mathrm{CO}-\mathrm{R_3}]_T}{1+K_3+K_4+K_3K_4} \quad (6)$$

Eq. 5 can be rewritten as follows:

$$\frac{-\mathrm{d}[\mathrm{R_1}-\mathrm{NH_2}]_T}{\mathrm{d}t} = k_{\mathrm{obs}}[\mathrm{R_1}-\mathrm{NH_2}]_T \quad (7)$$

which can be integrated to

$$\ln\frac{[\mathrm{R_1}-\mathrm{NH_2}]_T}{[\mathrm{R_1}-\mathrm{NH_2}]_{T0}} = -k_{\mathrm{obs}}t \quad (8)$$

If the ε-amino group in the Ac-Phe-Lys dipeptide acts as a nucleophile, then one must consider the ionization equilibrium of such a group (Fig. 4b), via constant K_5:

$$K_5 = \frac{[\mathrm{R_1}-\mathrm{NH_2}][\mathrm{H_3O^+}]}{[\mathrm{R_1}-\mathrm{NH_3^+}]}. \quad (9)$$

Taking into account the mass balance for Ac-Phe-Lys: $[R_1–NH_2]_T = [R_1–NH_2] + [R_1–NH_3^+]$ and for carbonyl compounds, and by using a reasoning similar to that used for PM, k_{obs} is now defined as:

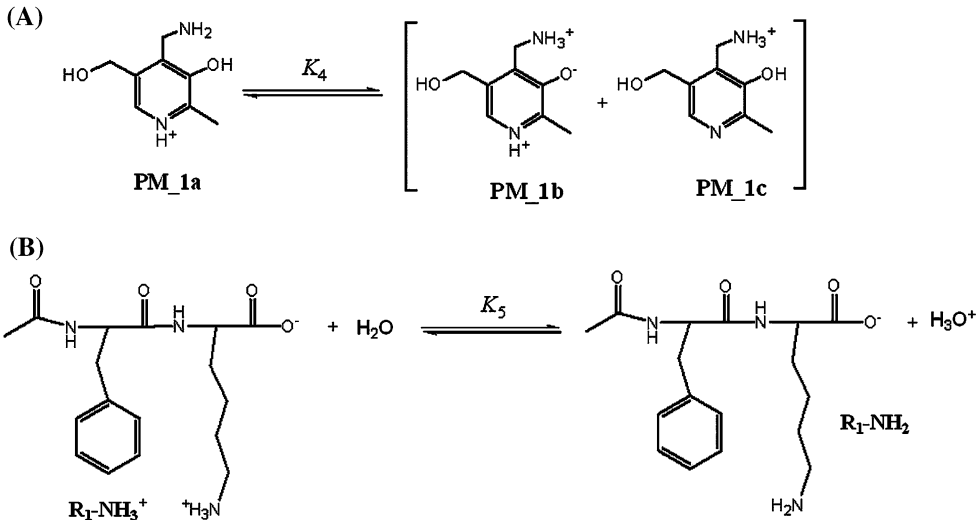

Fig. 4 **a** Equilibrium of ionic form PM_1 in PM. **b** Ionic equilibrium of Ac-Phe-Lys

