Modification of Proteins In Vitro by Physiological Levels of Glucose: Pyridoxamine Inhibits Conversion of Amadori Intermediate to Advanced Glycation End-products Through Binding of Redox Metal Ions

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1Abbreviations: : AGE, advanced glycation end-product; AG, aminoguanidine; ALE, advanced lipoxidation end-product; BSA, bovine serum albumin; CML, Nε-(carboxymethyl)lysine; DTPA, diethylenetriaminepentaacetic acid; FL, fructosyllysine; GO, glyoxal; GLA, glycolaldehyde; MGO, methylglyoxal; MOLD, methylglyoxal-lysine dimer; PM, pyridoxamine; RNase, bovine pancreatic ribonuclease A.
Hyperglycemic conditions of diabetes accelerate protein modifications by glucose leading to the accumulation of advanced glycation end-products (AGEs). The role of AGEs in the development of serious complications of diabetes and other diseases emphasizes the necessity for understanding the mechanisms of their formation and inhibition. We have investigated the conversion of protein-Amadori intermediate to protein-AGE and the mechanism of its inhibition by pyridoxamine (PM), a potent AGE inhibitor that has been shown to prevent diabetic complications in animal models. During incubation of proteins with physiological diabetic concentration of glucose, PM prevented the degradation of the protein glycation intermediate identified as fructosyllysine (Amadori) by 13C NMR using [2-13C]-enriched glucose. Subsequent removal of glucose and PM led to conversion of protein-Amadori to AGE Nε-carboxymethyllysine (CML). We utilized this inhibition of post-Amadori reactions by PM to isolate protein-Amadori intermediate and to study the inhibitory effect of PM on its degradation to protein-CML. We first tested the hypothesis that PM blocks Amadori-to-CML conversion by interfering with the catalytic role of redox metal ions that are required for this glycoxidative reaction. Support for this hypothesis was obtained by examining structural analogs of PM in which its known bidentate metal ion binding sites were modified and by determining the effect of endogenous metal ions on PM inhibition. We also tested the alternative hypothesis that the inhibitory mechanism involves formation of covalent adducts between PM and protein-Amadori. However, our 13C NMR studies demonstrated that PM does not react with the Amadori. Because the mechanism of interference with redox metal catalysis in post-Amadori AGE formation is operative under the conditions closely mimicking diabetic state, it may contribute significantly to PM efficacy in preventing diabetic complications in vivo. Inhibition of protein-Amadori degradation by PM also provides a simple procedure for the isolation of protein-Amadori intermediate, prepared at physiological levels of glucose for relevancy, to study both the biological effects and the chemistry of post-Amadori pathways of AGE formation.
Chemical modifications of circulating, cellular and matrix proteins by glucose are thought to be a major factor in pathogenesis of diabetes, atherosclerosis, and neurodegenerative diseases, as well as in the process of ageing (1-4). These modifications derive, in part, from glycation reactions, i.e. the reversible condensation of the aldehyde group of glucose with a protein amino group, forming a Schiff base followed by an essentially irreversible rearrangement to an Amadori intermediate (Fig.1). This intermediate undergoes cycles of condensations with additional amines, dehydration, and oxidative fragmentations to yield heterogeneous chemical compounds collectively referred to as advanced glycation end-products (AGEs)\(^1\). Formation of chemically stable AGEs in this Maillard reaction can permanently alter protein structure and function (for review, see ref. 5-7). They range from CML (the pathway is shown in Fig.1) to more complex structures such as pentosidine (a lysine-arginine crosslink) and pyralline (8-10). More AGEs have been identified in the recent years, among them inter-lysine cross-links crosslines (11) and vesperlysines (12). Some Maillard products are antigenic, and it has been shown that anti-AGE sera predominantly recognize CML attached to protein (13). A number of pathways of AGE formation include oxidative steps catalyzed by redox-active transition metal ions, as exemplified by the conversion of protein-Amadori intermediate to CML (Fig.1).

CML is a major AGE modification associated with different human pathologic states. It can be derived either directly from the glycated protein (Fig. 1), or from protein modification by small reactive carbonyl and dicarbonyl compounds that are either metabolites or autoxidation products of glucose, ascorbate, Schiff bases, Amadori intermediates or polyunsaturated lipids (14-16). CML-modified proteins have been found in plasma, renal tissues, retinas, and collagen of diabetic patients (17-22). CML is the predominant AGE in intracellular neurofibrillary deposits in patients with Alzheimer disease (23) and in macrophage-derived foam cells in human atherosclerotic plaques (24). Its concentration in human tissues increases significantly with age (25). CML-modified protein has also been reported to be a ligand for RAGE, an AGE receptor that mediates cellular pro-inflammatory responses (26).
We earlier reported a novel way to isolate ribose-Amadori intermediate using high concentration of ribose (27, 28). This ribose-derived intermediate, although not physiologically relevant, provided an experimental strategy to elucidate mechanisms of AGE formation and to search for inhibitors that block it. This led to discovery that pyridoxamine, a natural intermediate of vitamin B6 metabolism, is an efficient in vitro inhibitor of conversion of the Amadori intermediate to AGEs, particularly CML (28-30). Under in vivo conditions, pyridoxamine inhibited the accumulation of CML modifications in skin collagen and in retina, and prevented the development of early renal disease and retinopathy in the streptozotocin rat and db/db mouse models of diabetes (31-33). In non-diabetic Zucker obese (fa/fa) rats, the protection of PM against renal and vascular pathology was also accompanied by the inhibition of CML formation (34).

