Effects of Antioxidants on the Hydrogen Peroxide–Mediated Oxidation of Methionine Residues in Granulocyte Colony-Stimulating Factor and Human Parathyroid Hormone Fragment 13-34

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Purpose. The effects and mechanisms of different antioxidants, methionine, glutathione, acetylcysteine, and ascorbic acid ($AscH_2$), on the oxidation of methionine residues in granulocyte colony-stimulating factor (G-CSF) and human parathyroid hormone fragment 13-34 (hPTH 13-34) by hydrogen peroxide (H_2O_2) were quantified and analyzed.

Methods. The rates of oxidation of methionine residues in G-CSF were determined by peptide mapping analyses, and the oxidation of methionine residue in hPTH 13-34 was quantified by reverse-phase HPLC.

Results. At pH 4.5, free methionine reduces, glutathione and acetylcysteine have no obvious effect on, and $AscH_2$ promotes the rates of oxidation of methionine residues in G-CSF. The H_2O_2-induced oxidation rate constants for free methionine, acetylcysteine, and glutathione at pH 4.5 were measured to be 32.07, 1.00, and 1.63 $M^{-1}h^{-1}$, respectively, while the oxidation rate constant for Met^1, the most readily oxidizable methionine residue in G-CSF, is 13.95 $M^{-1}h^{-1}$. Therefore, the different effects of free methionine, acetylcysteine, and glutathione on the rates of oxidation of methionine residues in G-CSF are consistent with their different reactivity toward oxidation by H_2O_2. By using hPTH 13-34, the effect of $AscH_2$ on the H_2O_2-induced oxidation of methionine residue was quantified, and the mechanisms involved were proposed. Because of the presence of trace transition metal ions in solution, at low concentrations, $AscH_2$ is prone to be a prooxidant, increasing the hydroxyl radical (·OH) production rate via Fenton-type reactions. In addition to peroxide oxidation, these radicals lead to the degradation of hPTH 13-34 to smaller peptide fragments. At high concentrations, $AscH_2$ tends to act as an ·OH scavenger. EDTA inhibits ·OH production and thus eliminates the degradation of hPTH 13-34 by forming complexes with transition metal ions. However, the rate of oxidation of the methionine residue in hPTH 13-34 increases as the concentration of $AscH_2$ is increased from 0 to 200 mM, and the reason for this is still not clear.

Conclusions. Our results demonstrate that free methionine is an effective antioxidant to protect G-CSF against methionine oxidation at pH 4.5. Acetylcysteine and glutathione are not effective antioxidants at pH 4.5. Their oxidation rates at different pH values imply that they would be much more effective antioxidants than free methionine at alkaline conditions. $AscH_2$ is a powerful electron donor. It acts as a prooxidant in the conditions in this study and is unlikely to prevent oxidation by H_2O_2 in protein formulation, whether or not EDTA is present.

KEY WORDS: antioxidants; ascorbic acid; granulocyte colony-stimulating factor (G-CSF); human parathyroid hormone (hPTH); methionine oxidation.

INTRODUCTION

The oxidation of methionine residues in proteins is one of the major degradation pathways that must be addressed in therapeutic protein formulation and storage (1,2). Such oxidation can be divided into two categories: site-specific and non-site-specific oxidation (1). Site-specific oxidation is induced by transition metal ions, such as Fe^{3+} and Cu^{2+}, and non-site-specific oxidation is induced by light or the presence of oxidants in the protein formulation. Numerous studies have shown that the oxidation of methionine may affect the activity of a number of biologically active proteins including human parathyroid hormone (hPTH) (3), alpha 1-antitrypsin (4), antithrombin (5), vascular endothelial growth factor (6), leptin (7), and granulocyte colony-stimulating factor (G-CSF) (8). According to the U.S. Food and Drug Administration (FDA) guidelines (9), stabilization against methionine oxidation must be controlled in the product formulation of therapeutic proteins.

