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**PROTEIN SYNTHESIS
POST-TRANSLATION MODIFICATION
AND DEGRADATION:**

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A Post-Amadori Inhibitor Pyridoxamine Also Inhibits Chemical Modification of Proteins by Scavenging Carbonyl Intermediates of Carbohydrate and Lipid Degradation*

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Reactive carbonyl compounds are formed during auto-oxidation of carbohydrates and peroxidation of lipids. These compounds are intermediates in the formation of advanced glycation end products (AGE) and advanced lipoxidation end products (ALE) in tissue proteins during aging and in chronic disease. We studied the reaction of carbonyl compounds glyoxal (GO) and glycolaldehyde (GLA) with pyridoxamine (PM), a potent post-Amadori inhibitor of AGE formation *in vitro* and of development of renal and retinal pathology in diabetic animals. PM reacted rapidly with GO and GLA in neutral, aqueous buffer, forming a Schiff base intermediate that cyclized to a hemiaminal adduct by intramolecular reaction with the phenolic hydroxyl group of PM. This bicyclic intermediate dimerized to form a five-ring compound with a central piperazine ring, which was characterized by electrospray ionization-liquid chromatography/mass spectrometry, NMR, and x-ray crystallography. PM also inhibited the modification of lysine residues and loss of enzymatic activity of RNase in the presence of GO and GLA and inhibited formation of the AGE/ALE *N*^ε-(carboxymethyl)lysine during reaction of GO and GLA with bovine serum albumin. Our data suggest that the AGE/ALE inhibitory activity and the therapeutic effects of PM observed in diabetic animal models depend, at least in part, on its ability to trap reactive carbonyl intermediates in AGE/ALE formation, thereby inhibiting the chemical modification of tissue proteins.

Non-enzymatic modifications of proteins have been implicated in the pathogenesis of diabetes, atherosclerosis, and neurodegenerative diseases as well as in normal aging (1–4). These modifications can arise from direct exposure to reactive oxygen, chlorine, or nitrogen species or from reaction with low molecular weight reactive carbonyl compounds derived from carbohydrates, lipids, or amino acids (5–7). These carbonyl compounds react primarily with lysine and arginine residues, forming both adducts and cross-links in protein. Examples

include the formation of *N*^ε-(carboxymethyl)lysine (CML)¹ by reaction of GO or GLA with lysine, the formation of carboxyethyllysine by the reaction of MGO with lysine, the formation of argpyrimidine in the reaction between MGO and arginine, and lysine-lysine cross-links derived from the reactions of this amino acid with GO or MGO (8–11). Less reactive carbonyl compounds, such as glucose and other reducing sugars, can also react with proteins by forming intermediate Amadori adducts that may undergo further rearrangement, dehydration, and oxidation reactions to form stable AGEs, such as pentosidine and CML (12, 13). Carbonyl products of lipid peroxidation, malondialdehyde and hydroxynonenal, have been shown to react with protein lysine residues forming advanced lipoxidation end products (14). *In vivo*, the relative significance of different pathways of protein modification by carbonyl compounds would depend on the specific conditions, such as the level of oxidative stress and the status of carbonyl scavenging mechanisms.

Reactive carbonyl species are formed in a variety of metabolic reactions. Some are generated by non-oxidative pathways such as the formation of MGO by the spontaneous decomposition of triose phosphates or during anaerobic metabolism of acetone and amino acids (15). Other carbonyl species derive from oxidative reactions. For example, GO, MGO, and GLA are formed during the autoxidation of carbohydrates (8, 9, 16). Lipid peroxidation reactions can also produce GO and MGO (6, 17). Carbonyl compounds dehydroascorbate, acrolein, and MGO are also produced during the oxidation of ascorbate, hydroxyamino acids, and polyunsaturated fatty acids, respectively (5, 7, 18).

Because carbonyl modification reactions can alter protein structure and function and cause the formation of high molecular weight protein aggregates, they have been implicated in the development of a number of pathologies via a condition known as “carbonyl stress” (4, 19, 20). Therefore, inhibition of synthesis and/or trapping of free and protein-bound carbonyls presents an important avenue for drug development. Therapeutic agents such as aminoguanidine, L-arginine, OPB-9195, tenilsetam, and metformin have been proposed to trap reactive carbonyl compounds (21–25).

We have recently demonstrated that pyridoxamine, a natu-

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¹ The abbreviations used are: CML, *N*^ε-(carboxymethyl)lysine; AGE, advanced glycation end product; BSA, bovine serum albumin; DNPH, 2,4-dinitrophenylhydrazine; GO, glyoxal; GOPM, GO-pyridoxamine adduct; GLA, glycolaldehyde; GLAPM, GLA-pyridoxamine adduct; MGO, methylglyoxal; PM, pyridoxamine; RNase, bovine pancreatic ribonuclease A; ELISA, enzyme-linked immunosorbent assay; ESI-LC/MS, electrospray ionization-liquid chromatography/mass spectrometry; HPLC, high pressure liquid chromatography.