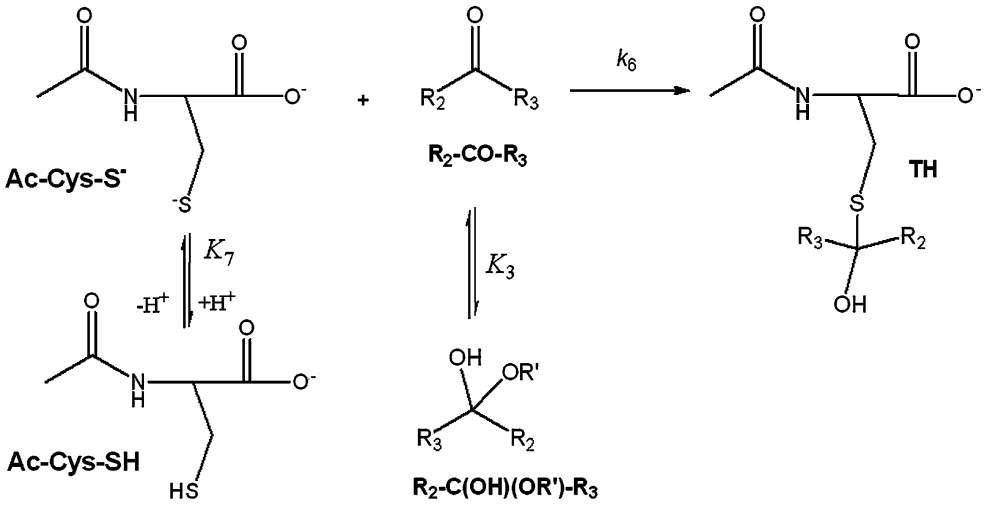

Fig. 5 Mechanism of the thiohemiacetal formation

$$k_{\text{obs}} = \frac{k_1 K_3 K_5 [\text{R}_2 - \text{CO} - \text{R}_3]_T}{[\text{H}_3\text{O}^+] + K_5 + [\text{H}_3\text{O}^+]K_3 + K_3 K_5}. \quad (10)$$

Experimentally, k_{obs} values were determined by using HPLC, measuring temporal changes the nucleophile concentration. Taking into account that the carbonyl concentrations used in the experiments were not the same we have defined the next second-order rate constant in order to compare the observed rate values:

$$k'_{\text{obs}} = \frac{k_{\text{obs}}}{[\text{R}_2 - \text{CO} - \text{R}_3]_T} \quad (11)$$

Determination of the thiohemiacetal formation rate constants

Figure 5 shows the general mechanism for the reaction between the thiol group in Ac-Cys and a carbonyl group. The temporal variation of the absorbance for each reaction was fitted to this kinetic scheme by using the DynaFit software (BioKin, Pullman, WA, USA) (Kuzmic 1996), which allowed the microscopic formation rate constant for the thiohemiacetal, k_6, to be calculated by numerical integration of the differential equations involved. Fitting required the prior determination of the molar absorption coefficient for each species, which are shown in Table 1. Data were fitted on the assumption of a negligible absorbance at both 286 and 305 nm for the R_2–CO–R_3 tautomer on the grounds of its low proportion in solution.

In order to compare the observed rate constants, the next differential equation was derived from a mathematical treatment similar to that previously shown for Ac-Phe-Lys, taking into account that the equilibrium corresponding to thiohemiacetal formation has been neglected since equilibrium was strongly displaced to products (Lo et al. 1994).

$$\frac{-\text{d}[\text{Ac} - \text{Cys} - \text{S}^-]}{\text{d}t} = k_{\text{obs}}[\text{Ac - Cys}] \quad (12)$$

where k_{obs} being defined as

Table 1 Molar absorption coefficients for Ac-Cys ionic species, hydrated carbonyl tautomers and thiohemiacetals

Compounds	ε_{286} (M^{-1} cm^{-1})	ε_{305} (M^{-1} cm^{-1})
Ac-Cys-SH	3.8 ± 0.5	0
Ac-Cys-S^-	9.8 ± 1.1	1.2 ± 0.2
Methylglyoxal	–	13 ± 1.9
Glyoxal	1.3 ± 0.2	–
Glycolaldehyde	1.4 ± 0.2	–
TH (Ac-Cys + methylglyoxal)	–	127 ± 8
TH (Ac-Cys + glyoxal)	20 ± 3	–
TH (Ac-Cys + glycolaldehyde)	20 ± 2	–

$$k_{obs} = \frac{k_6 K_3 K_7 [R_2 - CO - R_3]_T}{[H_3O^+] + K_7 + [H_3O^+]K_3 + K_3 K_7} \quad (13)$$

