Published in final edited form as:

Biochem Biophys Res Commun. 2011 August 5; 411(3): 574–579. doi:10.1016/j.bbrc.2011.06.188.

Pyridoxamine Protects Protein Backbone from Oxidative Fragmentation

Sergei Chetyrkin[#], Missy Mathis[§], W. Hayes McDonald[¶], Xavier Shackelford, Billy Hudson^{#,¶,‡}, and Paul Voziyan^{#,*}

[#]Department of Medicine, Vanderbilt University Medical Center, Nashville, TN 37232

[¶]Department of Biochemistry, Vanderbilt University Medical Center, Nashville, TN 37232

[‡]Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232

Abstract

Oxidative damage to proteins is one of the major pathogenic mechanisms in many chronic diseases. Therefore, inhibition of this oxidative damage can be an important part of therapeutic strategies. Pyridoxamine (PM), a prospective drug for treatment of diabetic nephropathy, has been previously shown to inhibit several oxidative and glycoxidative pathways, thus protecting amino acid side chains of the proteins from oxidative damage. Here, we demonstrated that PM can also protect protein backbone from fragmentation induced via different oxidative mechanisms including autooxidation of glucose. This protection was due to hydroxyl radical scavenging by PM and may contribute to PM therapeutic effects shown in clinical trials.

Keywords

diabetic complications; protein glycation; protein degradation; reactive oxygen species; hydroxyl radical; polypeptide backbone fragmentation; pyridoxamine

Introduction

Oxidative damage to proteins is one of the major pathogenic mechanisms in many chronic diseases including diabetes. Glucose itself can be a source of reactive oxygen species, such as hydroxyl radical, produced via oxidative pathways catalyzed by transition metal ions [1]. Hydroxyl radical can oxidize various protein amino acid side chains and also cause fragmentation of polypeptide backbone [2; 3; 4]. In particular, hydroxyl radical-mediated protein backbone fragmentation can occur next to proline, glutamic and aspartic acid, valine, leucine, and glycine residues as well as at random protein sites [3]. Thus, fragmentation of protein backbone can propagate damage beyond those specific sites, mostly lysine and arginine residues, that are preferentially targeted by classical glycoxidative reactions.

© 2011 Elsevier Inc. All rights reserved.

^{*}To whom correspondence should be addressed: Division of Nephrology, Vanderbilt University Medical Center, S-3223 MCN, 1161 21st Avenue South, Nashville, Tennessee 37232-2372. Tel.: 615-322-2089 Fax: 615-343-7156; paul.vozian@vanderbilt.edu.

[§]Current address: Aegis Sciences, 515 Great Circle Rd., Nashville, TN 37228

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Inhibition of oxidative damage to protein backbone can be a part of the strategies to protect protein functionality in diseases that involve oxidative and glycoxidative pathogenic pathways. Pyridoxamine (PM), a prospective drug for treatment of diabetic nephropathy [5]1, has been previously shown to inhibit several oxidative and glycoxidative pathways that can cause protein damage [6; 7; 8; 9; 10]. The goal of this study was to determine whether PM can also protect protein backbone from oxidative fragmentation. Using ribonuclease A, lysozyme, and serum albumin as models, we demonstrated that PM can inhibit fragmentation of protein backbone induced via different mechanisms including glucose autooxidation. This protection was due to hydroxyl radical scavenging by PM and may contribute to therapeutic effects demonstrated in PM clinical trials [5]1.

Materials and Methods

Materials

2-deoxy-D-ribose, dansyl chloride, and pyridoxamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Bovine pancreatic RNase A was from Worthington Biochemical (Freehold, NJ). All other chemicals were from Sigma-Aldrich.

Incubation conditions

Proteins (0.5 mg/mL) were incubated in 100 mM sodium phosphate buffer, pH 7.5 or in the same buffer containing 50 μ M CuSO₄ and 5 mM H₂O₂ without or with different concentrations of PM. After incubation at 37°C, EDTA was added to the samples to the final concentration of 0.1 mM. For generation of hydroxyl radical via either Fenton or xantine/xantine oxidase systems, reactions included: 100 mM sodium phosphate buffer, pH 7.5, 0.1 mM EDTA, 0.1 mM FeCl₃, 0.1 mM ascorbate (or 0.2 mM hypoxanthine), and 0.2 mM sodium salicylate. The reactions were initiated by adding 1 mM hydrogen peroxide (or 16 mU xanthine oxidase) followed by incubation for 90 min at room temperature. For glucose-induced fragmentation, samples were incubated with 100 mM glucose in 100 mM sodium phosphate buffer, pH 7.5 at 37°C. All incubations were performed in the dark; for glucose-treated samples, sodium azide (0.02%) was added to prevent bacterial growth.