One of the most common ways to protect proteins against oxidation is by adding antioxidants in protein formulations. Antioxidants are substances that are more readily oxidized than the proteins that they are used to protect, thereby reducing the concentration of oxidants in protein solution. Several examples of pharmaceutically acceptable antioxidants include glutathione, acetylcysteine, methionine, ascorbic acid ($AscH_2$), sodium bisulfite, and so forth (10–15). It should be emphasized that the optimum choice of antioxidants must be evaluated for different proteins. Some antioxidants can be problematic in the formulation of some proteins. For example, $AscH_2$ has been shown to promote rather than inhibit not only the site-specific oxidation of methionine in small peptides (16) but also the hydrogen peroxide induced non-site-specific oxidation of methionine in recombinant human ciliary neurotrophic factor (rhCNTF) (13). Because $AscH_2$ is a powerful electron donor, it can act as both an antioxidant and a prooxidant (17). In the presence of transition metal ions and oxygen, $AscH_2$ is readily oxidized to dehydroascorbic acid. It also generates highly reactive oxygen species, such as hydrogen peroxide and peroxyl radicals. These, in turn, may accelerate the oxidation of methionine (16). The mechanism by which $AscH_2$ promotes peroxide induced, non-site-specific oxidation of proteins is still not well understood. Knepp *et al.* (13) suggested that $AscH_2$ promotes the oxidation of rhCNTF by initiating the heterolytic cleavage of the oxygen-oxygen bond in hydrogen peroxide.

This study focuses on understanding the effects of different antioxidants on the non-site-specific oxidation of methionine residues in G-CSF and in the hPTH fragment 13-34 by using hydrogen peroxide (H_2O_2) as the oxidant. G-CSF is an important hematopoietic growth factor used to treat cancer patients undergoing chemotherapy in order to alleviate the depression of white blood cell levels produced by cytotoxic

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therapeutic agents (8,18). *Escherichia coli* produced G-CSF contains 175 amino acids, including four methionine residues: Met^{1}, Met^{122}, Met^{127}, and Met^{138} (8). Early studies have shown that each of the four methionine residues can be oxidized by hydrogen peroxide with distinct oxidation rates in the order of $Met^{1} > Met^{138} > Met^{127} > Met^{122}$ (8,19). When all the four methionine residues in G-CSF are oxidized, the biologic activity of G-CSF decreases to 3% (8). hPTH is another example of a therapeutic protein prone to oxidation. Previous studies have shown that the oxidation of methionine residues 8 and 18 severely impact the biologic activity of hPTH (3,20). In order to simplify the assay and facilitate our study, we used hPTH 13-34, which is a peptide fragment of hPTH and contains only one methionine residue: Met^{18}. Our goals in this investigation are to compare the stabilization effects of different antioxidants on the oxidation of proteins and peptides and to elucidate their mechanisms. Our previous data (19) have demonstrated that the methionine residues in G-CSF have minimum oxidation rates at pH 4.5. In addition, the formulation pH of G-CSF is 4.0 (21). Therefore, in this study we focused on the effectiveness of different antioxidants at pH 4.5.

MATERIALS AND METHODS

Materials

G-CSF (4 mg/ml in water, pH 4.0 via addition of HCl) was obtained from Amgen Inc. (Thousand Oaks, CA, USA). Human parathyroid hormone fragment 13-34 (hPTH 13-34) (P-2780), 30% (w/w) H_2O_2 solution (H-1009), ethylenediaminetetraacetic acid (EDTA) (E-1644, SigmaUltra), L-ascorbic acid (A-2218, USP), L-methionine (M-5308, USP/EP/JP), *N*-acetyl-L-cysteine (A-8199, SigmaUltra), and L-glutathione reduced (G-6529, SigmaUltra) were purchased from Sigma, Inc, St. Louis, MO, USA. All other reagents and chemicals used were of at least analytical grade, and HPLC grade water was used through the study.

Oxidation of G-CSF

Oxidation reactions were carried out with 0.3 mg/ml G-CSF, 60 mM H_2O_2, and 25 mM acetate (pH 4.5), incubated at 25°C. Antioxidants were present at final concentrations of 60 mM when used. The extent of oxidation of individual methionine residues in G-CSF was quantified by peptide mapping analyses using trypsin for Met^{1} and endoproteinase Glu-C for Met^{122}, Met^{127} and Met^{138} (19).