ral intermediate of vitamin B₆ metabolism, prevented the development of nephropathy in the rat model of diabetes (Baynes and co-authors, Refs. 26 and 27). Our earlier studies of the mechanism of action of PM suggest that it acts by inhibiting the conversion of intermediates in protein glycation reactions (Amadori compounds) to advanced glycation end products, such as CML (Hudson and co-authors, Refs. 28–30), and by trapping reactive intermediates formed during lipid peroxidation (31). In the present report, we extend our studies into the mechanism of action of PM by showing that it also inhibits protein modifications by GO and GLA, major products of sugar and lipid degradation. Pyridoxamine exerts this protective effect by competing with protein lysine residues for dicarbonyl and α -hydroxycarbonyl moieties of GO and GLA, respectively, to form relatively stable cyclic aminated derivatives, GOPM and GLAPM. Based on this work, we propose that the therapeutic effects of pyridoxamine observed in diabetic animal models (26, 27) are, in part, the result of its action in trapping a wide range of carbonyl intermediates in the pathway of protein modification by sugars and lipids. We suggest that pyridoxamine acts through this mechanism in addition to its inhibition of post-Amadori protein modifications established in our earlier studies (28–30).

EXPERIMENTAL PROCEDURES

Materials—D-Ribose, GLA, PM(HCl)₂, DNPH, lanthanum nitrate hexahydrate, yeast RNA, and BSA were purchased from Sigma. GO and Girard's reagent T were from Aldrich; RNase A was from Worthington Biochemical.

Incubation Conditions—All reactions were performed at 37 °C in 200 mM sodium phosphate buffer, pH 7.5. Sodium azide (0.02%) was added to prevent bacterial growth.

ELISA Detection of CML-BSA—The formation of CML in reactions between BSA and carbonyl compounds was measured by ELISA. The ELISA measurements used polyclonal anti-AGE antibody R618 (1:350) and were performed as described previously (29, 30, 32). CML has been identified as a dominant antigen for polyclonal antibodies against glycated proteins (33, 34). To determine the epitope specificity of our polyclonal antibody R618, we purified the antibody by affinity chromatography on CML-BSA-coupled Pierce AminoLink Plus column. The reactivity of purified antibody toward several AGE-modified proteins was identical to that of unpurified antibody,² indicating that CML was the primary epitope recognized by R618 polyclonal antibody.

Measurements of RNase Activity—RNase activity was determined by measuring the formation of acid-soluble oligonucleotide as described by Kalnitsky *et al.* (35), with some modifications. For the assay, 100 μ l of 3 μ g/ml RNase in 100 mM sodium acetate, pH 5.0, was mixed with 100 μ l of 1% yeast RNA in the same buffer. After the incubation at 37 °C for 5 min, the reaction was stopped by the addition of 100 μ l of an ice-cold solution of 0.8% lanthanum nitrate in 18% perchloric acid. Incubation tubes were kept on ice for 5 min to ensure complete precipitation of undigested RNA and then centrifuged at 12,000 $\times g$ for 10 min. An aliquot of the supernatant (20 μ l) was diluted to 1 ml with distilled water, and the amount of digested (solubilized) RNA was determined by measuring absorbance at 260 nm. The activity of RNase incubated either alone or with PM at 37 °C was monitored separately and used as the reference for each incubation time. This reference activity did not change significantly over the course of incubation.

Determination of Reactive Dicarbonyl and Carbonyl Groups—The concentration of GO was determined using Girard's reagent T (36). Briefly, an aliquot (5 μ l of 10 mM solution of GO) was mixed with 995 μ l of 120 mM sodium borate, pH 9.3. An aliquot of this mixture (200 μ l) was then added to 800 μ l of 100 mM Girard's reagent T in the same sodium borate buffer. After the reaction had reached equilibrium (10 min at room temperature), the amount of reacted dicarbonyl groups was determined by measuring absorbance at 326 nm (36).

The concentration of GLA was determined by the DNPH assay (37). Aliquots (20 μ l) of the samples containing 10 mM glycolaldehyde were mixed with 1 ml of 200 μ M DNPH in 1 M HCl. After 20 min at room temperature, the amount of reacted carbonyls was determined by meas-

uring absorbance at 380 nm (37).

Mass Spectrometry—ESI-LC-MS/MS was carried out in a positive ion mode on a Micromass Quattro LC mass spectrometer (Micromass, Beverly, MA) equipped with a Hewlett-Packard (Palo Alto, CA) series 1100 HPLC system and a Keystone (Bellefonte, PA) Aquasil C-18 microbore column. The solvent system consisted of 0.1% trifluoroacetic acid (solvent A) and methanol (solvent B), flow rate at 0.8 ml/min. The gradient was as follows: 0–2 min, 15% B; 2–40 min, 15–75% B, hold 5 min; 45–55 min, 75–15% B, hold 20 min. In some experiments, samples were analyzed by direct injection ESI-mass spectrometry. In this case, the carrier buffer consisted of 80% acetic acid and 20% methanol, flow rate at 0.03 ml/min.

