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Research Paper

Effects of Excipients on the Hydrogen Peroxide–Induced Oxidation of Methionine Residues in Granulocyte Colony-Stimulating Factor

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Purpose. The objective of this study was to elucidate the different mechanisms of action of different excipients on the oxidation of Met^{1}, Met^{122}, Met^{127}, and Met^{138} in granulocyte colony-stimulating factor (G-CSF) by using hydrogen peroxide as the oxidant.
Methods. The oxidation of Met^{1}, Met^{127}, and Met^{138} was quantified by peptide mapping analysis. The oxidation of Met^{122} has biphasic oxidation kinetics with a faster second phase. Therefore, the oxidation of Met^{122} was quantified by two different methods: peptide mapping analysis for the first phase of oxidation and direct reverse-phase HPLC for the second phase of oxidation.
Results. The current work reveals that the preferential excluding excipients sorbitol, sucrose, and trehalose, in the concentration range 0–30% (w/v), and the preferential binding excipients urea and guanidine hydrochloride, in the concentration range 0–0.8 M, do not affect the oxidation of methionine residues in G-CSF at pH 4.5. The chelating agents citrate and EDTA have different effects on the rates of oxidation of methionine residues in G-CSF. At low concentrations, citrate decreases the rates, while at high concentrations, citrate increases the rates. EDTA decreases the rates of oxidation of methionine residues in G-CSF, such that its effect becomes more and more as its concentration is increased from 0 to 200 mM. The efficacy of EDTA on the rates of oxidation of the four methionine residues in G-CSF follows the order $Met^{122} > Met^{127} > Met^{138} > Met^{1}$.
Conclusions. Our results indicate that EDTA can protect the methionine residues in G-CSF against oxidation induced by hydrogen peroxide. The more exposed the methionine residue is, the more difficult it is to be protected by EDTA. The mechanism may be due to the specific ion binding of EDTA to proteins.

KEY WORDS: chelating agents; excipients; granulocyte colony-stimulating factor (G-CSF); methionine oxidation; sugars and polyols.

INTRODUCTION

Methionine oxidation has been recognized to be an important cause of inactivation of therapeutic proteins and peptides during bioprocessing and storage (1,2). Examples of this effect have been reported for parathyroid hormone (3), alpha 1-antitrypsin (4), antithrombin (5), vascular endothelial growth factor (6), leptin (7), and granulocyte colony-stimulating factor (G-CSF) (8). Stabilizing therapeutic proteins and peptides against methionine oxidation is a challenge for formulation. One of the most common ways to protect therapeutic proteins and peptides against methionine oxidation is the use of excipients in formulation. Methionine oxidation can typically be divided into two categories: site-specific and non-site-specific (1). Site-specific oxidation is induced by transition metal ions such as Fe^{3+} and Cu^{2+}, and therefore can often be inhibited by adding chelating agents to form complexes with transition metal ions (9–11). Non-site-specific oxidation is induced by light or by contaminant oxidants like hydrogen peroxide (H_2O_2). There are few reports of the effect of excipients on non-site-specific oxidation (10,12). DePaz *et al.* (12) studied the excluding effect of sucrose on the methionine oxidation of subtilisin and found that sucrose inhibited the oxidation of the partially buried Met^{222} in subtilitsin, presumably by reducing the accessibility of the subtilisin active site to a borate-H_2O_2 complex. Andersson *et al.* (10) reported using ethylenediaminetetraacetic acid (EDTA) and polyethyleneimine (PEI) to protect lactate dehydrogenase (LDH) against peroxide-mediated oxidation. Although EDTA and PEI increased the half-life of LDH activity by 7- and 16-fold, respectively, they actually did not reduce the oxidation rate of the residual thiol groups at pH 7.2 (10).

In this investigation, we focused on conducting "accelerated oxidation studies" at relatively high H_2O_2 concentrations, as typically practiced in academia and industry, to compare the efficacy of different excipients against H_2O_2 induced non-site-specific methionine oxidation. In general, the reactivity of an individual methionine residue is greater if the

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residue is surface exposed compared to being buried (3,4,7,8, 13,14). Therefore, any excipients that can limit and reduce the exposure of methionine residues to potential oxidants may be used to protect proteins against non-site-specific oxidation. According to the protein-solvent preferential interaction theory of Timasheff (15–17), there are two types of excipients that may effectively inhibit oxidation: excipients that are preferentially excluded from the surface of the protein and excipients that bind specifically in the vicinity of sites that are oxidizable. In the first case, exclusion may shift the protein structure toward more compact state and therefore may lower the exposure of methionine residues to oxidants. In the latter case, binding may block a certain portion of the surface of a protein and therefore may limit the exposure of methionine residues to potential oxidants.