Oxidation of hPTH 13-34

Oxidation reactions were typically carried out with 2.6 μM hPTH 13-34, 5 mM H_2O_2, different concentrations of ascorbic acid ($AscH_2$) and EDTA, and 25 mM acetate buffer, pH 4.5, incubated at 25°C. The reaction was stopped by injecting 0.1 ml sample into a C_4 column (Vydac 214TP52; 300 Å, 5 μm, 2.1 mm i.d. × 25 cm, Hesperia, CA, USA), and the extent of oxidation of Met^{18} in hPTH 13-34 was quantified by reverse-phase HPLC (Beckman Coulter, System Gold, 126 Solvent Module, 168 Detector and 508 Autosampler, Fullerton, CA, USA). The mobile phases used were solvent A [0.1% (w/v) trifluoroacetic acid (TFA)] and solvent B [0.1% (w/v) TFA in 90% acetonitrile]. The column was initially equilibrated with 15% B at a flow rate of 0.2 ml/min. After sample injection, the separation was performed by a linear gradient of 15% B to 50% B for 35 min and then by isocratic elution at 50% B for 5 min with a UV detector set at 215 nm. Representative chromatographs of unoxidized and oxidized hPTH 13-34 are shown in Fig. 1. Two distinct peaks, 1 and 2, were obtained. Each peak was collected and subjected to mass spectrometry to measure the molecular weight of the molecule associated with it. Peak 1 is unoxidized hPTH 13-34 and peak 2 is oxidized hPTH 13-34 (O). Peak 2 corresponds to 16 more mass units than peak 1, representing the oxidation of Met^{18} in hPTH 13-34. No methionine sulfone or oxidation product of oxidized tryptophan or tyrosine was detected in these two peaks.

Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed at the MIT Biopolymers Laboratory, using a Voyager-DETM STR BioSpectrometry Workstation (PerSeptive Biosystems, Boston, MA, USA). Samples were dried in a Speed-Vac and then purified with ZipTipC18 (Millipore, Billeria, MA, USA), following the manufacturer's instruction. ®-Cyano-4-hydroxycinnamic acid was used as matrix. Measurements were made in the positive, linear mode, and the accelerating voltage was 20,000 V.

Oxidation of Free Methionine, Acetylcysteine, and Glutathione

Free methionine, acetylcysteine and glutathione (5–30 mM) were individually incubated with H_2O_2 (0.46 mM) in the

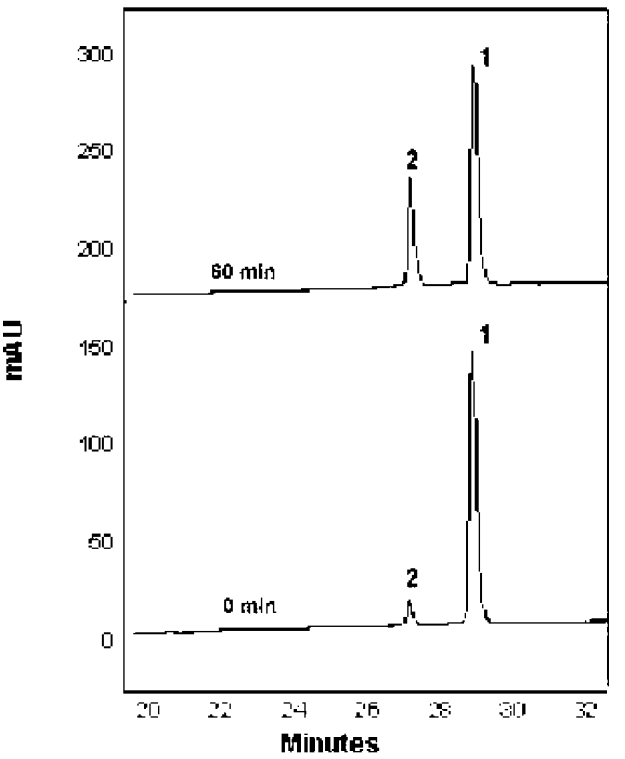

Fig. 1. Reverse-phase HPLC analyses of H_2O_2-oxidized hPTH 13-34 samples at various oxidation times. Peak 1: unoxidized hPTH 13-34; peak 2: oxidized hPTH 13-34 (O). Oxidation reactions were carried out with 2.6 μM hPTH 13-34, 5 mM H_2O_2, 25 mM acetate, pH 4.5 at 25°C.

pH range of 2 to 8 at 25°C. Oxidation rate constants were determined by measuring the decrease of H_2O_2 concentration, using a ferrous oxidation in xylenol orange (FOX) assay as described by Wolff (22).

RESULTS AND DISCUSSION

Effects of Different Antioxidants on the Oxidation of G-CSF

The effects of different antioxidants on the oxidation of the four methionine residues in G-CSF are shown in Fig. 2. It is obvious that different antioxidants have distinctly different effects. Among them, free methionine is the most effective agent in reducing the oxidation of methionine residues; acetylcysteine and glutathione have no obvious effects; and $AscH_2$ promotes rather than inhibits the oxidation of the methionine residues in G-CSF.