Pyridoxamine can form moderately stable complexes with a number of transition metal ions (35, 36). Recently, Baynes et al. reported that PM and other AGE inhibitors can inhibit copper-dependent oxidation of ascorbic acid and proposed that metal chelation may play a major role in the inhibition of glycoxidation reactions (37). However, it remains to be demonstrated whether or not the redox metal ion requirement and its inhibition by ligand binding are identical in these two reactions. It also has not been established whether other mechanisms of inhibition, such as covalent adduct formation of PM with the Amadori, could significantly contribute to AGE inhibition. In the present work, we demonstrate for the first time that PM can inhibit degradation of a glucose-derived protein-Amadori intermediate and have utilized this inhibition to isolate the glucose-derived Amadori at physiological glucose concentrations. The isolation of this intermediate has enabled the study of the mechanism of PM inhibition of post-Amadori pathway leading to CML formation, including demonstration by 13C NMR that no adducts are formed between PM and Amadori. Our overall results support the hypothesis that PM inhibits post-Amadori glycoxidation reactions by binding to required redox metal ions and interfering with their catalytic role in these reactions. Since this PM inhibition occurred in vitro under physiological diabetic concentrations of glucose, we propose that this PM mechanism contributes to inhibition of AGE formation by PM observed in diabetic animal models (31-33).
Experimental procedures

Materials. D-ribose, pyridoxamine dihydrochloride, 1-deoxy-1-morpholino-D-fructose, 3-hydroxypyridine, 4-aminomethylpyridine, and bovine serum albumin were purchased from Sigma-Aldrich. 5-Nitro-2-hydroxybenzylamine was synthesized by ChemSyn Laboratories (Lenexa, KS), and its structure and purity were confirmed by proton NMR and mass spectrometry. D-Glucose was purchased from Gibco BRL. RNase A was obtained from Worthington Biochemical Co. D-[2-13C]Glucose (99 atom-% 13C) was purchased from Omicron Biochemicals.

Preparation of glucose-derived and ribose-derived protein-Amadori. For the preparation of protein-Amadori intermediate under physiologically relevant conditions, BSA or RNase (20 mg/ml) was incubated with either 5 mM or 30 mM glucose and 20 mM pyridoxamine in 200 mM Na-phosphate pH 7.5 buffer, containing 0.02% sodium azide, at 37 °C for 80 days. In some cases, protein-Amadori intermediate was prepared using 100 mM glucose and the conditions described above. Accumulation of BSA-Amadori was followed using the nitroblue tetrazolium method described below. When the concentration of Amadori intermediate reached a maximum constant value, glucose and pyridoxamine were removed by dialysis against 100 volumes of the same Na-phosphate buffer with 6 buffer changes over a 24 h period. The buffer was equilibrated at 4°C and dialysis was carried out at this temperature. The complete removal of PM was confirmed by the absence of PM fluorescence (Ex=324 nm, Em=393 nm).

Ribose-Amadori modified BSA was prepared as described earlier (29). Briefly, protein was incubated in the presence of 500 mM ribose in 200 mM Na-phosphate pH 7.5 buffer at 37°C for 24 h. Sodium azide (0.02%) was added to the incubations to prevent bacterial growth. At the end of the incubation, free and Schiff-base ribose was removed by dialysis at 4°C.

Detection of protein-CML using ELISA. Modifications of protein lysine residues to CML were measured by ELISA using polyclonal anti-AGE antibody R618 as described earlier (28-30). Protein-CML was the antigenic epitope in our ELISA measurements (38).

Quantitative measurements of protein-Amadori. The formation of protein-Amadori was analyzed using a Sigma Fructosamine kit. The assay is based on the
ability of Amadori intermediates to reduce nitroblue tetrazolium leading to the formation of formazan that can be monitored spectrophotometrically at 550 nm. We modified the Sigma protocol by filtering the working solution prior to use in the assay. This modification significantly reduced the background signal and improved the reproducibility of the measurements. Aliquots (40 µl) of protein-Amadori (5 mg/ml) were mixed with 0.7 ml of Sigma Fructosamine assay working solution and incubated at 37°C for 10 min. The absorbance of each sample was measured and incubation at 37°C was resumed. After 10 min, a second absorbance measurement was taken. Amadori intermediate was quantified by comparing the differences in absorbance between two measurements for each sample with those of a 1-deoxy-1-morpholino-D-fructose standard. The protein concentration was determined using a second derivative analysis of the protein absorbance spectrum to minimize contributions from glycation-related spectral components (39).