In this study, we examined the oxidation of G-CSF, an important hematopoietic growth factor used to alleviate the depression of white blood cell levels produced by cytotoxic agents associated with chemotherapy (8,18). A variety of excipients, many of which have already been used in protein formulation and may potentially be used as preferential excluding or binding excipients, were compared specifically for their effects on methionine oxidation of G-CSF by using H_2O_2 as the oxidant. Our previous data (13) have demonstrated that the methionine residues in G-CSF have minimum oxidation rates at pH 4.5. This pH value is coincidently the same as the pH value used in another research group's published paper (8) and very close to pH 4.0, the formulation pH of the commercial G-CSF product (19). Therefore, in this study we focused on the effectiveness of different excipients at pH 4.5, choosing excess concentrations of H_2O_2, so that experiments were performed in the pseudo–first-order regime in reasonable amounts of time.

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), and 30% (w/w) H_2O_2 solution (H-1009), urea (U-4884, USP), guanidine hydrochloride (G-7153, SigmaUltra), ethylenediaminetetraacetic acid (EDTA) (E-1644, SigmaUltra), citric acid (C-7129), and sucrose (S-3929, USP/NF) were purchased from Sigma, Inc. (St. Louis, MO, USA) Sorbitol (S-112, NF) and α,α-trehalose dihydrate (T-104-1, high purity and low endotoxin) were purchased from FERRO Pfanstiehl Laboratories, Inc. (Waukegan, IL, USA). All other reagents and chemicals used were of at least analytical grade, and HPLC grade water was used through the study.

Oxidation of Met^{122} (First Phase) and $Met^{1,127,138}$

In the first phase of experiments, G-CSF was exposed to relatively mild oxidation conditions under various solution conditions. The typical reaction mixtures contained 0.1 or 0.3 mg/ml G-CSF and 30 or 60 mM H_2O_2 in acetate buffer (25 mM) at pH 4.5. Different excipients with different concentrations were added to the reaction mixtures in order to evaluate their effects on the oxidation of G-CSF. The reaction mixtures were incubated at 25°C. The sites and extent of oxidation of individual methionine residues in G-CSF were quantified by peptide mapping analyses using trypsin for Met^1 and endoproteinase Glu-C for Met^{122} at the first phase, Met^{127} and Met^{138} (13).

Oxidation of Met^{122} (Second Phase)

Oxidation of Met^{122} in the second phase was conducted under stronger oxidation conditions with 0.1 mg/ml G-CSF, 300 mM H_2O_2 and 25 mM acetate (pH 4.5), incubated at 25°C. Different excipients were present at different concentrations as specified. For the second phase of experiments, reverse-phase HPLC analyses of the intact protein, rather than peptide mapping, were performed to quantify oxidation. The oxidation reaction was stopped by injecting a 0.05 ml sample into a C_4 column (Vydac 214TP52; 300 Å, 5 μm, 2.1 mm i.d. × 25 cm), separating different oxidized forms of G-CSF by reverse-phase HPLC (Beckman Coulter, Fullerton, CA, USA, System Gold, 126 Solvent Module, 168 Detector and 508 Autosampler). 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 35% B at a flow rate of 0.2 ml/min. After sample injection, the column was washed with 35% B for 20 min. The separation was then performed by a linear gradient of 35% B to 70% B for 35 min, 70% B to 90% B for 5 min, and then by isocratic elution at 90% B for 20 min with a UV detector set at 215 nm. Representative chromatographs of unoxidized and oxidized G-CSF forms are shown in Fig. 1. One of our previous studies (13) has shown that peak 1 is the only oxidized form of G-CSF containing oxidized Met^{122}. By assuming that different G-CSF oxidized forms have similar extinction coefficient at 215 nm, the percentage of oxidized Met^{122} can be determined.