Determination of the Ac-Phe-Lys and Ac-Cys ionization equilibrium constants

The ionization equilibrium constant for the ε-amino (K_5) and carboxyl group (K_5') in Ac-Phe-Lys were determined by titrating a solution containing 12 mM dipeptide solution that was adjusted to pH 1.6 by addition of 1 M HCl. The ionization equilibrium constant for the thiol group in Ac-Cys (K_7) was determined by titrating a 12 mM peptide solution that was adjusted to pH 1.15 with 1 M HCl. The titrations were performed under the same working conditions employed in other tests, using a Titrino 718 pH-Stat from Metrohm. The titrant was a freshly made (0.120 ± 0.005) M NaOH solution that was standardized with potassium hydrogen phthalate. The titrations were done in triplicate and the results provided the following values for the constants: $K_5 = (8 \pm 1) \times 10^{-11}$ M and $K_5' = (4.0 \pm 0.3) \times 10^{-4}$ M for Ac-Phe-Lys, and $K_7 = (8.5 \pm 0.8) \times 10^{-10}$ M for the Ac-Cys.

Theoretical methodology

In order to justify the differences between the k_1 values for the reactions of PM and Ac-Phe-Lys, their structures were optimized by using PM3 Hamiltonian (Steward 1989) included in the software AMPAC v.8.0 (AMPAC 8, © 1992–2004 Semichem, Inc. P.O. Box 1649, Shawnee, KS 66222.). The solvent effect was included in the calculations by using the COSMO continuum method (Klamt 1995) as implemented in AMPAC. Charges on the nitrogen atom in the α-amino group of the PM and the nitrogen atom in the ε-amino group of the Ac-Phe-Lys were calculated by using Mulliken (1955) and Kollman methods (Besler et al. 1990).

Results and discussion

Reactions of pyridoxamine and Ac-Phe-Lys with carbonyl compounds

We studied the reactions of PM and Ac-Phe-Lys with acetone, acetaldehyde, glycolaldehyde, D-glucose, D-ribose, D-arabinose, glyoxal and methylglyoxal, and also the reactions between Ac-Phe-Lys with acetol, 1-deoxy-1-morpholino-D-fructose and *N*-(1-deoxy-D-fructos-1-yl)-L-tryptophan under physiological conditions in presence of $NaCNBH_3$.

Figure 6 shows the temporal variation of the chromatograms obtained at 321 nm for the reaction between PM and acetaldehyde. The initial chromatogram exhibited a major signal corresponding to PM (t_R 1.8 min). That a signal decreased with time as a single signal at t_R 3.4 min simultaneously appeared. HPLC–MS analysis of the reaction mixture provided an *m*/*z* value of 197.0 ($[M + H]^+$) for the compound giving the signal; this, together with the ^1H, ^{13}C NMR results, confirmed that the reaction product