Determination of pKₐ. The apparent pKₐ values for 5-nitro-2-hydroxybenzylamine were determined by spectrophotometric titration at 22°C using an Agilent 8453 UV-visible spectrophotometer. The value of pKₐ₁ (phenolic group), obtained by least squares curve fitting of absorbance titration data, was 6.12±0.01. These data indicate that at physiological pH, the phenolic group of 5-nitro-2-hydroxybenzylamine is present mostly in the deprotonated state, a condition favoring complex formation between PM and metal ions (35).

Solution ¹³C NMR. Amadori-RNase was prepared using D-[2-¹³C]glucose under experimental conditions described earlier. After the removal of free and Schiff-base [2-¹³C]glucose and PM by dialysis at 4 ºC, the buffer was replaced by 50 mM Na-phosphate buffer prepared with D₂O, pD 7.5, using a Centricon-3 centrifugation concentrator. NMR experiments were performed at 21 ºC on a Varian UnityPlus 600 MHz spectrometer. FID transients (20,000) were collected over spectral widths of 225 ppm (Fig. 6A, B) or 300 ppm (Fig. 6C-E). Larger spectral widths were used in Fig. 6C-E to determine whether the adducts between PM and acyclic [2-¹³C]-labeled Amadori intermediate may have formed upon incubation with 20 mM PM for 8 days at 37°C. No signals were detected at δ > 180 ppm in Fig. 6D, E compared to Fig. 6C. In all NMR experiments, the recycle time was 4.7 s, which was judged sufficient to ensure a return
to magnetization equilibrium between pulses. All NMR experiments were performed under waltz-16 decoupling conditions. The FIDs were zero-filled (final digital resolution 0.2 Hz/ data point), apodized with an exponential window function (line broadening = 15 Hz) and Fourier transformed to give the spectra shown in Fig. 6. Reprocessing the FIDs with a Gaussian-to-Lorentzian window function showed that the apparent singlets at ~ 97.7 ppm and ~ 95.5 ppm contained significant fine structure. At least 3 resonances were observed between 97.6 and 97.8 ppm, and at least 6 signals were observed between 95.3 and 95.6 ppm. Presumably this multiplicity is due to the small effect of protein site environments on Amadori chemical shifts.

Absorbance measurements. Single-wavelength absorbance measurements, absorbance spectra, and second derivative analyses were performed on a Hewlett Packard 8452A diode array spectrophotometer equipped with a Peltier temperature control unit.

Results

Reversible inhibition of CML formation by pyridoxamine. We initially described a method to prepare Amadori intermediate in the AGE pathways using high concentrations of ribose (27). This ribose-derived intermediate provided an experimental strategy to search for inhibitors that block AGE formation and led to the discovery of PM inhibition of post-Amadori reactions (28-30). In the present work, we have used PM to trap and identify glucose-derived protein-Amadori intermediate. As shown in Fig. 2A, PM completely inhibited formation of immunoreactive BSA-CML during the initial 35 days of incubation of BSA with 200 mM glucose. When PM and glucose were removed, the formation of BSA-CML from the putative intermediate was observed (Fig. 2A, triangles). After 35 days of incubation with glucose, significant accumulation of fructosyllysine (Amadori) intermediate was detected only in the sample containing PM (Fig. 2B, bar graph). The structural identity of protein Amadori intermediate was confirmed using $^{13}$C NMR as described vide infra in Fig. 6. Upon the removal of PM and glucose, BSA-Amadori degraded (Fig. 2B, inset) and converted to BSA-CML (Fig. 2A, triangles). It is noteworthy that CML is the major but not the only AGE product of degradation of Amadori intermediate. This complexity of post-Amadori
reactions may account for the apparent differences in kinetics of Amadori degradation and formation of Amadori-derived CML (Fig. 2).

The data in Fig. 2 present two important findings. 1) PM inhibits the formation of glucose-derived protein-CML by inhibiting degradation of protein-bound Amadori intermediate; 2) this PM property provides a strategy to specifically follow the formation and degradation of protein-Amadori intermediate.

We utilized the isolation of the glucose Amadori by PM to determine the kinetics of formation and degradation of BSA-Amadori under physiologically relevant concentrations of glucose found in the plasma of diabetic patients (>5 mM and up to 30 mM). As shown in Fig. 3A, incubation of BSA with 30 mM glucose at 37°C in the presence of PM resulted in significant increase in the rate of accumulation of BSA-Amadori intermediate compared to that at 5 mM glucose. The kinetics was characterized by t_{1/2} ~ 9 days, consistent with the notion that relatively short-lived proteins (t_{1/2} ~ days) can be modified by glucose in vivo (40, 41). The degree of modification of protein lysine residues in the albumin-Amadori prepared with diabetic concentration of glucose was about 9% (Fig. 3A), or ~5 lysine residues per molecule out of total 58 Lys. This result is in good agreement with preferential modification of 4 lysine residues found in albumin purified from diabetic patients (42).