Oxidation Rate Constants of Free Methionine, Acetylcysteine, and Glutathione

To understand why acetylcysteine and glutathione have no obvious effects on the oxidation of G-CSF, we measured the oxidation rate constants of free methionine, acetylcysteine and glutathione by H_2O_2 at different pH values. As shown in Fig. 3, pH has no obvious effect on the oxidation rate constant of free methionine. However, the oxidation rate constants of acetylcysteine and glutathione strongly depend on pH. At pH 4.5, the oxidation rate constants for free methionine, acetylcysteine and glutathione were measured to be 32.07, 1.00, and 1.63 $M^{-1}h^{-1}$, respectively, while the oxidation rate constant for Met^1, the most readily oxidizable methionine residue in G-CSF, is 13.95 $M^{-1}h^{-1}$. Therefore, at pH 4.5, in Fig. 2, free methionine can effectively protect G-CSF against methionine oxidation, but acetylcysteine and glutathione have no obvious effects. At pH 8.0, however, the oxidation rate constants of acetylcysteine and glutathione are even higher than 1000 $M^{-1}h^{-1}$ (data not shown).

It has been shown that the anionic forms of thiol-containing compounds such as acetylcysteine and glutathione are usually more easily oxidized than their neutral forms (23). The thiol groups of glutathione and acetylcysteine have pKa's of 8.8 and 9.5 respectively (23). Lowering the pH reduces the amount of the anionic form and thereby slows oxidation. On the other hand, the thio ether group of methionine cannot be protonated except at very low pH, and therefore its reactivity toward oxidation does not change significantly over the pH range of 2 to 8. This implies that methionine is a more effective antioxidant than acetylcysteine and glutathione at acidic conditions while acetylcysteine and glutathione are much more effective antioxidants at alkaline conditions.

Effect of $AscH_2$ on the Oxidation of hPTH 13-34 in the Absence of EDTA

The effect of $AscH_2$ concentration on the oxidation of hPTH 13-34 was first studied in solutions containing 2.6 μM hPTH 13-34, 5 mM H_2O_2, 25 mM acetate, pH 4.5, and 0, 1, 10, 100, 200 mM $AscH_2$, respectively. As shown in Fig. 4, in the