Determination of hydroxyl radical and the rate of hydroxyl radical scavenging

The level of hydroxyl radical was determined by measuring hydroxylation of salicylate (2-hydroxybenzoate) to 2,3-dihydroxybenzoate as described previously [11]. Rate constant of hydroxyl radical scavenging by PM was determined using competition method with 2-deoxy-D-ribose as a probe [12].

Gel-electrophoresis

Reducing SDS-PAGE was performed on either 12% gels or 8–16% gradient gels using Xcell II Mini-Cell (Novex). Following the electrophoresis, protein bands were visualized by staining with Coomassie R250 or by Western blot using anti-CML antibody [6]. Intensity of protein bands was quantified using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Determination of RNase fragmentation using RP-UPLC

RNase (0.5 mg/ml) was incubated in 100 mM sodium phosphate buffer, pH 7.5 (control) or in the same buffer containing 50 μ M CuSO₄ and 5 mM H₂O₂ without or with different

Edmund J. Lewis, Tom Greene, Samuel Spitaler, Samuel Blumenthal, Tomas Berl, Lawrence G. Hunsicker, Marc A. Pohl, Richard D. Rohde, Itamar Raz, Yair Yerushalmy, Yoram Yagil, Tommy Herskovitz, David K. Packham, Julia B. Lewis for The Collaborative Study Group, Chicago IL, USA. A Clinical Trial of Pyridoxamine in Patients with Type 2 Diabetes Mellitus and Overt Nephropathy (manuscript submitted to J. Am. Soc. Nephrol.)

concentrations of PM. After a 3-h incubation at 37°C, EDTA was added to the samples to the final concentration of 0.1 mM and protein was precipitated by incubation with 10% TCA for 1 h on ice followed by centrifugation. Supernatants containing fragmented RNase were removed, adjusted to neutral pH with NaOH, and stored at -80°C. For peptide and amino acid derivatization, 0.5 mL of supernatant was mixed with 0.5 mL of saturated Na-borate, pH 9.2 and 0.25 mL of freshly prepared solution of dansyl chloride in anhydrous acetonitrile (25 mg/mL) was added to the mixture. Samples were incubated in the dark for 20 min at room temperature and 3.75 mL of 20% aqueous solution of acetonitrile was added followed by 2-h incubation in the dark at room temperature to hydrolyze the excess of dansyl chloride. Samples were clarified by centrifugation, filtered through 0.2 µm filter and 10 µL aliquots were loaded onto Acquity BEH C₁₈ column (1×100 mm). Samples were eluted at the flow rate of 0.1 mL/min using 5% acetonitrile in 10 mM NH₄Ac (Buffer A) and 95% acetonitrile in 10 mM NH₄Ac (buffer B) and the following gradient: 0–0.25 min, 10% B; 0.25–7 min, 10–90% B; 7–8 min, 90% B; 8–8.25 min, 90–10% B; 8.25–13 min, 10% B. Eluted peaks were detected by fluorescence with λ_{ex} =340 nm and λ_{em} =520 nm.

Determination of RNase fragmentation using mass-spectrometry

RNase was incubated alone or with 100 mM glucose for 40 d as described above. RNase samples (5 µg) were injected onto Acquity UPLC BEH-C18 (2.1×50 mm) column and separated using Acquity UPLC system (Waters). The solvents were (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile. A gradient (5% B for 5 min; 5–30% B in 45 min; 30–50% B in 5 min; 50–70% B in 1 min; 70% B for 4 min; 70–5% B in 1 min; 5% B for 10 min) was used to separate fragmentation peptides from the intact protein, at a flow rate of 0.2 mL/min. The elution profile was monitored at 220 nm with UV detector. Following multiple chromatographic runs, the corresponding peptide fractions (from 5 to 40 min) were combined, lyophilized and analyzed by tandem mass-spectrometry.