The removal of unreacted glucose and PM by extensive dialysis at 4°C followed by incubation again at 37°C led to the disappearance of ~90% of protein-Amadori intermediate (FL) after 80 days (Fig. 3A). This process was accompanied by formation of BSA-CML. Different concentrations of freshly added PM inhibited this CML formation (Fig. 3B). This inhibition of post-Amadori protein-CML formation is consistent with the results of our earlier experiments using ribose-derived protein-Amadori intermediate (28, 30). At 1 mM PM (a 2.5-fold molar excess of PM over Amadori moiety), BSA-CML formation was completely inhibited (Fig. 3B). Even at the substoichiometric concentration of PM (0.1 mM), a significant inhibition was still evident (Fig. 3B). Importantly, this PM concentration is similar to a steady-state pyridoxamine level in plasma of PM-treated animals (31). The data in Fig. 3 establish that PM can inhibit CML formation under physiologically relevant solution conditions. Since PM prevented the degradation of protein-Amadori intermediate, the target of PM action must be post-
Amadori steps in the CML pathway that lead to irreversible oxidation (Fig. 1). We set out to establish which of these steps is targeted by PM.

**Role of metal ions in inhibition of the post-Amadori CML pathway by PM.** Pyridoxamine is known to form complexes with transition metal ions such as Fe$^{3+}$ and Cu$^{2+}$ and to also inhibit copper-dependent oxidation of ascorbic acid (35-37). We checked if PM interferes with the oxidative steps in the CML pathway through binding catalytic metal ions (Fig. 1). The redox metal ion catalysts required for CML formation occur naturally in the Na-phosphate buffer used in our experiments (43). Electron pairs on oxygen and nitrogen of the aminomethyl and phenol moieties of PM participate in bidentate coordination with the metal ion (Fig. 4). If pyridoxamine-metal complex formation is critical for the inhibition of the conversion of protein-Amadori intermediate to protein-CML, then modification of these PM functional groups would abolish inhibition.

We utilized ribose-derived BSA-Amadori, a model that exhibits a more rapid rate of Amadori-to-CML conversion due to the higher abundance of reactive acyclic form of ribose-Amadori compared to glucose-Amadori (28, 30). We tested three PM structural analogs to determine their inhibitory potential. 3-Hydroxypyridine and 4-aminomethylpyridine lack one of the two moieties critical for bidentate complex formation with the metal ion (Fig. 4A and B). These PM analogs showed no inhibition, even at very high concentrations (Fig. 4A and B). On the other hand, 5-nitro-2-hydroxybenzylamine, a compound that has both 3-hydroxy and 4-aminomethyl groups, showed strong inhibition similar to that reported previously for PM (28-30), even though its other aromatic ring differs from that of PM (Fig. 4C). These results suggest that PM inhibits the post-Amadori CML pathway through complex formation with catalytic metal ions. This conclusion is further supported by the effects of addition of CuCl$_2$ to incubations of protein-Amadori and PM (Fig. 5). In these experiments, metal ion contaminants naturally present in phosphate buffer were not removed prior to incubation. As shown in Fig. 5, the increase in metal concentration significantly diminished the inhibitory effect of PM on protein-CML formation in a concentration-dependent fashion, consistent with the importance of metal ion complexation in the PM inhibition of post-Amadori reactions.
Lack of reactivity of PM with protein-Amadori intermediate. We demonstrated recently that PM forms relatively stable five-ring adduct structures with carbonyl groups of low molecular weight compounds such as glycolaldehyde (38). Since the protein-bound Amadori moiety, a ketoamine, contains a carbonyl group (Fig. 1), we checked whether the amino group of PM may form a stable adduct with an acyclic Amadori carbonyl. In such AGE inhibition mechanism, originally hypothesized for aminoguanidine (44) but later refuted (45), the adduct formation would inhibit the conversion of the reactive form of the protein-Amadori intermediate to protein-CML. The formation of a stable adduct with PM, whether covalent or reversible, would necessarily shift the tautomeric equilibrium towards the acyclic form of the Amadori intermediate, thus depleting the cyclic forms (Fig. 1).