X-ray Crystallography—Colorless crystals of GOPM (0.28 \times 0.20 \times 0.16 mm) or GLAPM (0.24 \times 0.20 \times 0.08 mm) were coated in inert oil, mounted on the end of a thin glass fiber, and transferred to the cold stream of a Bruker SMART APEX charged coupled device-based diffractometer system (molybdenum K α radiation, λ = 0.71073 Å). The x-ray intensity data were measured at 190°K. Crystal quality and initial unit cell parameters were determined based on reflections taken from a set of three scans measured in orthogonal regions of reciprocal space. Subsequently a hemisphere of frame data was collected with a scan width of 0.3° and an exposure time of 20 s per frame. The first 50 frames were recollected at the end of the data set to monitor crystal decay. The raw data frames were integrated into reflection intensity files using the software SAINT+ (38), which also applied corrections for Lorentz and polarization effects.

The unit cell parameters were as follows: for GOPM, a = 18.3459 Å, b = 14.8104 Å, c = 12.8368 Å, α = 90°, β = 119.123°, γ = 90°; for GLAPM, a = 17.5912 Å, b = 8.7332 Å, c = 18.6798 Å, α = 90°, β = 93.8200°, γ = 90°. The final unit cell parameters are based on the least squares refinement of 3867 and 5943 reflections (for GOPM and GLAPM, respectively) with $I > 5\sigma(I)$. Analysis of the data showed negligible crystal decay during data collection. No correction for absorption was applied. GOPM and GLAPM crystallized in the space group C2/c as determined by the systematic absences in the intensity data. The structure was solved by a combination of direct methods and difference Fourier syntheses and refined by full matrix least squares against F² using the SHELXTL software (39). The protonated GOPM species is situated about a crystallographic inversion center. The asymmetric unit therefore contains half of the GOPM cation and one trichloroacetate anion. For the diprotonated GLAPM cation, the asymmetric unit also contains one-half of the cation and one trichloroacetate anion. All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were placed in idealized positions and refined using a riding model except for H1n bound to N1, which was located and refined with an isotropic displacement parameter.

Solution NMR—NMR data were collected on a Varian Inova 500 MHz instrument using deuterated dimethyl sulfoxide as solvent. Structural characterization of GOPM was provided by gradient-enhanced heteronuclear multiple quantum coherence, gradient-enhanced heteronuclear multiple quantum-multiple bond coherence, distortionless enhancement by polarization transfer, ¹H, and ¹³C NMR.

Absorbance Measurements—Absorbance and absorbance spectra were measured using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a Peltier temperature control unit. The concentration of modified proteins was determined using second derivative analysis of absorbance spectra to exclude a contribution from carbonyl modification-related spectral components (40).

RESULTS

Reaction of PM with GO and GLA—Because PM possesses a nucleophilic amino group (see Fig. 6), it has the potential to react with carbonyl compounds. We therefore measured the loss of reactive carbonyl and dicarbonyl groups in solutions of 10 mM GO or 10 mM GLA during the course of incubation with 15 mM PM. In our earlier studies, this concentration of PM was effective at inhibiting the formation of CML from the protein-Amadori intermediate *in vitro* (28–30).

PM reacted with both GO and GLA and trapped these carbonyl compounds (Fig. 1). The reaction of PM with GO was notably faster than with GLA ($t_{1/2}$ = 0.94 and 5.0 h, respectively). Because GLA can be oxidized to GO (9), it was important to establish whether oxidation was necessary for the reaction of PM with GLA. The rate of GLA oxidation was relatively slow under our experimental conditions. After 72 h of incubation,

² B. Cussimano, R. G. Khalifah, and B. G. Hudson, unpublished data.

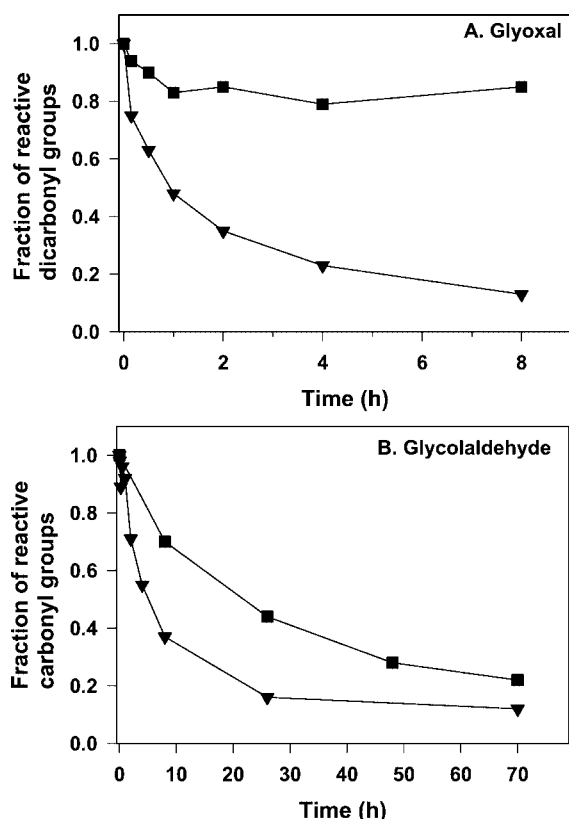


FIG. 1. PM forms adducts with GO and GLA. Samples of 10 mM glyoxal (A) or 10 mM glycolaldehyde (B) were incubated with 15 mM pyridoxamine (triangles) or 15 mM lysine (30 mM amino group) (squares). The incubations were carried out at 37 °C in 200 mM sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide. The loss of carbonyl moieties in the course of the reaction was measured spectrophotometrically using either Girard's reagent T or DNPH as described under "Experimental Procedures." Parallel experiments with carbonyl compounds incubated under the same conditions but without PM or lysine were used as references for calculating the relative amount of reactive carbonyl groups. Note the different time scales in panels A and B.