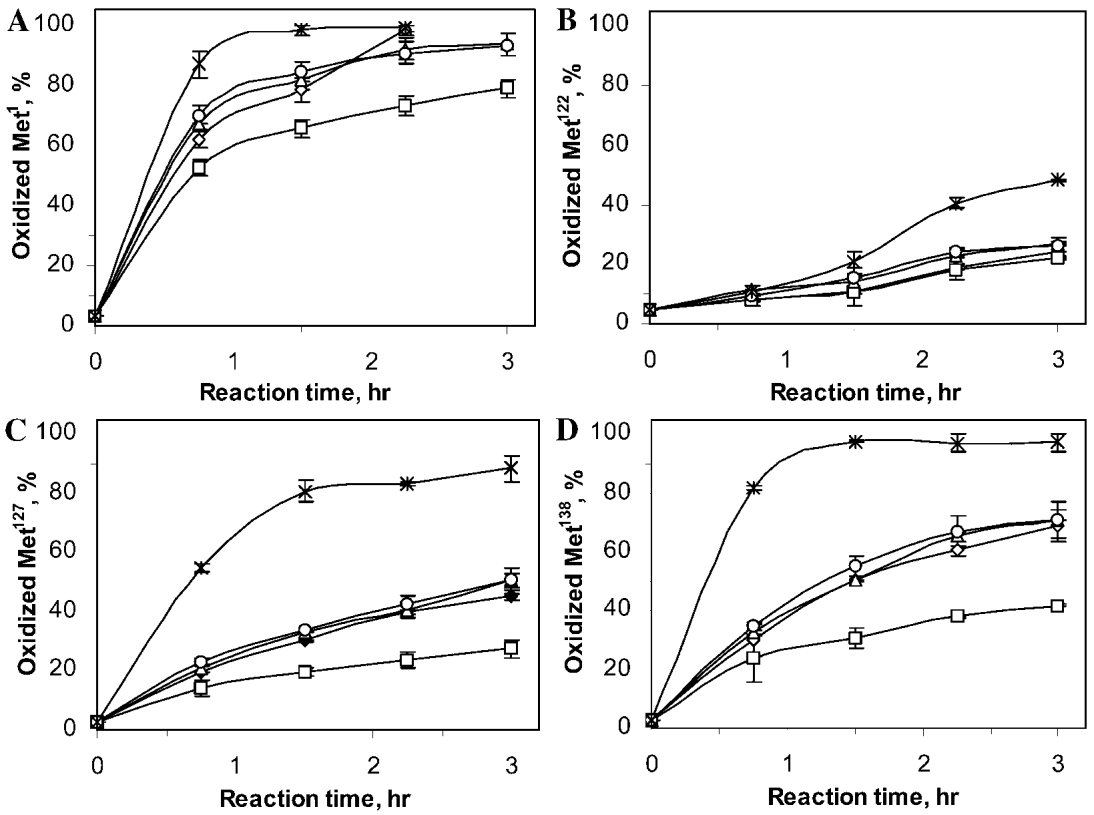

Fig. 2. Effects of different antioxidants on the oxidation of methionine residues in G-CSF: (A) Met^1, (B) Met^{122}, (C) Met^{127}, and (D) Met^{138}. (◇) control; (□) methionine; (△) glutathione; (○) acetylcysteine; (✻) ascorbic acid. Oxidation reactions were carried out with 0.3 mg/ml G-CSF, 60 mM H_2O_2, 25 mM acetate, at pH 4.5 and 25°C. The concentrations of antioxidants are 60 mM.

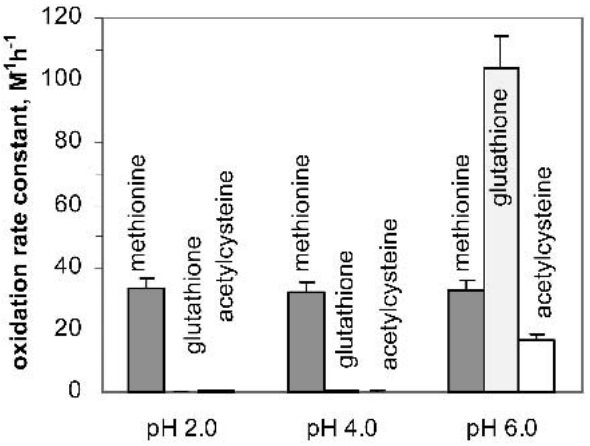

Fig. 3. Bimolecular oxidation rate constants of free methionine, glutathione, and acetylcysteine with hydrogen peroxide at different pH values (25°C)

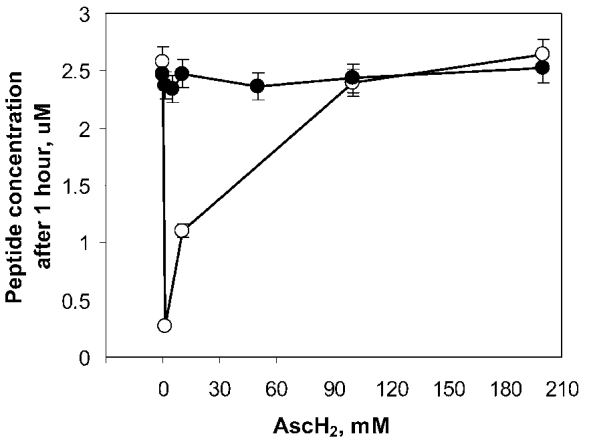

Fig. 4. Effect of the concentration of $AscH_2$ on the mass balance of hPTH 13-34: without EDTA (○) and with 6 mM EDTA (●). Experiments were carried out with 2.6 μM hPTH 13-34, 5 mM H_2O_2, 25 mM acetate, at pH 4.5 and 25°C. The peptide concentrations plotted represent the sum of oxidized hPTH 13-34 (O) and unoxidized hPTH 13-34 detected after 1 h.

absence of EDTA the total recovery of unoxidized hPTH 13-34 and oxidized hPTH 13-34 (O) was strongly dependent on the $AscH_2$ concentration. It decreases at all concentrations of $AscH_2$, but more rapidly at lower concentrations of $AscH_2$. As an example, Fig. 5 shows a detailed comparison of the profiles obtained from reverse-phase HPLC analyses. When hPTH 13-34 is present with 5 mM H_2O_2, there exist only two peaks: peak 1 for unoxidized hPTH 13-34 and peak 2 for oxidized hPTH 13-34 (O) (curve A in Fig. 5). However, when hPTH 13-34 is present with both 1 mM $AscH_2$ and 5 mM H_2O_2 (curve B in Fig. 5), there exist additional small peaks, such as peaks 3 and 4. In addition, the areas of peaks 1 and 2 in curve B are much smaller than those of peaks in curve A. This suggests that in addition to methionine oxidation, other degradation pathways occur in the oxidation system, such as cutting of the peptide.