This possibility was investigated using $^{13}$C NMR and RNase as the model protein, since resonance assignments for the Amadori moieties have been established previously (46). Like with BSA, RNase-Amadori intermediate was forming in the presence of 30 mM glucose and PM, $t_{1/2}$~22 d (data not shown). For the $^{13}$C NMR experiments the RNase-Amadori intermediate was prepared with 30 mM D-[2-$^{13}$C]glucose and also with 100 mM D-[2-$^{13}$C]glucose to achieve a higher degree of lysine modification and improve the signal-to-noise ratio. The $^{13}$C NMR spectra of RNase-[2-$^{13}$C]-Amadori (Fig. 6A, C) were in good agreement with previously reported resonance assignments for RNase-[2-$^{13}$C]-Amadori in the anomeric carbon region (46). Characteristic resonances (Fig. 6C) arising from the prevailing cyclic $\beta$-pyranose forms (at 95.5, 96.1 and 97.7 ppm) of Amadori intermediates on the $\varepsilon$-amino group of lysine-41, on $\varepsilon$-amino groups of remaining lysines, and on the N-terminal $\alpha$-amino group were observed. Corresponding signals arising from the minor $\alpha$- and $\beta$-furanose forms of Amadori intermediates on similar amino groups were also detected (101.9, 102.8 (broad) and 104.1 ppm for $\alpha$; 98.9 ($\varepsilon$-amino group) and 101.4 ppm ($\varepsilon$-amino group of Lys-41) for $\beta$). The signal at 95.8 ppm previously assigned (46) to the $\alpha$-pyranose Amadori on $\varepsilon$-amino groups may overlap with that at 95.5 ppm assigned to $\beta$-pyranose Amadori on similar groups. Neither the unmodified RNase nor PM contributed to the anomeric region of $^{13}$C NMR spectrum (data not shown). In RNase-Amadori prepared under physiological diabetic conditions (30 mM glucose), the observed ratio of modified
ε-amino to α-amino groups of ~1 appears significantly smaller than reported in RNase-Amadori prepared with 250 mM glucose (46).

In the presence of either 3 mM or 20 mM PM, no significant change in the intensities of resonances corresponding to cyclic Amadori forms was observed even after prolonged incubation (8 days). Importantly, in the experiments shown in Fig. 6B, 6D and 6E, the same PM-to-Amadori molar ratio was used at which PM inhibited conversion of protein-Amadori to protein-CML (Fig. 3B, data for 0.1 mM and 1mM PM). If the PM inhibitory effect was due to Amadori-PM adduct formation, it would cause a shift of the C2 Amadori signals out of the anomeric region of the $^{13}$C NMR spectrum, thus decreasing their intensities. The observed lack of intensity changes and the absence of additional $^{13}$C NMR signals suggests that Amadori-PM adducts, if formed, are produced in minor amounts. The content of these adducts would be substantially less than 5% of total protein-Amadori as determined by the sensitivity of $^{13}$C NMR experiments (Fig. 6). Therefore, the formation of such adducts cannot significantly contribute to PM inhibition of Amadori-to-CML conversion.

Discussion

Glucose is a ubiquitous natural metabolite present in all human tissues. In its aldehyde form it reacts non-enzymatically with nucleophilic groups on proteins, preferentially with ε-amino groups of lysines and N-terminal α-amino groups. An early product of this reaction, protein-Amadori, is converted to a variety of stable advanced glycation end-products or AGEs. Although this reaction is very slow due to the low abundance of the reactive aldehyde form of glucose at equilibrium (0.003%), the gradual accumulation of stable AGEs alters protein conformation and function. This protein modification by glucose is significantly accelerated under hyperglycemic conditions of diabetes and is considered one of the major factors in the development of diabetic complications.

Our previous work showed that pyridoxamine, unlike other AGE inhibitors such as aminoguanidine, blocks the conversion of ribose-derived Amadori intermediate to protein-CML (28-30). In the present work, we identified the nature of the protein-bound intermediate formed from glucose in the presence of PM and investigated the
mechanism of inhibition of post-Amadori protein-CML formation by PM. In the presence of PM and diabetic levels of glucose (30 mM), the CML precursor was identified as protein-Amadori intermediate using $^{13}$C NMR (Fig. 6). When glucose and PM were removed, the protein-bound Amadori moiety underwent oxidative degradation and formed CML, indicating that PM acted on one of the post-Amadori steps of AGE formation (Fig. 3). The absence of reversible or irreversible adducts between protein-Amadori intermediate and PM was deduced also using $^{13}$C NMR (Fig. 6). The slow physiologically relevant kinetics of degradation, however, presented an experimental challenge. Therefore, once the inhibitory effects of PM were established under the physiological solution conditions, we utilized the experimental system based on ribose-derived Amadori that we introduced earlier, which allows for significantly higher rates of post-Amadori reactions (28-30).