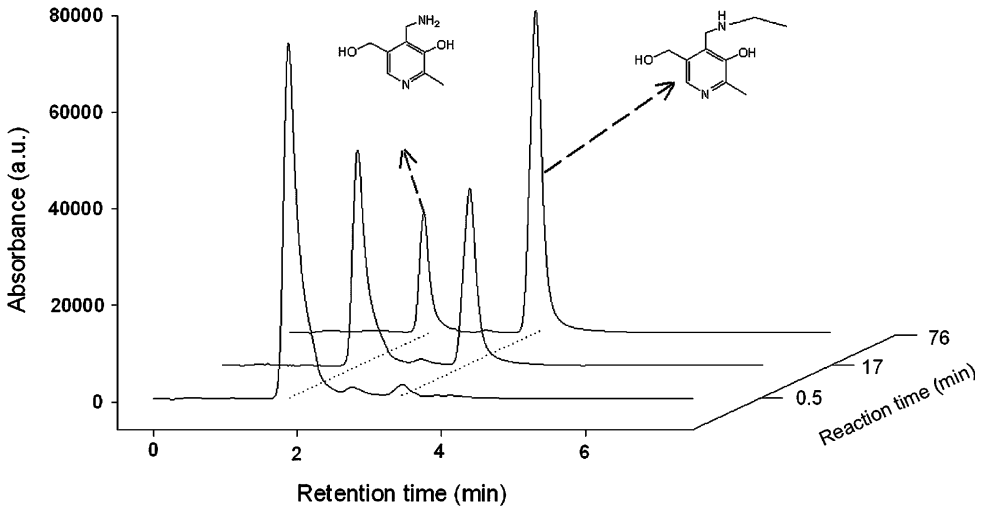

Fig. 6 Time-dependent HPLC chromatograms for the reaction between 0.1 mM PM and 3 mM acetaldehyde in the presence of $NaCNBH_3$ in a phosphate-buffered medium at pH 7.4 at 37°C as obtained with UV/Vis detection at 321 nm

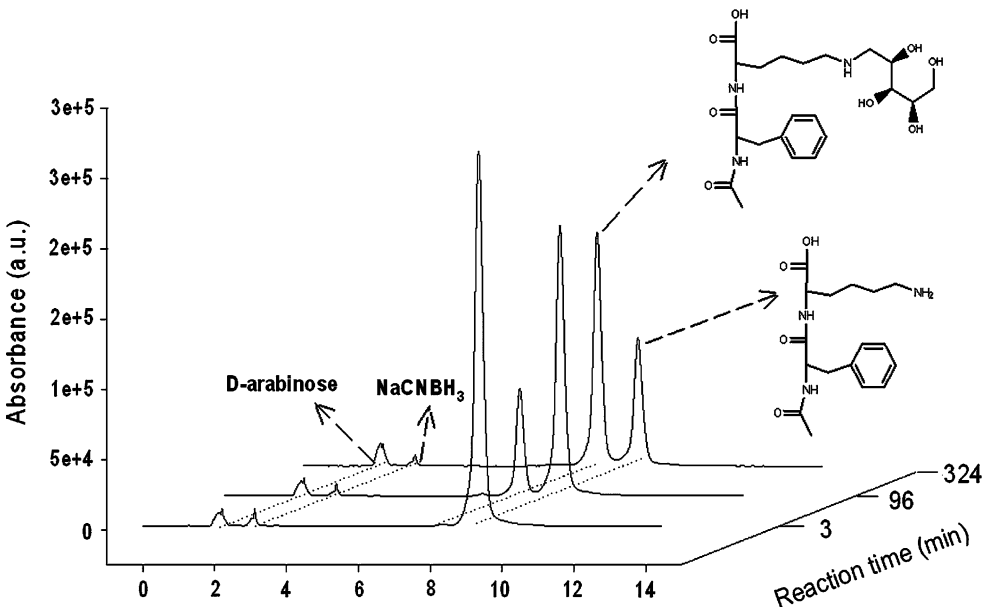

Fig. 7 Time-dependent HPLC chromatograms for the reaction between 5 mM Ac-Phe-Lys and 200 mM D-arabinose in the presence of $NaCNBH_3$ in a phosphate-buffered medium at pH 7.4 at 37°C as obtained with UV/Vis detection at 230 nm

was 4-((ethylamino)methyl)-5-(hydroxymethyl)-2-methylpyridin-3-ol.

Figure 7 shows the temporal variation of the chromatograms for the reaction between Ac-Phe-Lys and D-arabinose. The initial chromatogram obtained at 230 nm exhibited two weak signals at t_R 2.2 min and at t_R 3.1 min corresponding to D-arabinose and $NaCNBH_3$, respectively, in addition to a major signal at t_R 9.3 min corresponding to Ac-Phe-Lys. The area under both minor signals remained unchanged throughout the reaction since both reactants were present in excess amounts. On the other hand, the area under the major signal decreased with time as a new signal at t_R 8.2 min simultaneously appeared; based on the HPLC–MS results (*m/z* 470.4, $[M + H]^+$) and the ^1H, ^{13}C NMR spectra, the product giving such a signal was 6-(2,3,4,5-tetrahydroxypentylamino)-2-(2-acetamido-3-phenylpropanamido)hexanoic acid.