when ~90% of GLA had reacted with PM (Fig. 1B), only 21% of GLA had been converted to GO, as determined in separate incubations using the Girard's T assay (data not shown). Thus, PM appeared to react directly with both GLA and GO, as was also confirmed below by structural analysis of reaction products. Importantly, the reactivity of PM with GO was significantly greater than that of free amino acid lysine even though the lysine amino groups were at 2-fold excess over the amino groups of PM (Fig. 1A). In a slower reaction with GLA, PM still trapped this carbonyl compound more rapidly than did lysine (Fig. 1B).

To purify the GOPM adduct, we took advantage of its low solubility. Under the conditions of our experiments (10 mM GO and 15 mM PM), a visible precipitate formed during the course of reaction and was isolated by centrifugation at different incubation times. Although the absorbance of the solution gradually decreased, it still exhibited absorbance maxima characteristic of PM at neutral pH (256 and 324 nm) as shown in Fig. 2A. The GOPM precipitate (2 mg) was dissolved in 3 ml of 0.5% trichloroacetic acid, and an aliquot of this solution was then diluted 200-fold into sodium phosphate buffer, pH 7.5. The spectrum of GOPM complex was significantly different from that of PM itself: the short wavelength maximum shifted to 282 nm and its relative intensity was increased (Fig. 2B). Reactions of PM and GLA proceeded more slowly but also yielded a precipitate. Similar changes in absorbance spectra were also

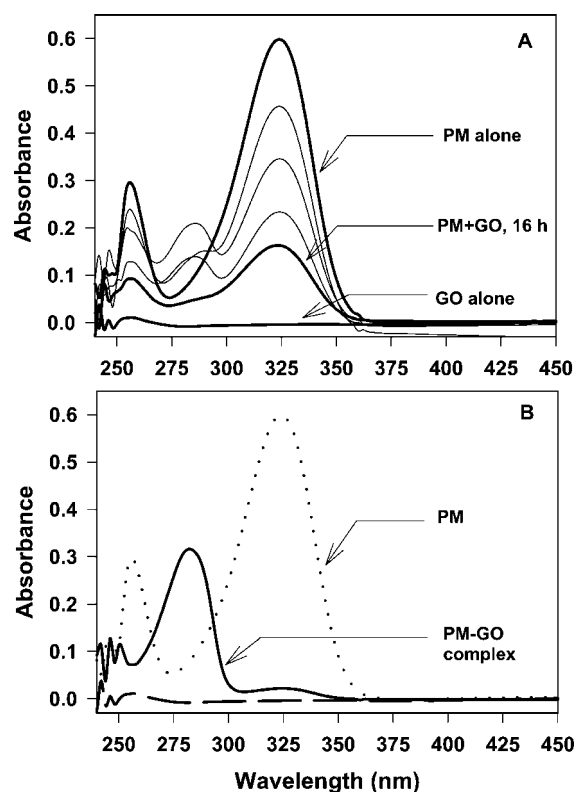


FIG. 2. Effect of GO on the absorbance spectrum of PM in solution. PM (15 mM) was incubated with GO (10 mM) in 200 mM sodium phosphate buffer, pH 7.5, at 37 °C (A). Insoluble complex was removed at various times by brief centrifugation, and the absorbance spectrum of the supernatant was recorded following 200-fold dilution into phosphate buffer. Thin lines represent the absorbance spectrum of the supernatant solution after 0.5, 2, and 4 h of incubation of PM with GO. After 16 h of incubation, no further spectral change was observed. Complex collected by centrifugation was washed with water, vacuum-dried, and dissolved in 0.5% trichloroacetic acid (B). Solution pH was adjusted to pH 7.5 by 200-fold dilution into 200 mM sodium phosphate buffer, and the absorbance spectrum was recorded. The presence of traces of trichloroacetic acid did not affect the absorbance spectrum of pyridoxamine in phosphate buffer.

observed, suggesting the formation of similar products from GO and GLA (data not shown).

Structures of GOPM and GLAPM Adducts—A direct injection electrospray ionization mass spectrometry analysis of GOPM and GLAPM produced protonated molecular ions $[M+H]^+$ with $m/z = 417$ and 385 , respectively (Fig. 3, A and B). The other prominent ions present in the spectra of GOPM and GLAPM ($m/z = 209$ and 193 , respectively) were doubly charged molecular ions $[M+2H]^{2+}$ based on the spacing of ^{13}C isotope satellites of these ions at 0.5 atomic mass units and by their identical HPLC elution time with $[M+H]^+$ ions. Fragmentation mass spectra (MS/MS) of all ions yielded peaks at $m/z = 169$ and 152 , characteristic of the molecular ion and deamination product of PM, respectively (data not shown). To unequivocally establish novel structures of GOPM and GLAPM, we have performed solution 1H and ^{13}C NMR and x-ray crystallography experiments. The results (Table I and Fig. 3, A and B, insets) were consistent with the structures shown in Fig. 3C.