We utilized the ribose-derived Amadori to demonstrate that pyridoxamine inhibits the conversion of protein-Amadori intermediate to protein-CML primarily by binding with catalytic metal ions and blocking the oxidative steps in the pathway (Fig. 1). This conclusion is based on several experimental observations: pyridoxamine did not react with the carbonyl group of protein-Amadori (Fig. 4); the removal of either one of the PM pyridine ring substituents that participate in bidentate metal ion coordination caused a complete loss of PM inhibition of post-Amadori CML formation (Fig. 5); the inhibitory effect of PM can be diminished by increasing concentration of metal ions (Fig. 6). Importantly, this inhibition occurred at PM concentrations similar to those reported in preclinical studies of PM efficacy (31) and with the level of protein modification similar to that found in diabetic patients (Fig. 3).

The inhibition of degradation of important protein glycation intermediate by PM, which can be reversed by removal of PM, provides an experimental tool to study the formation and the degradation of protein-Amadori intermediate. Blocking of the post-Amadori reactions enables the rates and levels of formation of protein-Amadori intermediate to be easily measurable, even at low physiologically relevant levels of glucose. Upon the removal of PM and glucose, post-Amadori AGE pathways could be investigated without the interference from glucose or Schiff-base oxidation products that may contribute to AGE formation (47, 48). Often, high concentrations of glucose (up to
1 M) are used in protein glycation reactions in vitro. These reaction conditions lead to high levels of protein modifications by glucose and enhance additional modifications of protein by reactive products of glucose degradation. As an illustration, the amount of albumin-Amadori formed at 200 mM glucose was about 7-fold greater than that formed at more physiological 30 mM glucose (Fig. 2 and 3). The level of Amadori and AGE moieties formed at high non-physiological concentration of glucose is likely to have different impact on structure, function and cellular effects of the proteins compared to more subtle modifications occurred at physiologically relevant glucose concentrations. In our experiments, the formation of albumin-Amadori at diabetic glucose concentration (30 mM) was consistent with elevated levels of glucose-modified proteins in diabetes (40, 41), while the degree of modification of lysine residues was in good agreement with that found in albumin purified from diabetic patients (42). Thus the inhibition by PM provides a simple method for isolation of protein-Amadori intermediates, prepared at physiological levels of glucose, for studies of its biological effects in cell culture or in vitro experiments.

Under in vivo conditions, Cu$^{2+}$ and Fe$^{3+}$ are the most important metal ion catalysts of oxidative reactions. In blood plasma and in cellular cytoplasm virtually all of these ions are sequestered in specific metal transporters and other metalloproteins (49, 50). However, copper metalloproteins such as ceruloplasmin and Zn,Cu-superoxide dismutase and the iron metalloproteins such as hemoglobin and myoglobin have been shown to undergo significant conformational change and even fragmentation during glycation reactions, causing the release of bound metal (51-53). Pyridoxamine can form moderately stable complexes with a number of transition metal ions but has a preference for Cu$^{2+}$ and Fe$^{3+}$ (36, 54). By forming such complexes, PM can reduce catalytic activity of metal ions released from damaged proteins.

Our results demonstrate that metal binding appears to be critical for PM inhibition of post-Amadori AGE formation. However, the observation that aminoguanidine also binds catalytic metal ions (37) but does not inhibit post-Amadori AGE formation (28) indicates that there are other factors required for efficient inhibition of post-Amadori reactions by PM. For example, it has been suggested that metal ions could also be bound by CML clusters on glycated proteins or by native proteins, such as serum
albumin. Such bound metal ions may retain a significant redox activity for catalysis of oxidative reactions (55-58). Uniquely, PM may form ternary complexes with such protein-bound metal ions, making them significantly less active as oxidation catalysts.

Previously, we and others have demonstrated that PM can prevent protein modifications by chemically trapping free low molecular weight reactive carbonyl products of glucose and lipid degradation (38, 59-61, Fig. 7). In the present study, we determined that PM inhibition of post-Amadori CML formation (28-30) is due primarily to its interference with metal ion catalysis (Fig. 7). It is noteworthy that metal ion complexation may also contribute to PM inhibition of AGEs derived from free low molecular weight carbonyl compounds (Fig. 7). Some of these reactions, such as the formation of CML from glycolaldehyde, require oxidative steps and can be catalyzed by redox metal ions (14). In this case, complexation of metal ions could be complementary to the PM carbonyl trapping mechanism as suggested by our previous work (38).

It is clear that the effective inhibition of protein AGE modifications has an important therapeutic potential. In recent reports, PM alleviated renal pathology in the streptozotocin rat and spontaneous db/db mouse models of diabetes (31-33), and it showed successful progress through safety and efficacy clinical trials for diabetic nephropathy (62). It is possible that different inhibitory mechanisms such as scavenging of toxic carbonyl metabolites from carbohydrates (38, 59) and lipids (60, 61), and interfering with metal ion catalysis (37 and this study) may operate in vivo. Supporting this notion, PM treatment of diabetic animals has resulted in lower levels of protein-CML, as well as MOLD, a dicarbonyl-derived protein crosslink (31, 32, 59). The relative importance of these mechanisms is difficult to estimate because pyridoxamine action in vivo will be influenced by factors such as local tissue and intracellular concentrations of redox metal ions, reactive carbonyls, and PM itself. It may also be affected by competition from endogenous carbonyl and metal scavengers. However, the greater efficacy of PM, compared to carbonyl scavengers, in diabetic animal studies (31) suggests that the inhibition of degradation of protein-Amadori via interference with redox metal ion catalysis may play a more important role in vivo.