The oxidation of the methionine residue in hPTH 13-34 and the degradation of hPTH 13-34 and oxidized hPTH 13-34 (O) can be written simply by the following two reactions:

$$\text{hPTH13-34} \xrightarrow{k_1^{app}} \text{hPTH13-34 (O)} \quad \text{(a)}$$

$$[\text{hPTH 13-34} + \text{hPTH 13-34 (O)}] \xrightarrow{k_2^{app}} \textit{degraded products} \quad \text{(b)}$$

The pseudo first-order kinetics of these two reactions can then be described as

$$\frac{d[P]}{dt} = -(k_1^{app} + k_2^{app})\,[P] \quad \text{(c)}$$

$$\frac{d[P + P(O)]}{dt} = -k_2^{app}\,[P + P(O)] \quad \text{(d)}$$

where [P] is concentration of unoxidized hPTH 13-34, μM; [P + P(O)] is total concentration of unoxidized hPTH 13-34 and oxidized hPTH 13-34 (O), μM; t is reaction time, h; k_1^{app} is apparent pseudo first-order rate constant of oxidation, h^{-1}; and k_2^{app} is apparent pseudo first-order rate constant of degradation, h^{-1}.

The effect of $AscH_2$ concentration on the rate constants of oxidation and degradation is shown in Fig. 6. In the absence of EDTA, the rates of both oxidation and degradation increase when the concentration of $AscH_2$ is changed from 0 to 1 mM, and they decrease as the concentration of $AscH_2$ is increased above 1 mM, up to 200 mM.

The reason that $AscH_2$ does not protect G-CSF and hPTH 13-34 against oxidation by H_2O_2 is probably due to its prooxidant properties (17,24,25). Early papers in the litera-

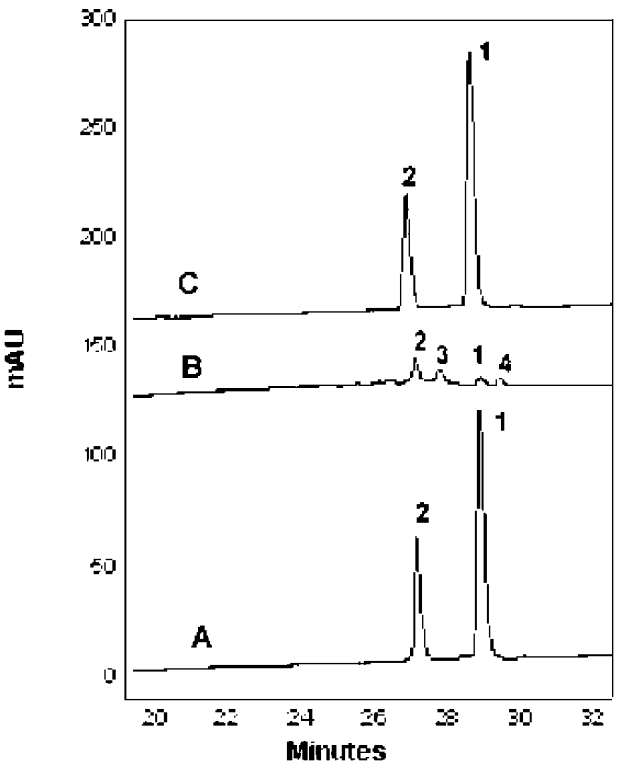

Fig. 5. Comparison of reverse-phase HPLC analyses of H_2O_2-oxidized hPTH 13-34 samples at different combinations of H_2O_2/$AscH_2$/EDTA. (A) 5 mM H_2O_2; (B) 5 mM H_2O_2 + 1 mM $AscH_2$; (C) 5 mM H_2O_2 + 1 mM $AscH_2$ + 6 mM EDTA. Peak 1: unoxidized hPTH 13-34; peak 2: oxidized hPTH 13-34 (O). Oxidation reactions were carried out with 2.6 μM hPTH 13-34, 25 mM acetate, pH 4.5 at 25°C for 1 h.