RESULTS AND DISCUSSION

Oxidation of G-CSF by H_2O_2

Escherichia coli produced G-CSF contains 175 amino acids, including four methionine residues: Met^1, Met^{122}, Met^{127}

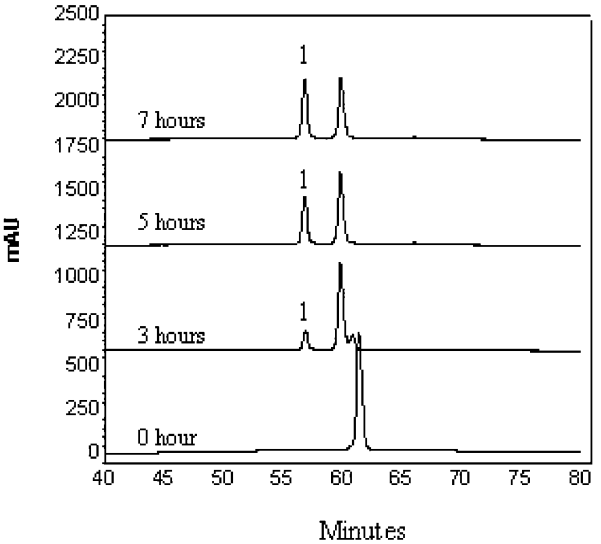

Fig. 1. Reverse-phase HPLC analyses of H_2O_2-oxidized forms of G-CSF at various oxidation times. Peak 1: G-CSF with all four methionines oxidized. Oxidation reactions were carried out with 300 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C.

and Met^{138} (8). Early studies have shown that each of the four methionine residues can be oxidized to methionine sulfoxide by H_2O_2. Besides methionine sulfoxide, no methionine sulfone or oxidation product of other amino acid residues was detected (8,13). The oxidation rates of the four methionine residues follow the order of $Met^1 > Met^{138} > Met^{127} > Met^{122}$ (8,13). This order is correlated with their different exposures to solvent (8,13): Met^1 is very exposed and in a flexible region of the protein, Met^{138} is solvent accessible, Met^{127} is more structurally hindered and less accessible to solvent than Met^{138}, and Met^{122} is located at a hydrophobic region. In addition, we observed biphasic oxidation kinetics with a faster second oxidation phase for Met^{122} (Fig. 2), as Lu *et al.* did (8). Lu *et al.* (8) suggested that the biphasic kinetics of Met^{122} was caused by the oxidation of Met^{127}, which may induce a local conformational change of G-CSF that allows the neighboring Met^{122} to become more solvent exposed, leading to faster oxidation.

Effects of Sugars and Polyols

Sugars and polyols are common excipients used to stabilize the conformation of proteins. Their stabilization effect is widely explained as being the result of preferential exclusion (2). Among various sugars and polyols, sucrose and trehalose are the most often-used stabilizers (2). Sorbitol is included in the formulation of Amgen's G-CSF product, NEUPOGEN (19). Therefore, we used sorbitol, sucrose, and trehalose to represent sugars and polyols in our studies. The effect of sorbitol concentration on the oxidation of individual methionine residues in G-CSF was first studied in solutions containing 30 mM H_2O_2, 0.1 mg/ml G-CSF, 25 mM acetate (pH 4.5) and 0, 2.5, 5, 7.5, 10, 20, 30% (w/v) sorbitol. As shown in Figs. 3A and 3B, sorbitol has no significant effect on the rates of oxidation of methionine residues in G-CSF. By contrast, sorbitol slightly increased the oxidation of Met^1 (Fig. 3A) and Met^{138} (Fig. 3B). The same phenomena were observed when sucrose was used (Figs. 3C and 3D). Because the oxidation of Met^{127} can induce a local conformational change of G-CSF and increase the exposure of Met^{122} to the solvent (8), we thought that the oxidation of Met^{122} at the second phase might be more sensitive to the conformational change of G-CSF caused by preferential excluding excipients. Further experiments were carried out in order to study the oxidation of Met^{122} (second phase) in solutions containing 300 mM H_2O_2, 0.1 mg/ml G-CSF, 25 mM acetate (pH 4.5), and 30% (w/v) sorbitol, sucrose, and trehalose, respectively. However, no decrease in the rate of oxidation of Met^{122} was observed either (Fig. 4). On the contrary, the addition of sorbitol, sucrose, or trehalose slightly increased the rate of oxidation of Met^{122} (Fig. 4). It seems that excipients that are preferentially excluded from the surface of G-CSF cannot prevent the oxidation of methionine residues in the protein, whether the methionine residue is buried (Met^{122}) or exposed (Met^1).