The data-dependent scanning was performed using a LTQ Orbitrap (Thermo Fischer Scientific, San Jose, CA) mass spectrometer equipped with an Eksigent AS1 autosampler and an Eksigent 1D+ HPLC pump attached directly to the instrument's nanospray source. The peptides were separated on a capillary tip, 100 µm 18 cm, packed with C₁₈ resin (Jupiter C₁₈, 3 µm, 300Å, Phenomenex, Torrance, CA) using an inline vented trapping column that was 100 µm × 6 cm. The flow rate during the solid phase extraction phase of the gradient was 2.5 µL/min, and during the separation phase it was 500 nL/min. Mobile phase A was 0.1% formic acid, while mobile phase B was acetonitrile with 0.1% formic acid. A 95 minute gradient was performed with a 15 minute washing period (100% A for the first 10 minutes followed by a gradient to 98% A at 15 minutes) to allow for removal of any residual salts. After the initial washing period, a 60 minute gradient was performed in which the first 35 minutes was a slow, linear gradient from 98% A to 75% A, followed by a faster gradient to 10% A at 65 minutes and an isocratic phase at 10% A at 75 minutes. MS/MS spectra of the peptides were obtained using data-dependent scanning in preview mode, which consisted of one full MS spectrum (mass range of 400–2000 amu) followed by five MS/MS spectra.

The data generated from the data-dependent LC-MS/MS experiments were analyzed using Sequest [13]. Identified peptide m/z values were included in the list for targeted analysis. The whole range of LC profile was divided by several segments 2–5 min wide and mass spectrometer was set to monitor full m/z range followed by 10 to 14 (one scan in full range 400–2000 m/z) values depending on retention time for particular peptide with an isolation window of 3 m/z. The peak containing the peptide of interest was extracted from the chromatogram and the area of the peak was determined using Xcalibur software.

Statistical analysis

Data were expressed as mean $\pm$ S.D., and statistical analysis was performed using Student's *t* test for unpaired samples or ANOVA followed by post hoc Student-Newman-Keuls comparisons. Differences were considered statistically significant if *p* values were less than 0.05.

Results and Discussion

Pyridoxamine protects proteins from oxidative degradation

Incubation of RNase, lysozyme or BSA in the presence of copper-Fenton system caused degradation of the protein bands on SDS-PAGE (Fig. 1). The absence of visible accumulation of distinct lower molecular weight bands upon protein degradation is consistent with random fragmentation of protein backbone and characteristic of hydroxyl radical-induced damage [14]. Lysozyme was more resistant to degradation compared to BSA and RNase (Fig. 1). In the presence of PM, very little degradation occurred in all the proteins tested (Fig. 1).

Oxidative fragmentation of RNase backbone and protection by PM

We further investigated protective effect of PM against oxidative protein degradation using RNase as a model. Under our solution conditions, about half of RNase degraded within 0.5 h; the protein was totally degraded within 24 h (Fig. 2A). In the presence of PM, RNase was completely protected from degradation within the initial 3 h of incubation; even after 24-h incubation only about 30% of protein was degraded (Fig. 2A). Protective effect of PM was concentration-dependent at different incubation times (Fig. 2B).

To determine protein backbone fragmentation directly, we have isolated RNase peptide fragments and modified their amino groups with dansyl chloride. These derivatized peptides were analyzed using RP-UPLC. We have detected multiple RNase fragments within the retention time region of 5.7–7.7 min (Fig. 2C, arrows) and other regions of chromatogram (not shown). Only very minimal baseline fragmentation was detected in the control sample (Fig. 2C). PM inhibited RNase fragmentation in a concentration-dependent manner (Fig. 2C), similar to that determined by SDS-PAGE (Fig. 2B).

Glucose-induced RNase backbone fragmentation and protection by PM

Hydroxyl radical is produced during glucose autooxidation [15]; thus, glucose can cause protein backbone fragmentation under oxidative conditions. Indeed, glucose-induced protein degradation was demonstrated using gel-electrophoresis in proteins that are prone to oxidative damage due to bound catalytic transition metal ions [1; 14; 16]. However, direct evidence of polypeptide backbone fragmentation is lacking. Here, we employed mass-spectrometry to determine glucose-induced protein backbone fragmentation and protective effect of PM.

Glucose caused significant degradation of RNase after 40-day incubation which was inhibited in the presence of PM (Fig. 3A). When degradation peptides were isolated and analyzed using LC-MS/MS, level of C-terminal RNase peptide PYVPVHFDASV was increased ~15-fold in glucose-treated samples compared to controls (Fig. 3 B–D), thus providing direct evidence of glucose-induced protein backbone fragmentation. There was no increase in the level of this peptide when RNase was treated with glucose and PM (Fig. 3D). Other RNase fragmentation peptides were also present at the elevated levels in glucose-treated samples and inhibited by PM as determined by comparison of total ion current chromatograms (data not shown). However, these peptides were not identified by Sequest algorithm most likely because they contained lysine, arginine or other residues modified by

glucose or glucose autooxidation products. Apparently, the degree of PM inhibition was different for different fragmentation peptides since overall PM protection of RNase was about 66 % after 40-d incubation (Fig. 3A).