The reactions of PM and Ac-Phe-Lys with the other carbonyl compounds were studied by using HPLC to monitor the decrease in the chromatographic signal at 321 nm for PM and that at 230 nm for Ac-Phe-Lys. All chromatograms exhibited a new major signal with time which, based on the HPLC–MS and ^1H, ^{13}C NMR results, was assigned to a secondary amine as reduction product of the Schiff base formed.

The concentration changes in PM and Ac-Phe-Lys as a function of time were fitted to a *pseudo* first-order kinetic (Eq. 8) and used to determine the first (k_{obs}) and second-order (k'_{obs}) observed rate constants which are shown in Table 2. The kinetic rate constants for the Schiff bases formation between the amino group in PM and the different carbonyl compounds, k_1, were obtained from Eq. 6, and those for the reaction between the ε-amino group in Ac-Phe-Lys and the same electrophiles, from Eq. 10. The equilibrium constants for the different carbonyl compounds (K_3, Table 2) and $K_4 = 0.036$ for the equilibrium between the reactive specie in PM (PM_1a) and the other species present at the working pH (Fig. 4a) (Adrover et al. 2007) were used in the calculations. The dissociation rate constant for the ε-amino group in Ac-Phe-Lys (K_5, Fig. 4b) was obtained from titration (see Exp.)

The results clearly reveal that the amino groups in PM and Ac-Phe-Lys react with the carbonyl groups in glycating compounds to form a Schiff base. The k_1 values for the reactions of Ac-Phe-Lys (Table 2) are 2–5 times greater than those for PM, possibly as a result of differences in reactivity between their amino groups. Thus, unlike Ac-Phe-Lys, the amino group in PM (PM_1a) can form an intramolecular hydrogen bond with the phenol proton at 3′ and the bond probably reduces its negative charge density. This hypothesis was confirmed by optimizing the geometries of species PM_1a and the R_1-NH_2 tautomer of Ace-Phe-Lys by using a semi-empirical method involving the PM3 Hamiltonian and introducing the solvent effect via the COSMO method. Based on the calculated Mulliken and Kollman charges, the electronic charge on the nitrogen atom in the amino group of Ac-Phe-Lys is ca. 0.20e greater than that for the amino group of PM, which confirms the previous hypothesis.

k_1 values indicate that the α-oxoaldehydes are the most reactive of all carbonyl compounds studied; thus, k_1 is one order of magnitude greater for glyoxal than it is for methylglyoxal. The k_1 values for the α-hydroxyaldehydes (viz. glycolaldehyde and the aldoses) are one order of

Table 2 Kinetic constants for the reactions of PM and Ac-Phe-Lys with the carbonyl compounds studied

Compound		PM			Ac-Phe-Lys		
	K_3	k_{obs} (h^{-1})	k'_{obs} (M^{-1} h^{-1})	k_1 (M^{-1} h^{-1})	k_{obs} (h^{-1})	k'_{obs} (M^{-1} h^{-1})	k_1 (M^{-1} h^{-1})
Acetone	9.9×10^{1}[a]	0.27	1.4	3.9×10^{1}	0.073	0.36	1.8×10^{2}
Acetol	4.9×10^{1}[b]	0.58[c]	2.9	8.5×10^{1}[c]	0.17	0.86	4.3×10^{2}
1-deoxy-1-morpholino-D-fructose	5.0×10^{-2}[c]	0.027[c]	0.14	8.1×10^{1}[c]	0.0037	0.018	1.9×10^{2}
N-(1-deoxy-D-fructos-1-yl)-L-tryptophan	5.0×10^{-2}[c]	0.027[c]	0.14	8.1×10^{1}[c]	0.0031	0.015	1.6×10^{2}
Acetaldehyde	8.0×10^{-1}[d]	1.4	470	3.0×10^{4}	0.16	54	6.0×10^{4}
Glycolaldehyde	8.6×10^{-2}[e]	1.8	600	2.2×10^{5}	0.34	110	7.0×10^{5}
Glucose	2.0×10^{-5}[f]	0.058	0.29	3.7×10^{5}	0.0092	0.046	1.0×10^{6}
Ribose	4.0×10^{-4}[f]	1.1	5.4	3.9×10^{5}	0.19	0.86	1.2×10^{6}
Arabinose	3.0×10^{-4}[f]	0.62	3.4	2.9×10^{5}	0.11	0.54	8.7×10^{5}
Glyoxal[i]	1.8×10^{-5}[g]	0.13	6.5	1.8×10^{7}	0.021	1.1	3.0×10^{7}
Methylglyoxal	1.7×10^{-3}[h]	0.66	66	1.1×10^{6}	0.11	11	3.1×10^{6}

