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

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Running title: Mechanism of action of AGE inhibitor pyridoxamine

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Reactive carbonyl compounds are formed during autoxidation 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 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 5-ring compound with a central piperazine ring, which was characterized by ESI-LC/MS, 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 Nε-(carboxymethyl)lysine during reaction of GO and GLA with BSA. 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 crosslinks in protein. Examples include the formation of N\(^\epsilon\)-(carboxymethyl)lysine (CML)\(^1\) by reaction of GO or GLA with lysine; the formation of carboxyethyllysine by 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 sugars, can also react with proteins by forming intermediate Amadori compounds 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, MDA and HNE, have been shown to react with protein lysine residues forming ALEs (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 formation of MGO by 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 autooxidation 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 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 formation of high molecular weight protein aggregates, they have been implicated in the development of a number of pathologies via 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 natural intermediate of vitamin B₆ metabolism, prevented the development of nephropathy in rat model of diabetes (Baynes and co-authors, ref. 26, 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, ref. 28-30), and by trapping reactive intermediates formed during lipid peroxidation (31). In the present paper we extend our studies into the mechanism of action of PM by showing that it also inhibits the 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 aminal derivatives, GOPM and GLAPM. Based on this work, we propose that the therapeutic effects of pyridoxamine observed in diabetic animal models (26, 27) are the result of its action in trapping a wide range of carbonyl intermediates in the pathway of protein modification by sugars and lipids.

Experimental Procedures.

Materials. D-Ribose, GO, PM(HCl)₂, DNPH, lanthanum nitrate hexahydrate, yeast RNA, and BSA were purchased from Sigma (St. Louis, MO). GO and Girard’s Reagent T were from Aldrich (Milwaukee, WI); RNase A was from Worthington Biochemical (Freehold, NJ).

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

ELISA detection of CML-BSA. Formation of CML, in reactions between BSA and carbonyl compounds, were 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). In order 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 towards several AGE-modified proteins was identical to that of unpurified antibody, 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 µl of 3µg/ml RNase in 100 mM Na-acetate, pH 5.0 was mixed with 100 µl 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 µl of an ice-cold solution of 0.8% lanthanum nitrate in 18% perchloric acid. Incubation tubes were kept on ice for 5 min to insure complete precipitation of undigested RNA and then centrifuged at 12,000 g for 10 min. An aliquot of the supernatant (20 µl) 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 µl of 10 mM solution of GO) was mixed with 995 µl of 120 mM Na-borate, pH 9.3. An aliquot of this mixture (200 µl) was then added to 800 µl of 100 mM Girard’s reagent T in the same Na-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 µl) of the samples containing 10 mM glycolaldehyde were mixed with 1 ml of 200 µM DNPH in 1 M HCl. After 20 min at room temperature, the amount of reacted carbonyls was determined by measuring 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,
Aquasil C-18 microbore column. The solvent system consisted of 0.1% trifluoroacetic acid (solvent A) and methanol (solvent B), flow rate 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, carrier buffer consisted of 80% acetic acid and 20% methanol, flow rate 0.03 ml/min.

**X-ray crystallography.** Colorless crystals of GOPM (0.28x0.20x0.16 mm) or GLAPM (0.24x0.20x0.08 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 CCD-based diffractometer system (Mo Kα 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 re-collected at the end of the data set to monitor crystal decay. The raw data frames were integrated into reflection intensity files using 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(s)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 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 dimethylsulfoxide 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 
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 (t1/2=0.94 h 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 
icubation, 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 (256 nm and 324 nm) as shown in Fig. 2A. The GOPM precipitate (2 mg) was dissolved in 3 ml of 0.5% TCA, an aliquot of this solution was then diluted 200-fold into Na-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 observed, suggesting 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 spacing of 13C isotope satellites of these ions at 0.5 amu 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). In order to unequivocally establish novel structures of GOPM and GLAPM we have performed solution 1H- and 13C-NMR and X-ray crystallography experiments. The results (Table 1 and Fig. 3 A and B, insets) were consisted 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 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 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 amino groups 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 case of GO, compared to GLA (Fig. 4 A and B), consistent with lower reactivity of PM towards GLA (Fig. 1).