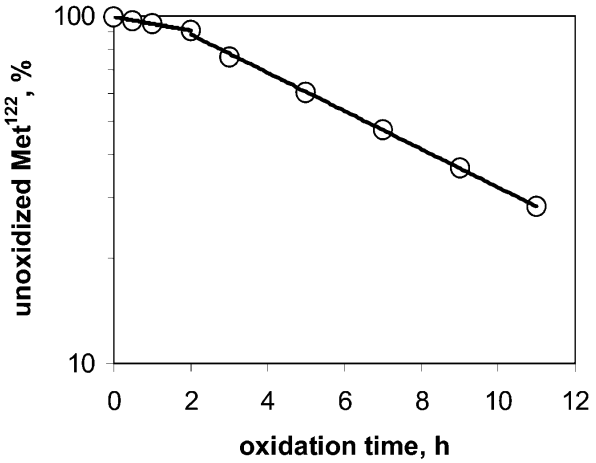

Fig. 2. Biphasic oxidation kinetics of Met^{122}. Oxidation reactions were carried out with 300 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C.

Effects of Urea and Guanidine Hydrochloride (GdnHCl)

Both urea and GdnHCl are well-known protein denaturants. At high concentrations, urea and GdnHCl increase the oxidation rates of individual methionines in proteins. For example, Met^{170} in human growth hormone is located in a buried position and can only be oxidized by H_2O_2 when the molecule is unfolded by 8 M urea (1). Our results also show that for equivalent amounts of time and conditions, the percentages of oxidized methionine residues were 95.0% for Met^1, 90.7% for Met^{122}, 88.5% for Met^{127}, and 90.1% for Met^{138} when 6 M GdnHCl was present with 0.1 g/L G-CSF and 30 mM H_2O_2 at pH 4.5 for 1 h, while the percentages of oxidized methionine residues were 47.9% for Met^1, 4.2% for Met^{122}, 9.6% for Met^{127}, and 19.5% for Met^{138} when no GdnHCl was present.

However, urea and GdnHCl are also excipients known to preferentially bind to the peptide backbone of proteins (2). Bhuyan (20) reported that low concentrations of GdnHCl and urea stabilized proteins although their stabilizing effect was overwhelmed by their effect on protein unfolding when the concentrations of urea and GdnHCl were increased further. Based on their preferential binding characteristics (2) to and stabilization effect (20) on proteins, we chose urea and GdnHCl as representatives of preferential binding excipients. Experiments were performed in solutions containing 60 mM H_2O_2, 0.3 mg/ml G-CSF, 25 mM acetate (pH 4.5), and 0, 0.2, 0.4, 0.6, 0.8 M urea or 0, 0.2, 0.4, 0.6, 0.8 M GdnHCl, respectively. As shown in Fig. 5, up to 0.8 M urea has no significant effect on the rates of oxidation of methionine residues in G-CSF, while GdnHCl slightly increases the rate of oxidation. Further studies demonstrated that 0.6 M urea increased the rate of oxidation of Met^{122} (second phase) (Fig. 6).

Although urea and GdnHCl may preferentially bind to the peptide backbone and block a certain portion of the surface of protein, as denaturants they may also shift protein structure toward a less compact state. In combination, the exposure of methionine residues to oxidants may not be reduced and therefore no decrease of oxidation was observed, even when the concentration of urea or GdnHCl was low.

Effects of EDTA and Citrate

Chelating agents have often been used as metal scavengers to inhibit site-specific oxidation by forming complexes with transition metal ions, such as Fe^{3+} and Cu^{2+} (9–11). Because proteins usually have exposed positively charged residues on their surface, chelating agents may bind to these residues. Therefore, we assumed that chelating agents might