[a] (Greenzaid et al. 1967), [b](Glushonok et al. 2003), [c](Adrover et al. 2008), [d](Schuchmann and von Sonntag 1988), [e](Beeby et al. 1987), [f](Dworkin and Miller 2000), [g](Schweitzer et al. 1998), [h](Creighton et al. 1988)

[i] It has been assumed that glyoxal presents two equivalent nucleophilic sites

magnitude greater than that for acetaldehyde; this suggests that the presence of an α-hydroxyl group increases the reactivity of the carbonyl group. As can also be seen from Table 2, the k_1 values for the ketones are 3–4 orders of magnitude smaller than those for the aldehydes, as expect from the decreased reactivity of the carbonyl group in ketones relative to aldehydes.

Table 2 also shows the observed second-order rate constants (k'_{obs}) for the reactions of PM and Ac-Phe-Lys with the different carbonyl compounds at pH 7.4. As can be seen, the values for the reactions of PM with acetol and acetone are roughly 3 times greater than those for Ac-Phe-Lys with these compounds. The rate constants differ by a factor of 8 for the reactions with the Amadori compounds and by one of 6 for those with an aldehyde or α-oxoaldehyde. As previously shown for k_1, the increased value of k'_{obs} for PM is not a result of a higher nucleophilicity of its amino group, but rather of differences in the proportion of free amino group between PM -3.5% (Adrover et al. 2007) and Ac-Phe-Lys (0.007%) at physiological pH. The increased proportion of free amino group in PM relative to Ac-Phe-Lys can be ascribed to the presence of the phenol group at position 3′ of the pyridine ring in the former. Based on these results, PM can competitively inhibit the glycation of terminal ε-amino residues in proteins as has been described in a previous work (Voziyan and Hudson 2005).

Reactions of Ac-Cys with carbonyl compounds

Most research into protein glycation has focussed on the formation of AGEs on ε-amino and guanidine side chains in Lys and Arg residues of proteins (particularly extracellular proteins). In recent years, it has been reported that thiol groups in Cys side chains have also be found to be reactive towards carbonyl groups. This process is increased in intracellular space, which is a reducing environment containing abundant enzymes and proteins; unlike the extracellular space, these conditions favour the presence of Cys in its reduced form. For this reason, the thiol group is a perfect target for protein glycation caused by small-sized carbonyl compounds such as glyoxal, glycolaldehyde and methylglyoxal (Thorpe and Baynes 2003).

Based on the foregoing, and on recent reports that the previous aldehydes can form AGEs by reaction with Cys protein residues both in vitro (Zeng and Davies 2005) and in vivo (Alderson et al. 2006), we expanded the initial study by comparing the reactivity of the thiol group in Ac-Cys with that of the amino groups in Ac-Phe-Lys and PM. To this end, we studied the reactions of Ac-Cys with glycolaldehyde, glyoxal and methylglyoxal at physiological pH and temperature. Also, we studied the reaction of Ac-Cys with methylglyoxal at two other pH values in order to assess the influence of this variable on the reaction kinetics. The addition of the thiol group to a carbonyl group resulted in the reversible formation of a thiohemiacetal as structurally confirmed by ^{1}H-NMR and HPLC–MS spectroscopy, which revealed that the reaction, as previously reported by Lo et al. (1994), was completely displaced to this product. The thiohemiacetals formed in each reaction were identified by injecting the reaction mixture into the HPLC–MS instrument; in fact, the $[M–H]^-$ signal thus detected corresponded to a thiohemiacetal. Thus, m/z was 222.1, 220.3 and 234.2 for the products of Ac-Cys with glycolaldehyde, glyoxal and methylglyoxal, respectively.