The pathogenicity of protein-Amadori intermediate itself remains an open question. Damaging effects of early protein glycation intermediates in diabetic animals
and cell culture systems have been reported (63). However, the transient nature of such intermediates makes it difficult to attribute these effects to specific chemical structures. The observed effects may reflect the conversion of these early intermediates to the chemically stable AGEs or may be mediated by the byproducts of this conversion, such as superoxide. Interestingly, the level of the circulating protein-Amadori intermediates remained unchanged in diabetic animals treated with PM (31), even though their conversion to AGEs was blocked. These data suggest the existence of mechanisms that confer a rapid catabolism of protein-Amadori intermediate in vivo. Regardless of whether this intermediate is pathogenic itself or simply a precursor to pathogenic AGEs, the rate of its accumulation at diabetic glucose concentration suggests that proteins with relatively short lifetimes (days) may undergo modifications in diabetes (Fig. 3).

The combination of different inhibitory mechanisms may place PM at an advantage over the compounds functioning primarily as either scavengers of metal ions or carbonyl trapping agents. For instance, PM reactivity towards dicarbonyl compounds is significantly lower than that of aminoguanidine (P. A. Voziyan and B. G. Hudson, unpublished data), but PM exhibits greater efficacy in animal models (31). PM also exhibits relatively weak metal binding, with a stability constant for Cu^{2+} several orders of magnitude lower than that of strong chelators such as EDTA or DTPA (50). Hence, PM is a potent inhibitor of formation of protein-AGEs that is less likely to interfere with metabolic pathways that are not the intended targets.

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Figure legends

Figure 1. Pathway of formation of glucose-derived protein-Amadori and protein-CML (from ref. 5, 47, and 64). Reversible condensation of the aldehyde group of glucose with a protein amino group results in formation of a Schiff base followed by essentially irreversible rearrangement to an Amadori intermediate. The Amadori intermediate enolizes to the 2,3-enediol in a reaction catalyzed by phosphate anions (65). The 2,3-enediol undergoes spontaneous autoxidation involving the formation of superoxide, catalyzed by transition metal ions; hydrogen peroxide, formed by superoxide dismutation, regenerates the catalytic metal oxidation state. The putative dicarbonyl product of 2,3-enediol autoxidation undergoes further oxidative degradation, producing Nε-carboxymethyllysine and D-erythronic acid.

Figure 2. Reversible inhibition of the formation of glucose-derived BSA-CML by pyridoxamine. (A) BSA (5 mg/ml) was incubated with 200 mM glucose and 20 mM pyridoxamine in 200 mM Na-phosphate buffer containing 0.02% sodium azide, pH 7.5, at 37°C for 35 days. Glucose and pyridoxamine were removed by dialysis at 4°C; the complete removal of PM was confirmed by the absence of a fluorescence signal at Ex=324 nm, Em=393 nm. After dialysis, the protein solution was again incubated at 37°C in the same buffer for up to 120 days (triangles). An aliquot from this sample was incubated continuously without the removal of PM or glucose (squares). Control sample did not contain PM (circles). Modifications of protein lysine residues to CML were measured by ELISA as described under Experimental procedures. Each data point represents an average of two measurements. (B) After 35 days of incubation, the content of fructosyllysine in the samples was determined using the nitroblue tetrazolium method described under Experimental procedures. Inset: after the complete removal of glucose and PM, the protein solution was again incubated at 37°C in the same buffer for up to 120 days followed by the determination of fructosyllysine. Each data point represents an average of two measurements.
Figure 3. Kinetics of formation and degradation of BSA-Amadori at physiological levels of glucose. (A) BSA (20 mg/ml) was incubated with either 5 mM (open circles) or 30 mM glucose (filled circles) and 20 mM pyridoxamine in 200 mM Na-phosphate buffer containing 0.02% sodium azide, pH 7.5, at 37°C. Accumulation of BSA-Amadori (fructosyllysine, FL) was followed using the nitroblue tetrazolium method described under Experimental procedures with the baseline readings (BSA alone incubated under the same conditions) subtracted. For the incubations with 30 mM glucose: when the concentration of Amadori reached a maximum constant value, glucose and pyridoxamine were removed by dialysis at 4°C; the complete removal of PM was confirmed by the absence of a fluorescence signal at Ex=324 nm, Em=393 nm. After dialysis, the protein solution was again incubated at 37°C in the same buffer and degradation of Amadori-BSA was followed for up to 160 days. (B) BSA-Amadori was prepared with 30 mM glucose as described above. After dialysis, BSA-Amadori (5 mg/ml) was again incubated at 37°C in the same buffer either without PM (circles) or with different concentrations of freshly added PM: 0.1 mM (diamonds), 1 mM (squares) or 15 mM (triangles). Inverted triangles represent the data from incubations of unmodified BSA. Modifications of protein lysine residues to CML were measured by ELISA as described under Experimental procedures. Each data point represents an average of two measurements.