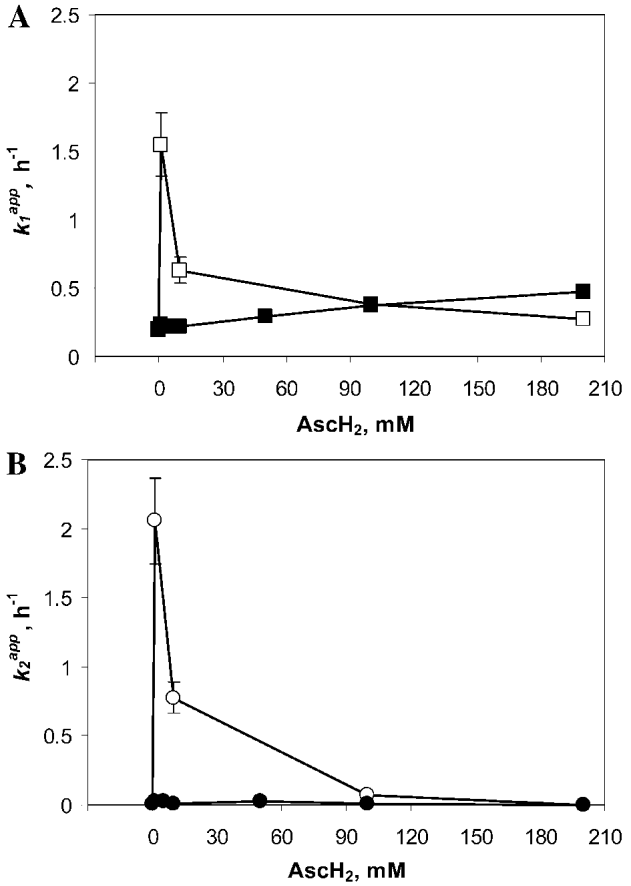

Fig. 6. Effect of $AscH_2$ concentration on the oxidation and degradation of hPTH 13-34. (A) Apparent pseudo first-order rate constant of oxidation (k_1^{app}): without EDTA (□) and with 6 mM EDTA (■); (B) apparent pseudo first-order rate constant of degradation (k_2^{app}): without EDTA (○) and with 6 mM EDTA (●). Experiments were carried out with 2.6 μM hPTH 13-34, 5 mM H_2O_2, 25 mM acetate, at pH 4.5 and 25°C.

ture have suggested that the prooxidant properties of $AscH_2$ are attributed to its recycling of transition metals, such as iron and copper, in Fenton-type reactions to produce hydroxyl radical (·OH) (26–31). $AscH_2$ is a di-acid with pKa's of 4.1 and 11.8 (32). At pH 4.5, 71% of $AscH_2$ will be present as $AscH^-$. The possible chemistry of this redox transition metal (M) cycling process to produce ·OH can be described by the following equations, in which equation (g) is the sum of equations (e) and (f)

$$AscH^- + M^{n+1} \rightarrow M^{n+} + Asc\cdot^- + H^+ \quad \text{(e)}$$

$$M^{n+} + H_2O_2 \rightarrow M^{n+1} + OH^- + \cdot OH \quad \text{(Fenton reaction)} \quad \text{(f)}$$

$$AscH^- + H_2O_2 \xrightarrow{\text{metal-ions}} Asc\cdot^- + H_2O + \cdot OH \quad \text{(g)}$$

Because ·OH actively oxidizes and degrades peptides and proteins, ascorbic acid is expected to promote rather than prevent oxidation and degradation when the concentration of $AscH_2$ is low. However, when the concentration of $AscH_2$ is high, extra $AscH_2$ can function as an antioxidant, being an ·OH scavenger, as shown in reaction (h).

$$AscH^- + \cdot OH \rightarrow H_2O + Asc\cdot^- \quad \text{(h)}$$

This "crossover" effect for $AscH_2$ as prooxidant or antioxidant (29) can be used to explain the phenomena observed in Figs. 4–6: higher rates of oxidation and degradation of hPTH 13-34 occur at lower concentrations of $AscH_2$, and lower rates of oxidation and degradation of hPTH 13-34 occur at higher concentrations of $AscH_2$. Our results in Figs. 4–6 also imply that our oxidation system had trace amount of metal ions, although the reagents and chemicals used were of at least analytical grade, and the water used was of HPLC grade. This implication is supported by early papers in the literature that demonstrated that trace catalytic metals up to 20 μM are ubiquitous in buffer solutions and typical concentrations of adventitious iron are 1–10 μM while that of copper is ~0.1 μM (29,33).

Effect of $AscH_2$ on the Oxidation of hPTH 13-34 in the Presence of EDTA

In order to reduce the effect of metal ions, we introduced the chelating agent EDTA. The effect of $AscH_2$ concentration on the oxidation of hPTH 13-34 was then studied in solutions containing 6 mM EDTA, 2.6 μM hPTH 13-34, 5 mM H_2O_2, 25 mM acetate (pH 4.5), and 0, 1, 5, 10, 50, 100, 200 mM $AscH_2$, respectively. We found that in the presence of 6 mM EDTA, the total recovery of unoxidized hPTH 13-34 and oxidized hPTH 13-34 (O) was nearly independent of the $AscH_2$ concentration (Fig. 4); thus k_2^{app} is close to zero (Fig. 6B) through the $AscH_2$ concentration range of 0 to 200 mM. Reverse-phase HPLC analyses also demonstrated that besides peaks 1 and 2, no other peaks were detected (curve C in Fig. 5) and that the areas of peaks 1 and 2 in curve C are almost the same as those in curve A. This implies that EDTA effectively reduces the production of ·OH and therefore prevents the degradation of peptide. However, surprisingly, k_1^{app} increases with increasing concentrations of $AscH_2$ in the presence of EDTA (Fig. 6A).