PM Inhibition of Chemical Modification of Proteins by GO and GLA—To investigate the efficiency of protection of protein from carbonyl stress, we evaluated the effect of PM on the chemical modification of RNase A and BSA by GO and GLA *in vitro*. BSA contains 58 lysines per molecule, and its chemical modification can be followed by ELISA measurement of CML. RNase, on the other hand, has two active site lysines, Lys-7 and Lys-41 (41); therefore, the enzyme loses activity on reaction

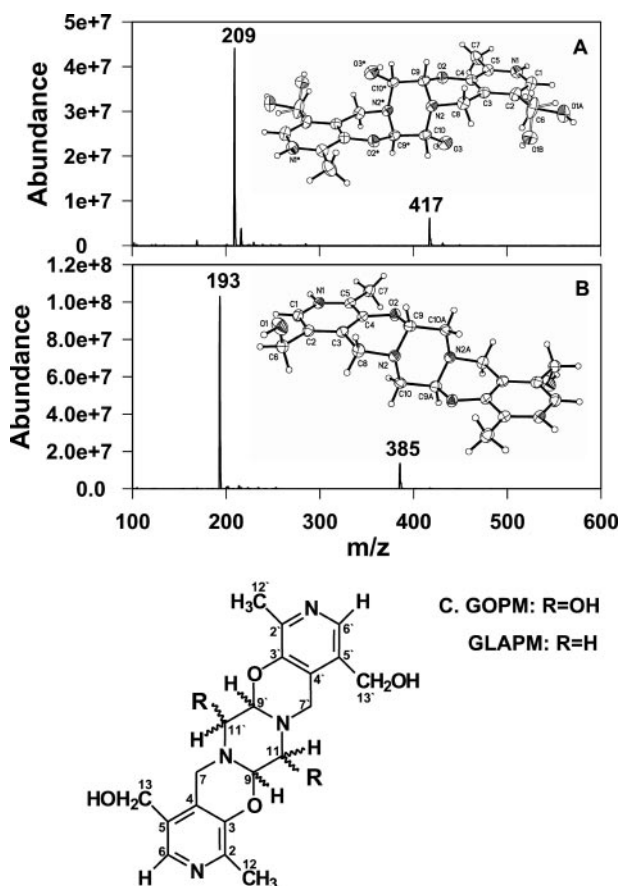


FIG. 3. ESI-mass spectra and structures of GOPM (A) and GLAPM (B) adducts. Purified adducts were dissolved in 0.5% acetic acid and analyzed by the direct injection ESI-mass spectrometry. Structures of GOPM and GLAPM adducts were analyzed by x-ray crystallography as described under "Experimental Procedures" (A and B, insets). C, chemical structures of GOPM and GLAPM consistent with the data of mass spectrometry, x-ray crystallography, and solution NMR. For ^1H and ^{13}C NMR chemical shifts of GOPM, see Table I.

TABLE I
 ^1H and ^{13}C NMR chemical shifts of GOPM

Carbon atom ^a	^1H NMR	^{13}C NMR
	<i>ppm</i>	<i>ppm</i>
C-2		144.2
C-3		148.4
C-4		127.6
C-5		130.8
C-6	7.89 (1H,s)	138.1
C-7	4.60 (1H,d), 4.18 (1H,d)	45.12
C-9	4.70 (1H,d)	86.1
C-11	4.59 (1H,d), 4.9 (1H,d)	80.46, 87.63
C-11 (-OH)	5.54 (1H,d)	
C-12	2.67 (3H,s)	18.26
C-13	4.45 (2H,d)	58.18
C-13 (-OH)	5.12 (1H,t)	

^a For carbon atom numbering, see Fig. 3C.

with carbonyl compounds. The incubations were carried out at equimolar concentrations of GO or GLA, PM, and protein lysines (6.7 mM) to compare the reactivity of PM and protein lysines with carbonyl compounds.

Although the inhibition of RNase activity by GO and GLA occurred with different kinetics, only about 20% of enzyme activity was detected after 288 h of incubation with either carbonyl compound (Fig. 4, A and B). When RNase was incubated with these carbonyl compounds, but in the presence of PM, a protection of enzyme activity was observed. The protection was more prominent in the case of GO as compared with