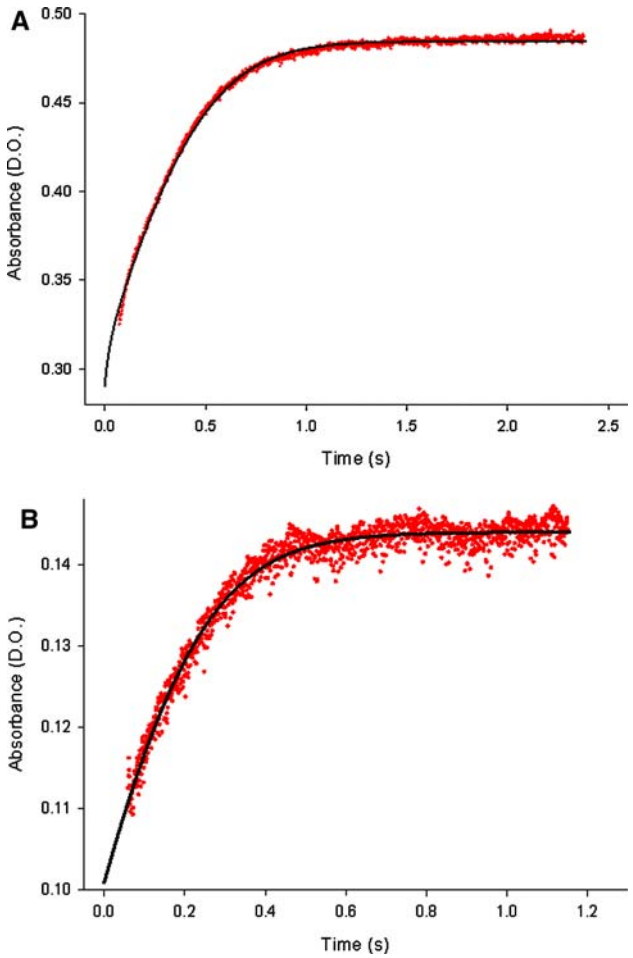

Fig. 8 **a** Temporal variation of the absorbance at 305 nm in the reaction between 2mM Ac-Cys and 20 mM methylglyoxal in a phosphate-buffered medium at pH 7.4 at 37°C. **b** Temporal variation of the absorbance at 286 nm in the reaction between 2.6 mM Ac-Cys and 66.6 mM glyoxal in a phosphate-buffered medium at pH 7.4 at 37°C

The kinetics of the thiohemiacetal formation was monitored by using stopped-flow spectroscopy with UV/Vis detection to measure absorbance changes as a function of time. Figure 8a shows the variation of the absorbance at 305 nm for a reaction mixture initially containing 2 mM Ac-Cys and 20 mM methylglyoxal, and Fig. 8b that at 286 nm for a mixture consisting of 2.6 mM Ac-Cys and 66.6 mM glyoxal. The same behaviour was observed with glycolaldehyde. The increase in absorbance with time was ascribed to the formation of the thiohemiacetal.

The temporal variation of the absorbance was fitted to the scheme of Fig. 5 by non-linear multivariate regression with DynaFit software. The fitted curves were used to calculate k_6 (Table 3) and the molar absorption coefficient for each thiohemiacetal at the corresponding wavelength (ε_{TH}) (Table 1). In addition, Eq. 13 was used to calculate k'_{obs} (Table 3).