The mechanism by which EDTA arrests proteolysis is not likely related to the inactivation of a contaminating metalloprotease, which should have also been active in the absence of $AscH_2$ (Fig. 5). Therefore, EDTA specifically prevents the further degradation of the oxidation byproducts generated at low $AscH_2$ concentrations by metal chelation. Chelating agents have long been known to either increase or decrease the activities of transition metal ions through the formation of chelate-metal ion complexes (34–36). In the presence of EDTA, reactions (e), (f), and (g) can be rewritten as:

$$AscH^- + (EDTA - M^{n+1}) \rightarrow (EDTA - M^{n+}) + Asc\cdot^- + H^+ \quad \text{(i)}$$

$$(EDTA - M^{n+}) + H_2O_2 \rightarrow (EDTA - M^{n+1}) + OH^- + \cdot OH \quad \text{(Fenton reaction)} \quad \text{(j)}$$

$$AscH^- + H_2O_2 \xrightarrow{\text{EDTA-(metal-ions)}} Asc\cdot^- + H_2O + \cdot OH \quad \text{(k)}$$

According to Welch *et al.* (36), because EDTA is a chelator that contains oxygen ligands, it tends to stabilize Fe^{3+}. Including EDTA in the reaction system would slow the Fe^{3+} to Fe^{2+} reduction reaction [reaction (i) would be slower than reaction (e)] and accelerate the Fenton reaction [reaction (j) would be faster than reaction (f)]. For example, Van der Zee and Van den Broek (37) reported that addition of 50 μM

EDTA into a 6 mM $AscH_2$, 0.2 M Na-phosphate buffer (pH 7.4) reduced the concentration of the ascorbate free radical (Asc·) by 43%. On the whole, the total redox cycling process would be slowed [reaction (k) would be slower than reaction (g)] and the yield of ·OH would thus decrease. Therefore, EDTA can effectively prevent the degradation of hPTH 13-34 through the formation of complexes with transition metal ions. However, the mechanism of EDTA/H_2O_2/$AscH_2$ on the enhancement of methionine oxidation of hPTH 13-34 at high concentrations of $AscH_2$ is still unclear. Perhaps the effectiveness of EDTA chelation on the prooxidant behavior of $AscH_2$ is only observed at low concentrations of $AscH_2$, and the intrinsic oxidation observed at higher concentrations of $AscH_2$ remains unaffected. This intrinsic oxidation at higher concentrations of $AscH_2$ could be due to $AscH_2$ induced conformational changes both with and without EDTA. Additional work in this area is still needed.

CONCLUSIONS

At pH 4.5, the antioxidants methionine, glutathione, acetylcysteine and $AscH_2$ have different effects on the oxidation of methionine residues in G-CSF by H_2O_2. Free methionine reduces, glutathione and acetylcysteine have no obvious effect on, and $AscH_2$ promotes the rates of oxidation. The different effects of free methionine, acetylcysteine and glutathione on the rates of oxidation of methionine residues in G-CSF are consistent with their different effects on oxidation by H_2O_2 at pH 4.5.

$AscH_2$ is a powerful electron donor. It acts as both an antioxidant and a prooxidant. Because of the presence of trace transition metal ions in solution, at low concentrations, $AscH_2$ is prone to be a prooxidant, increasing the rate of production of ·OH via Fenton-type reactions, and at high concentrations, $AscH_2$ tends to be an antioxidant, acting as an ·OH scavenger. EDTA inhibits the production of ·OH by forming complexes with metal ions. However, in the presence of EDTA, the rate of oxidation of the methionine residue in hPTH 13-34 increases as the concentration of $AscH_2$ is increased from 0 to 200 mM, and the reason for this is still not clear.

Our results also imply that free methionine is a more effective antioxidant than acetylcysteine and glutathione at acidic conditions, while acetylcysteine and glutathione are much more effective antioxidants than free methionine at alkaline conditions, based on their oxidation rate constants at different pH values. Ascorbic acid is not suggested to be an effective antioxidant for H_2O_2 oxidation processes, whether or not EDTA is present.

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