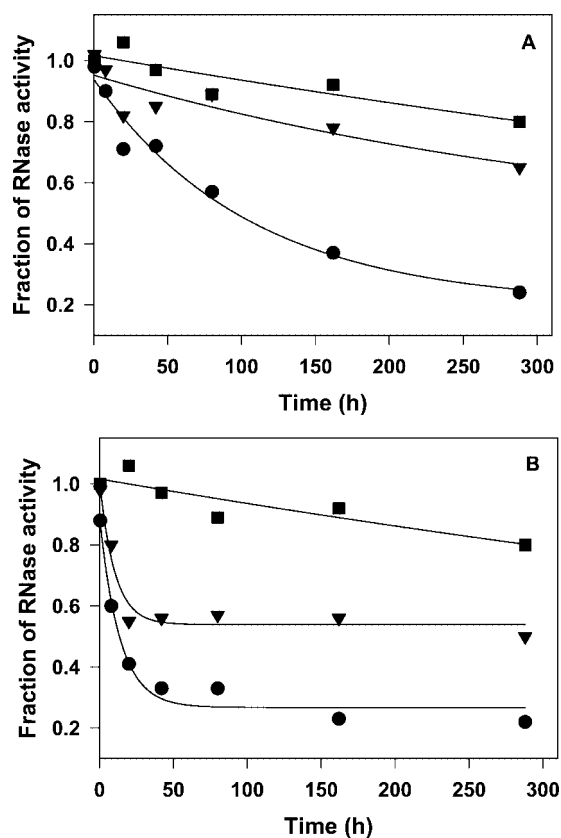


FIG. 4. PM inhibits inactivation of RNase by GO (A) and GLA (B). RNase (8.3 mg/ml, 6.7 mM amino groups) and corresponding reactive carbonyl compound (6.7 mM) were incubated either in the absence (circles) or presence (triangles) of 6.7 mM pyridoxamine. For the controls, RNase was incubated alone without any additives (squares). The incubations were carried out at 37 °C in 200 mM sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide. At the indicated times, aliquots were withdrawn, and RNase activity was determined as described under "Experimental Procedures." Each point represents an average of duplicate measurements.

GLA (Fig. 4, A and B), which is consistent with the lower reactivity of PM toward GLA (Fig. 1).

Because PM reacted more rapidly with GO or GLA as compared with lysine (Fig. 1), we predicted that PM would also inhibit the modification of protein lysine residues by reactive carbonyl compounds, specifically the formation of CML, a common product of the reaction of proteins with GO and GLA (9). As shown in Fig. 5, PM inhibited the GO- and GLA-induced formation of CML-BSA. In contrast to the results of experiments with RNase, PM was more effective in inhibiting the formation of CML from GLA as compared with GO. This was also evident at lower molar ratios of PM to carbonyl compounds (Fig. 5, A and B, insets). Because the reaction between PM and GLA is slower when compared with the reaction between PM and GO (Fig. 1), these results imply the participation of an additional inhibitory mechanism that is different from carbonyl scavenging. Interestingly, Glomb and Monnier (9) have found that the conversion of GO to CML does not include a metal-catalyzed oxidative step, whereas the GLA-induced CML-BSA synthetic pathway has an alternative metal-catalyzed oxidative step and thus depends, in part, on the presence of transition metal ions. Transition metal ions, such as Cu^{2+} , occur naturally in the sodium phosphate buffer that was used in our experiments (42). These ions were catalytically active under our experimental conditions because chelation with diethylenetriaminepentaacetic acid partially inhibited GLA-induced but not GO-induced CML formation (data not shown),

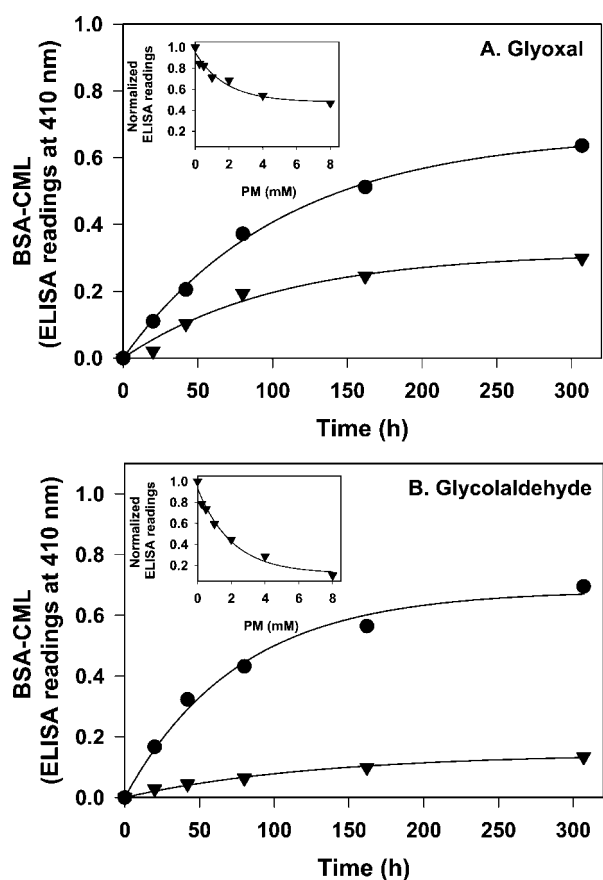


FIG. 5. PM inhibits formation of CML during exposure of BSA to GO and GLA. Carbonyl compounds (6.7 mM) and BSA (7.5 mg/ml, 6.7 mM amino groups) were incubated alone (circles) or with 6.7 mM pyridoxamine (triangles) for the indicated times. The incubations were carried out at 37 °C in 200 mM sodium phosphate buffer, pH 7.5, containing 0.02% sodium azide. CML-modified BSA was measured by ELISA as described under "Experimental Procedures." Each point represents an average of duplicate measurements. Inset, inhibition of formation of CML-BSA by different concentrations of PM, measured after 288 h of incubation.

which is consistent with the mechanism proposed by Glomb and Monnier (9). Because PM itself can chelate divalent metal ions (43, 44), this property may contribute to more efficient inhibition of GLA-induced CML formation by PM.