Glyoxal was also the aldehyde exhibiting the highest reactivity in the formation of the thiohemiacetal; thus, its k_6 is ca. one order of magnitude greater than that for methylglyoxal and two than that for glycolaldehyde. Also, the k_6 values are roughly four orders of magnitude greater than the k_1 values for PM and Ac-Phe-Lys. The increased value of k_6 relative to k_1 in all cases is the result of the increased nucleophilicity of the thiolate group relative to the amino group by effect of the negative charge on the sulphur atom.

The k'_{obs} values for the amino groups were four orders of magnitude smaller than those for the thiol group in Ac-Cys. The difference resulted from the high nucleophilicity of the thiol group. The variation of pH in the reaction between Ac-Cys and methylglyoxal reveals the pH-dependence of k_{obs}, and the pH-independence of k_6, and validates the employed methodology.

There are other protein residues in addition to Lys, and Cys side chains which can facilitate glycation, albeit to a lesser extent. Back in 1977, Dworschák and Örsi (1977) concluded that the indole N in Trp can react with trioses, but not with hexoses. In a subsequent work, the condensation product of the NH-indole group in Trp with D-xylose and D-glucose was isolated, but only in acid media (Nyhammar and Pernemalm 1985). However, small molecules such as glyoxal, methylglyoxal and acetaldehyde can react with the NH-indole group in Trp to give highly stable products at physiological pH. The reaction was studied by Saito et al. (1986) in phosphate buffer at pH 6.5 at 50°C; based on their results, the observed second-order rate constant for the reactions of Ac-Trp with acetaldehyde, glyoxal, and methylglyoxal were estimated to be 0.004, 0.008 and 0.017 $M^{-1}h^{-1}$, respectively. A comparison of these results with k'_{obs} for PM and Ac-Phe-Lys reveals that the values for Ac-Trp are four orders of magnitude smaller—the effect of the temperature difference excluded.

Table 3 Kinetic constants for the reactions of Ac-Cys with the carbonyl compounds studied

Reaction	pH	k_6 (M^{-1} h^{-1})	k'_{obs}(M^{-1} h^{-1})
Ac-Cys + methylglyoxal	6.2	$(5.5 \pm 0.8) \times 10^{10}$	$(1.2 \pm 0.3) \times 10^{5}$
Ac-Cys + methylglyoxal	7.4	$(4.2 \pm 0.7) \times 10^{10}$	$(1.7 \pm 0.4) \times 10^{6}$
Ac-Cys + methylglyoxal	8.5	$(4.1 \pm 0.8) \times 10^{10}$	$(1.5 \pm 0.3) \times 10^{7}$
Ac-Cys + glycolaldehyde	7.4	$(4.6 \pm 0.3) \times 10^{9}$	$(6.7 \pm 1.1) \times 10^{6}$
Ac-Cys + glyoxal[a]	7.4	$(2.2 \pm 0.4) \times 10^{11}$	$(4.2 \pm 0.8) \times 10^{4}$

[a] It has been assumed that glyoxal presents two equivalent nucleophilic sites

Therefore, PM might inhibit the Trp side chain modification by means of scavenger radical (Chetyrkin et al. 2008b) and carbonyl species.

Conclusions

As shown in this work, the kinetic rate constant for the reaction of the amino group in PM with the carbonyl group in various electrophiles, k_1, is similar to that for the amino group in Ac-Phe-Lys. On the other hand, that for the thiolate group in Ac-Cys is four orders of magnitude greater, such a large difference being a result of the nucleophilicity of the reactive group.

The observed second-order rate constant, k'_{obs}, for PM at physiological pH is roughly five times greater than that for Ac-Phe-Lys. This difference is the result of the higher proportion of reactive form of PM at pH 7.4. Therefore, PM can efficiently compete with amino groups of Lys side chains in proteins. The k'_{obs} values for Ac-Cys are five orders of magnitude greater than those for PM; therefore, the efficiency of PM as a competitive inhibitor for Ac-Cys must be virtually zero.

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