Figure 4. Inhibition of post-Amadori CML formation by structural analogs of PM. Ribose-derived BSA-Amadori intermediate was prepared as described under Experimental procedures. After the removal of sugar by extensive dialysis at 4°C, incubation at 37°C was resumed in the presence of the indicated concentrations of 3-hydroxypyridine (A), 4-aminomethylpyridine (B), and 5-nitro-2-hydroxybenzylamine (C). CML was measured using ELISA; each experimental point represents an average of two measurements. Note the differences in the concentration range used in (A) and (B) compared to that in (C).
Figure 5. Inhibition of post-Amadori CML formation by pyridoxamine at different metal concentrations. For the preparation of protein-Amadori intermediate, BSA (10 mg/ml) was incubated with 0.5 M ribose in 200 mM Na-phosphate buffer containing 0.02% sodium azide, pH 7.5, at 37°C for 24 h. After the removal of sugar by an extensive dialysis at 4°C, incubation at 37°C was resumed in the presence of the indicated concentrations of PM in buffer alone (circles), buffer containing 10 µM CuCl₂ (triangles) or buffer containing 100 µM CuCl₂ (diamonds). After 48 h, BSA-CML was measured by ELISA; each symbol represents an average of two measurements.

Figure 6. ¹³C NMR spectra of RNase-[2-¹³C]-Amadori with and without PM. Amadori-RNase was prepared using either 30 mM D-[2-¹³C]glucose (A and B) or 100 mM D-[2-¹³C]glucose (C, D and E) and experimental conditions described earlier. After removal of the free [2-¹³C]glucose and PM by dialysis, the buffer was replaced by 50 mM Na-phosphate buffer (pD 7.5), prepared with D₂O. NMR experiments were performed on a Varian UnityPlus 600 MHz NMR spectrometer at 21°C, collecting 20,000 transients per spectrum (see Experimental Procedures). (A) and (C) Spectra of RNase-[2-¹³C]-Amadori without PM using two samples at 4.1 mM (A; Sample 1) and 3.8 mM (C; Sample 2) protein concentration. (B) Spectrum of Sample 1 after the addition of 3 mM PM and incubation for 24 h (final protein concentration = 4.0 mM). (D) and (E) Spectra of Sample 2 after incubation with 20 mM PM for 1 day (D) or 8 days (E). RNase concentration was 3.1 mM in D and E. Abbreviations pyr and fur, respectively, designate the pyranose and the furanose forms of the protein-Amadori intermediate.

Figure 7. A model for inhibition of AGE/ALE formation by PM. The numbers represent steps in AGE pathways inhibited by PM. Step 1. PM prevents conversion of protein-Amadori to protein-CML via complexation of catalytic metal ions (present work). Step 2. PM inhibits protein modifications by scavenging free carbonyl products of glucose and lipid degradation (38, 59-61). Step 3. PM may also inhibit metal-catalyzed oxidative steps in protein modifications by low molecular weight carbonyl compounds (ref. 38 and present work).
Voziyan et al., Fig. 3

**A**

Incubation time (d) vs. FL (mol/mol Lys)

- 30 mM Glucose and 20 mM PM removed

**B**

Incubation time (d) vs. BSA-CML (ELISA readings at 410 nm)

- 30 mM Glucose and 20 mM PM removed
Voziyan et al., Fig. 4

A. ELISA Reading (410 nm) vs. Time (days)
- none
- 3 mM 3-HP
- 15 mM 3-HP
- 50 mM 3-HP

B. ELISA Reading (410 nm) vs. Time (days)
- none
- 3 mM 4-AMP
- 15 mM 4-AMP
- 50 mM 4-AMP

C. ELISA Reading (410 nm) vs. Time (days)
- 3 mM NHBA
- 1 mM NHBA
- 0.5 mM NHBA
- 0.1 mM NHBA
- none

Pyridoxamine metal complex (from ref. 35 and 36)
Reducing sugars
Protein-Amadori adducts
Protein

Reactive carbonyl compounds

Lipids

Protein-Amadori adducts

PM-carbonyl adducts

AGE/ALE-modified proteins

Voziyan et al., Fig. 7
Modification of proteins In vitro by physiological levels of glucose: Pyridoxamine inhibits conversion of amadori intermediate to advanced glycation end-products through binding of redox metal ions

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