DISCUSSION

Advanced protein glycation reactions, originally studied because of their role in the "browning" of food products, are now considered to be one of the major sources of protein modifications in chronic diseases and during normal aging (1–3). Although new AGEs are being discovered continuously, the general outline of the chemistry of AGE synthesis is relatively well understood. In the Maillard reaction, sugars and proteins interact directly. The Schiff base product of this reaction rearranges to an Amadori intermediate that, in turn, is converted to AGEs (see Fig. 7, step 1), most prominently to CML (13), which is detected at an increased concentration in animal and human tissues in diabetes, neurodegenerative diseases, and aging (45–48). AGE modifications have been implicated as a source of structural and functional damage of proteins in diseases such as diabetes (49–51). Sugar or protein-sugar intermediates can also degrade, largely through sugar autoxidation (Wolff pathway, Ref. 52) or through the degradation of the Schiff base intermediate (Namiki pathway, Ref. 53), to produce low molecular weight carbonyl compounds. Reactive carbonyls are also produced during lipid peroxidation reactions (see Fig.

7). These electrophilic compounds can react directly with proteins to form adducts with lysine or arginine side chains (9, 54), as schematically shown in Fig. 7, steps 2 and 3. Steady-state levels of reactive carbonyls such as GO and MGO are increased in the plasma of diabetic animals and in patients with diabetes and uremia (55–58).

The carbonyl stress hypothesis emphasizes the role of carbonyl compounds, derived from different sources, in the induction of pathogenic protein modifications (4, 19, 20). In this study, we have demonstrated that PM can protect model proteins from carbonyl stress by chemically trapping low molecular weight carbonyl compounds. A proposed mechanism of formation of GOPM and GLAPM is shown in Fig. 6. Reaction between PM and GO begins with nucleophilic attack of the primary amine of PM on a carbonyl group of GO (Fig. 6A, step 1). The tetrahedral carbinolamine eliminates water to yield an imine (Schiff base), which undergoes nucleophilic attack by the phenolate anion aromatic hydroxyl to form a six-membered hemiaminal ring (Fig. 6A, steps 2–4). This monomeric intermediate then condenses with a second molecule of the intermediate to form the final product, GOPM (Fig. 6A, steps 5 and 6). GLA and PM react along a similar pathway to form a similar product (Fig. 6B). However, after the formation of the six-membered ring, this reaction is more likely to proceed via an aziridine intermediate at neutral pH. An aziridine is formed between the secondary amine of PM and the methylene carbon of GLA, resulting in a partial positive charge on the methylene carbon (Fig. 6B, step 5). The electrophilic nature of the methylene carbon makes it a candidate for nucleophilic attack by another molecule of the intermediate (Fig. 6B, step 6). As in the formation of GOPM, two molecules of the intermediate condense to form the final product, GLAPM (Fig. 6B, step 7).

It is important to note that the inhibitory effects of PM are not limited to scavenging of low molecular weight carbonyl products of glycation reactions. As demonstrated in our earlier works (28–30), PM strongly inhibits the conversion of post-Amadori intermediate to CML. In more recent work, Onorato *et al.* (31) showed that PM inhibits advanced lipoxidation reactions by trapping lipid-derived intermediates. Thus, under *in vitro* conditions, PM appears to inhibit the principal steps that lead to chemical modification of proteins by low molecular weight carbonyl compounds derived from either sugars or lipids, in addition to its inhibition of the formation of AGEs derived from Amadori adducts (Fig. 7).

Clearly, the efficacy of PM *in vivo* will be influenced by factors such as the nature of carbonyl species and local tissue concentrations of reactive carbonyls and PM, as well as the concentrations of endogenous carbonyl scavengers. However, even very small amounts of reactive carbonyls that exceed the capacity of endogenous carbonyl scavenger systems (*e.g.* glyoxalase pathway) may lead, over a long time, to high levels of protein modifications. By trapping the excess of reactive carbonyls, PM may provide a significant protective effect. It is important to note that the steady-state concentration of PM reaches $\sim 100 \mu\text{M}$ in the plasma of PM-treated animals (26, 27), whereas the concentrations of GO, MGO, malondialdehyde, and hydroxynonenal are in the nanomolar to low micromolar range (58, 59). Thus, the plasma concentration of PM is sufficient to scavenge the ambient concentration of major reactive carbonyl intermediates. Because the metastable adducts formed between PM and GO or GLA are small when compared with proteins, they would be rapidly eliminated from the body in urine. Therefore, the dynamic equilibrium between GO/GLA adducts to PM and to protein amino groups would lead to the gradual depletion of these reactive carbonyl compounds from plasma. The trapping reaction by PM would also be favored by