Mechanism of PM inhibition of protein backbone fragmentation

We have explored the mechanism of inhibition of protein backbone fragmentation by PM. First, we demonstrated that production of hydroxyl radical was absolutely required to induce RNase backbone fragmentation. Only a complete copper-Fenton system caused RNase degradation, while neither Cu^{2+} nor hydrogen peroxide alone had any effect (Fig. 4A). To determine whether PM acts via scavenging of hydroxyl radical, we performed experiments in the presence of strong metal chelating agent EDTA. EDTA-bound transition metal ions can participate in redox catalysis and hydroxyl radical production but cannot be sequestered by PM [2]. PM inhibited hydroxylation of salicylate by either Fenton or xantine/xantine oxidase reaction systems in the presence of EDTA (Fig. 4B), which is consistent with hydroxyl radical scavenging mechanism. PM reaction rate with hydroxyl radical was relatively high with the rate constant $k=(2.61\pm0.24)\times10^9 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 4C), a value which is comparable to those of many biological molecules which have high reactivity towards hydroxyl radical [17].

In this study, we demonstrated that PM can protect the integrity of polypeptide backbone under oxidative and glycoxidative conditions. Direct evidence of protein backbone fragmentation and PM protection was provided through identification of degradation peptides using RP-UPLC and mass-spectrometry.

Protein backbone fragmentation by hydroxyl radical can potentially occur via direct attack at the α -carbon site, via attack at the proline side chain, or via radical transfer from either β - or γ -carbons of the side chains [3]. Our data suggest that the fragmentation occurred, at least in part, via abstraction of hydrogen at the α -carbon site since the N-terminus of the RNase degradation peptide PYVPVHFDASV remained intact (Scheme and Fig. 3). In this case, mechanism involving attack at proline side chain can be excluded because backbone cleavage occurred at the N-terminal side of the proline residue [3].

Glucose-induced fragmentation of protein backbone may propagate protein damage beyond the sites that are traditional targets of glycoxidation reactions, thus exacerbating pathogenic consequences. In diabetes, there is an increase in circulation levels and excretion of glycation free adducts, which are degradation products of glycated and AGE-modified proteins with suggested pathogenic role [18]. These free adducts are thought to be produced via intracellular proteolytic degradation mechanisms [18]. However, it is possible that glucose may also facilitate formation of these free adducts in the extracellular environment via oxidative protein backbone fragmentation mechanism.

Inhibition of oxidative protein backbone fragmentation by PM involved hydroxyl radical scavenging. Thus, PM can inhibit production of hydroxyl radical from hydrogen peroxide [10; 19] as well as scavenge it directly. Mechanism of hydroxyl radical scavenging by PM can potentially operate either via phenolic hydrogen donation or through hydroxylation of pyridinium ring [19; 20]. In the previous studies, PM has been shown to inhibit various glycoxidative reactions including post-Amadori AGE formation and tryptophan oxidation [6; 7; 10]. Protection by PM against protein backbone fragmentation is in line with these findings. In the oxidative diabetic environment, this mechanism may contribute to therapeutic effects demonstrated in PM clinical trials [5]1.

Highlights

► Autooxidation of glucose can cause fragmentation of protein backbone. ► This mechanism could contribute to protein damage in diabetes and to pathogenesis of diabetic complications. ► Pyridoxamine, a prospective drug for treatment of diabetic nephropathy, can inhibit glucose-induced fragmentation of protein backbone.

Acknowledgments

This work was supported by the grant DK65138 from the National Institutes of Health. Mr. Xavier Shackelford was supported by the summer student research grant DK65123 from the National Institutes of Health.