Because PM reacted more rapidly with GO or GLA compared to 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 reaction of proteins with GO and GLA (9). As shown in Fig. 5, PM inhibited 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, compared to 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 compared to the reaction between PM and GO (Fig. 1), these results imply the participation of additional inhibitory mechanism that is different from carbonyl scavenging. Interestingly, Glomb and Monnier have found that the conversion of GO to CML does not include a metal-catalyzed oxidative step, while 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 (9). Transition metal ions, such as Cu$^{2+}$, occur naturally in Na-phosphate buffer that was used in our experiments (42). These ions were catalytically active under our experimental conditions, since chelation with DTPA partially inhibited GLA-induced but not GO-induced CML formation (data not shown), 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 (Fig. 7, step 1), most prominently to CML (13) which is detected at increased concentration in animal and human tissues in diabetes, neurodegenerative diseases, and in aging (45-48). AGE modifications have been implicated as source of structural and functional damage of proteins in disease such as diabetes (49-51). Sugar or protein-sugar intermediates can also degrade, largely through sugar autoxidation (Wolff pathway, 52) or through the degradation of the Schiff base intermediate (Namiki pathway, 53), to produce low molecular weight carbonyl compounds; reactive carbonyls are also produced during lipid peroxidation reactions (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 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 paper, 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, PM also inhibits the conversion of post-Amadori intermediate to CML (28-30). In more recent work, Onorato et al. showed that PM inhibits advanced lipoxidation reactions by trapping lipid-derived intermediates (31). 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, as well as 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, local tissue concentrations of reactive carbonyls and PM, as well as concentration 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 ~100 µM in plasma of PM-treated animals (26, 27), while the concentrations of GO, MGO, MDA, and HNE 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 compared to 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 gradual depletion of these reactive carbonyl compounds from plasma. The trapping reaction by PM would also be favored by 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-MS³, as predicted by the reaction mechanism proposed in Fig. 6. Recently, Baynes and co-authors have reported that PM can alleviate nephropathy and other diabetes-related complications in STZ-diabetic rats. In agreement with our proposed mechanism of action (Fig. 7), PM treatment has resulted in lower levels of CML in 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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References:

Footnotes:

1 Abbreviations: AGE, advanced glycation end product; ALE, advanced lipoxidation end product; BSA, bovine serum albumin; CML, N\textsuperscript{\epsilon}-(carboxymethyl)lysine; DNPH, 2,4-dinitrophenylhydrazine; DTPA, diethylenetriaminepentaacetic acid; GO, glyoxal; GLA, glycolaldehyde; GOPM, glyoxal-pyridoxamine adduct; GLAPM, glycolaldehyde-pyridoxamine adduct; HNE, hydroxynonenal; MDA, malondialdehyde; MGO, methylglyoxal; PM, pyridoxamine; RNase, bovine pancreatic ribonuclease A.

2 Cussimanio, Khalifah, and Hudson, unpublished data.

3 Metz and Baynes, unpublished data
Table 1. $^1$H and $^{13}$C NMR chemical shifts of GOPM

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$^a$ For carbon atom numbering see Fig. 3 C.
Figure legends.

Figure 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 Na-phosphate buffer, pH 7.5 containing 0.02% sodium azide. The loss of carbonyl moieties in the course of reaction was measured spectrophotometrically using either Girard’s reagent T or DNPH as described in Experimental Procedures. Parallel experiments with carbonyl compounds incubated under the same conditions but without PM or lysine were used as references for calculating of relative amount of reactive carbonyl groups. Note the different time scales in panels A and B.

Figure 2. Effect of GO on the absorbance spectrum of PM in solution. (A) PM (15 mM) was incubated with GO (10 mM) in 200 mM Na-phosphate buffer, pH 7.5 at 37°C. 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. (B) Complex collected by centrifugation was washed with water, vacuum dried, and dissolved in 0.5% trichloracetic acid. Solution pH was adjusted to pH7.5 by 200-fold dilution into 200 mM Na-phosphate buffer and absorbance spectrum was recorded. The presence of traces of trichloracetic acid did not affect the absorbance spectrum of pyridoxamine in phosphate buffer.

Figure 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 in Experimental Procedures (panels 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 ¹³C NMR chemical shifts of GOPM see Table 1.
Figure 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 Na-phosphate buffer, pH 7.5 containing 0.02% sodium azide. At the indicated times, aliquots were withdrawn and RNase activity was determined, as described in Experimental Procedures. Each point represents an average of duplicate measurements.

Figure 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 Na-phosphate buffer, pH 7.5 containing 0.02% sodium azide. CML-modified BSA was measured by ELISA as described in 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.

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

Figure 7. A model of “carbonyl stress”-induced protein modifications and mechanism of PM inhibition. Numbers represent the sites of inhibition by PM. In previous work we have shown that (1) PM is a potent inhibitor of 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 (2) sugars or (3) lipids, inhibiting the AGE and ALE modifications of proteins.
Voziyan et al, Fig. 1.
Voziyan et al., Fig. 2.

(A) Absorbance spectra for PM alone, PM+GO, 16 h, GO alone, PM-GO complex.

(B) Absorbance spectra for PM and PM-GO complex.
Voziyan et al. Fig. 3.
Voziyan et al., Fig. 4.
A. Glyoxal

B. Glycolaldehyde
Reducing sugars

Protein-Amadori adducts

Reactive carbonyl compounds

GO, GLA, MGO

GO, MGO, MDA, HNE

AGE- and ALE-modified proteins

Protein