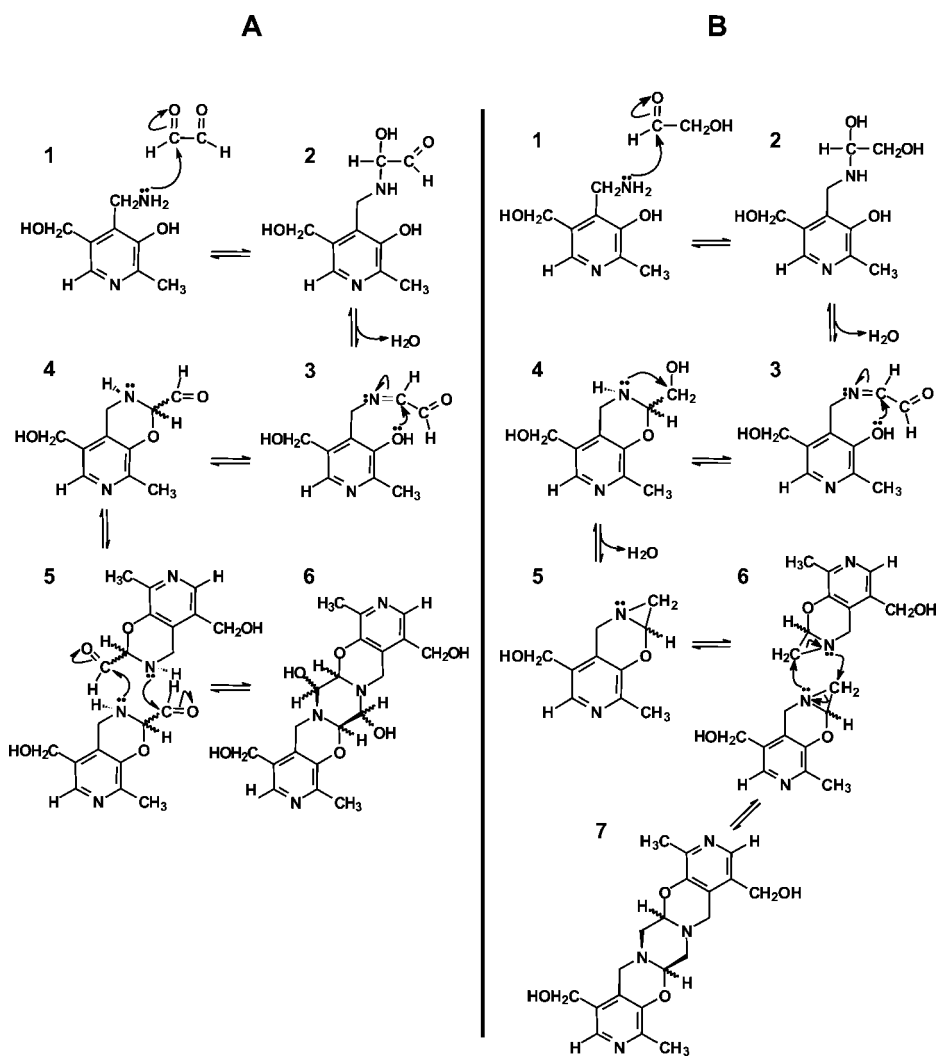


FIG. 6. Proposed mechanisms of adduct formation between PM and GO (A) or PM and GLA (B). Step 1, a nucleophilic attack of the primary amine of PM on a carbonyl group; steps 2–4, the formation of a six-membered hemiaminal ring; steps 5–7, condensation of two molecules to form the final five-ring product. See under “Discussion” for details.

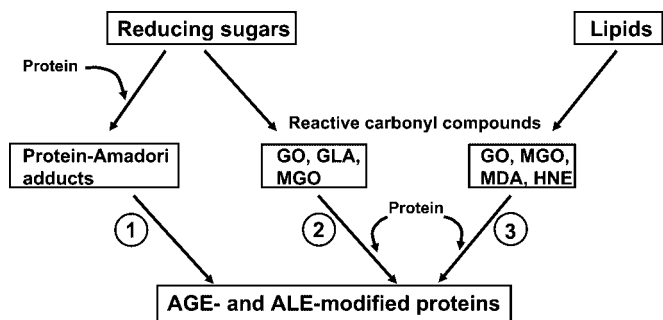


FIG. 7. A model of carbonyl stress-induced protein modifications and mechanism of PM inhibition. The numbers represent the sites of inhibition by PM. In previous work (28–30), we have shown that (step 1) PM is a potent inhibitor of the formation of AGE-modified proteins from protein-Amadori precursors. In the present work, we show that PM also traps reactive low molecular weight carbonyl compounds derived from either (step 2) sugars or (step 3) lipids, inhibiting the AGE and advanced lipoxidation end product modifications of proteins. HNE, hydroxynonenal; MDA, malondialdehyde.

the stabilization of GOPM and GLAPM in the hemiaminal form. Interestingly, when PM and GO were incubated at concentrations of 100 μM , the reaction product consistent with Schiff base or cyclic hemiaminal was detected by ESI-LC-MS,³ as predicted by the reaction mechanism proposed in Fig. 6. Recently, Baynes and co-authors (26, 27) have reported that

PM can alleviate nephropathy and other diabetes-related complications in streptozotocin-diabetic rats. In agreement with our proposed mechanism of action (Fig. 7), PM treatment has resulted in lower levels of CML in the skin collagen of diabetic rats (26, 27). Moreover, the hexanoic acid amide derivative of PM was quantified by mass spectrometry in urine from PM-treated rats, indicating that pyridoxamine traps reactive intermediates of lipid peroxidation *in vivo* (27).

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³ T. O. Metz and J. W. Baynes, unpublished data.

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