List of Abbreviations

AGE	advanced glycation end-product
BSA	bovine serum albumin
CML	N ^ε -(carboxymethyl)lysine
PM	pyridoxamine
RNase	bovine pancreatic ribonuclease A

References

- Hunt JV, Dean RT, Wolff SP. Hydroxyl radical production and autoxidative glycosylation. Glucose autoxidation as the cause of protein damage in the experimental glycation model of diabetes mellitus and ageing. *Biochem J.* 1988; 256:205–212. [PubMed: 2851978]
- Halliwell, B.; Gutteridge, JMC. Free radicals in biology and medicine. 3rd edition ed. London: Oxford University Press; 1999.
- Xu G, Chance MR. Hydroxyl radical-mediated modification of proteins as probes for structural proteomics. *Chem Rev.* 2007; 107:3514–3543. [PubMed: 17683160]
- Riedle B, Kerjaschki D. Reactive oxygen species cause direct damage of Engelbreth-Holm-Swarm matrix. *Am J Pathol.* 1997; 151:215–231. [PubMed: 9212747]
- Williams ME, Bolton WK, Khalifah RG, Degenhardt TP, Schotzinger RJ, McGill JB. Effects of Pyridoxamine in Combined Phase 2 Studies of Patients with Type 1 and Type 2 Diabetes and Overt Nephropathy. *Am J Nephrol.* 2007; 27:605–614. [PubMed: 17823506]
- Booth AA, Khalifah RG, Todd P, Hudson BG. In vitro kinetic studies of formation of antigenic advanced glycation end products (AGEs). Novel inhibition of post-Amadori glycation pathways. *J Biol Chem.* 1997; 272:5430–5437. [PubMed: 9038143]
- Voziiyan PA, Khalifah RG, Thibaudeau C, Yildiz A, Jacob J, Serianni AS, Hudson BG. 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. *J Biol Chem.* 2003; 278:46616–46624. [PubMed: 12975371]
- Voziiyan PA, Metz TO, Baynes JW, Hudson BG. A post-Amadori inhibitor pyridoxamine also inhibits chemical modification of proteins by scavenging carbonyl intermediates of carbohydrate and lipid degradation. *J Biol Chem.* 2002; 277:3397–3403. [PubMed: 11729198]
- Onorato JM, Jenkins AJ, Thorpe SR, Baynes JW. Pyridoxamine, an inhibitor of advanced glycation reactions, also inhibits advanced lipoxidation reactions. Mechanism of action of pyridoxamine. *J Biol Chem.* 2000; 275:21177–21184. [PubMed: 10801874]
- Chetyrkin SV, Mathis ME, Ham AJ, Hachey DL, Hudson BG, Voziiyan PA. Propagation of protein glycation damage involves modification of tryptophan residues via reactive oxygen species: inhibition by pyridoxamine. *Free Radic Biol Med.* 2008; 44:1276–1285. [PubMed: 18374270]
- Halliwell, B.; Gutteridge, JMC. Hydroxyl radicals assayed by aromatic hydroxylation and deoxyribose degradation. In: Greenwald, RA., editor. Handbook of methods for oxygen radical research. Boca Raton: CRC Press, Inc.; 1986. p. 177-180.

12. Halliwell B, Gutteridge JM, Aruoma OI. The deoxyribose method: a simple "test-tube" assay for determination of rate constants for reactions of hydroxyl radicals. *Anal Biochem.* 1987; 165:215–219. [PubMed: 3120621]
13. Liebler DC. Proteomic approaches to characterize protein modifications: new tools to study the effects of environmental exposures. *Environ Health Perspect.* 2002; 110 Suppl 1:3–9. [PubMed: 11834459]
14. Ookawara T, Kawamura N, Kitagawa Y, Taniguchi N. Site-specific and random fragmentation of Cu,Zn-superoxide dismutase by glycation reaction. Implication of reactive oxygen species. *J Biol Chem.* 1992; 267:18505–18510. [PubMed: 1326527]
15. Hunt JV, Wolff SP. Oxidative glycation and free radical production: a causal mechanism of diabetic complications. *Free Radic Res Commun.* 1991; 12–13(Pt 1):115–123.
16. Islam KN, Takahashi M, Higashiyama S, Myint T, Uozumi N. Fragmentation of ceruloplasmin following non-enzymatic glycation reaction. *J Biochem.* 1995; 118:1054–1060. [PubMed: 8749326]
17. Buxton GV, Greenstock CL, Helman WP, Ross AB. Critical-Review of Rate Constants for Reactions of Hydrated Electrons, Hydrogen-Atoms and Hydroxyl Radicals (.Oh/.O-) in Aqueous-Solution. *J Phys Chem Ref Data.* 1988; 17:513–886.
18. Thornalley PJ. Glycation free adduct accumulation in renal disease: the new AGE. *Pediatr Nephrol.* 2005; 20:1515–1522. [PubMed: 16133053]
19. Voziyan PA, Hudson BG. Pyridoxamine as a multifunctional pharmaceutical: targeting pathogenic glycation and oxidative damage. *Cell Mol Life Sci.* 2005; 62:1671–1681. [PubMed: 15905958]
20. Miyata T, van Ypersele de Strihou C, Ueda Y, Ichimori K, Inagi R, Onogi H, Ishikawa N, Nangaku M, Kurokawa K. Angiotensin II receptor antagonists and angiotensin-converting enzyme inhibitors lower in vitro the formation of advanced glycation end products: biochemical mechanisms. *J Am Soc Nephrol.* 2002; 13:2478–2487. [PubMed: 12239236]

Figure 1.

Pyridoxamine protects proteins from oxidative degradation. Proteins (0.5 mg/mL) were incubated in 100 mM sodium phosphate buffer, pH 7.5 with the copper-Fenton system with or without 10 mM PM at 37°C. At the indicated times, aliquots were removed and analyzed by SDS-PAGE as described under Experimental Procedures. Numbers on the left of the gel show molecular weights in kDa.

Figure 2.

Protection by PM against RNase backbone fragmentation. RNase (0.5 mg/mL) was incubated in 100 mM sodium phosphate buffer, pH 7.5 with the copper-Fenton system with or without PM at 37°C. At the indicated times, aliquots were taken and analyzed SDS-PAGE as described under Experimental Procedures. (A) Time course of RNase degradation and protection by PM. (B) Concentration dependence of PM protection after 1-h and 3-h incubations. Numbers below the gel indicate relative band intensity. (C) Direct evidence of RNase backbone fragmentation and PM protection by RP-UPLC. RNase (0.5 mg/ml) was incubated in 100 mM sodium phosphate buffer, pH 7.5 (dotted line) or in the same buffer containing 50 μ M CuSO_4 and 5 mM H_2O_2 without (solid line) or with different concentrations of PM (1, 2, and 4 mM, gray solid lines; 10 mM, dashed line) at 37°C for 3 h. RNase fragments were isolated, derivatized, and analyzed using RP-UPLC as described under Experimental Procedures. Arrows indicate dansylated RNase peptides. Peaks corresponding to dansylated PM (asterisks) were identified using dansylated PM standard (data not shown).

Figure 3.

Glucose-induced fragmentation of RNase polypeptide backbone and protection by PM. RNase (8 mg/mL) was incubated alone, with 100 mM glucose or with 100 mM glucose and 5 mM PM in 150 mM sodium phosphate buffer, pH 7.5 at 37°C for 40 d. (A) Protein samples were analyzed using SDS-PAGE followed by Coomassie staining. (B) Degradation peptide products were separated from the intact RNase using RT-UPLC and then analyzed by LC-MS/MS as described under Experimental Procedures. Total ion chromatogram of RNase fragmentation peptides from glucose-treated sample and extracted ion chromatograms of PYVPVHFDASV peptide ($MH^{2+}=615.8113$ Da) from control, glucose-treated, and glucose-treated + PM samples. RT indicates peptide retention time and MA indicates the integrated area under the peak. (C) The MS/MS spectrum of the C-terminal RNase fragmentation peptide PYVPVHFDASV. (D) Relative amounts of PYVPVHFDASV peptide in control, glucose-treated and glucose-treated + PM samples. Each bar represents the mean $\pm$ SD ($n=3$).

Figure 4.

Mechanism of protection of protein backbone by PM. (A) RNase (0.5 mg/mL) was incubated in 100 mM sodium phosphate buffer, pH 7.5 with CuSO₄, H₂O₂ or the complete copper-Fenton system at 37°C. At the indicated times, aliquots were taken and analyzed by SDS-PAGE as described under Experimental Procedures. (B) Fenton or xanthine/xanthine oxidase reaction systems were incubated with or without PM as described under Experimental Procedures. Hydroxyl radical-mediated oxidation of salicylate to 2,3-dihydrobenzoate was measured as described under Experimental Procedures. Symbols represent the mean $\pm$ SD (n=3). (C) PM reaction with hydroxyl radical was characterized using the deoxyribose method as described under Experimental Procedures. The rate constant was calculated from linear regression plots of three experiments.

Scheme.

Protein backbone cleavage by hydrogen abstraction at the α -carbon site (adopted from [3]).