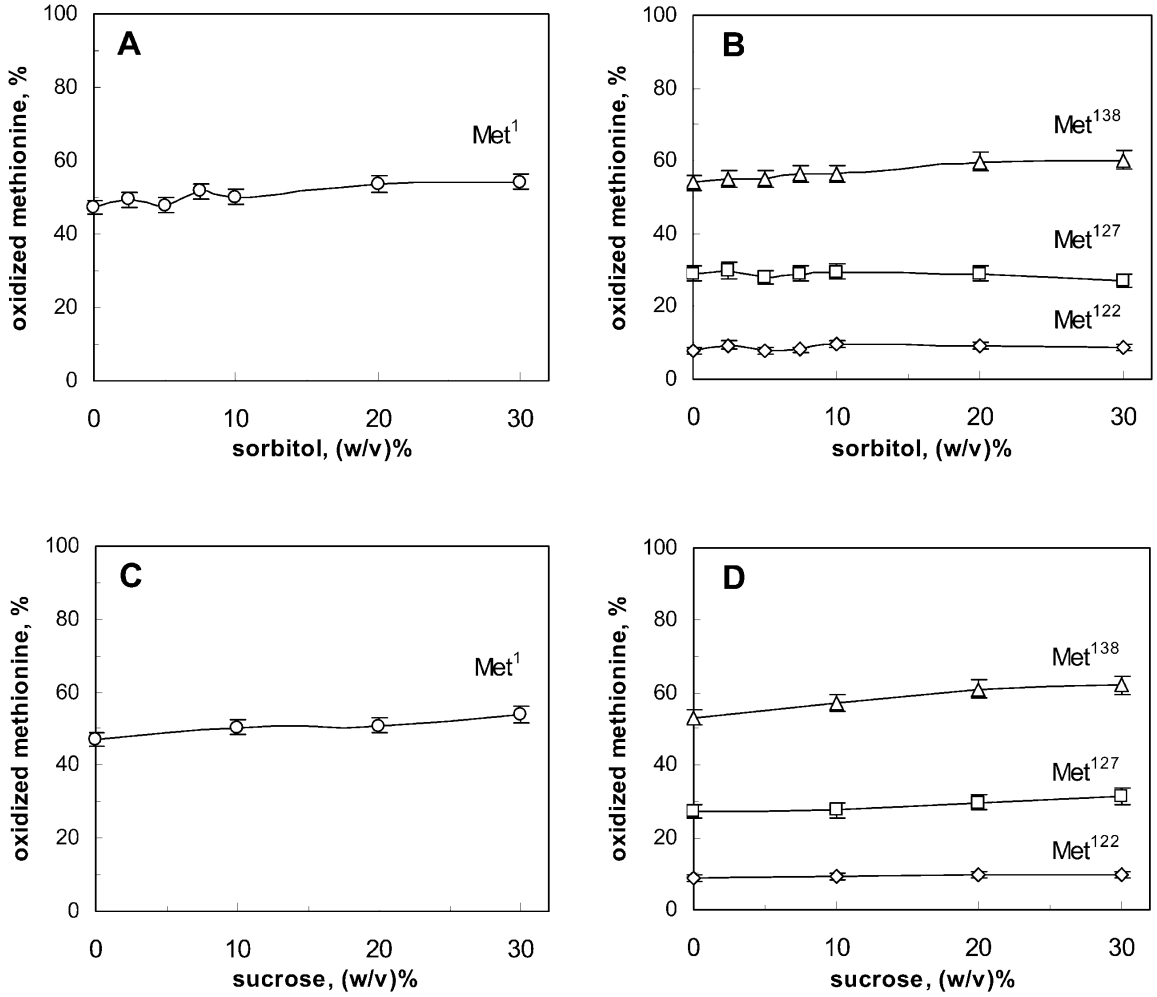

Fig. 3. Effect of sorbitol and sucrose concentration on the oxidation of individual methionine residues in G-CSF. (A), (B): sorbitol; (C), (D) sucrose. Oxidation reactions were carried out with 30 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C for 1 h (Met^1) or 4 h ($Met^{122,127,138}$). Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

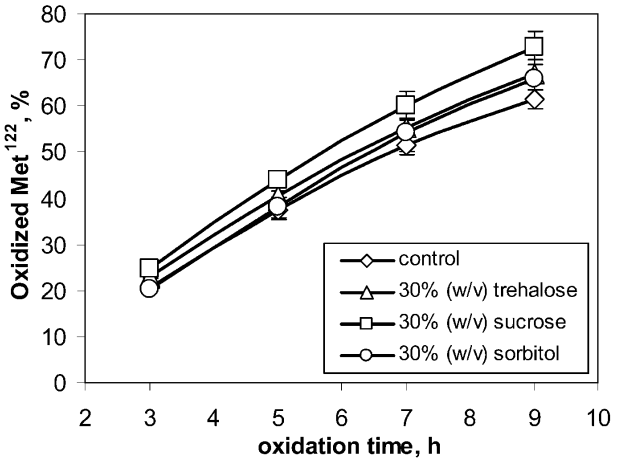

Fig. 4. Effect of different sugars and polyols on the oxidation of Met^{122} in G-CSF. Oxidation reactions were carried out with 300 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C. Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

potentially be used as preferential binding excipients to protect proteins against oxidation.

The most frequently used chelating agents in pharmaceutical formulations are EDTA and citric acid (1). The effect of EDTA concentration on the oxidation of Met^{122} (second phase) was first studied in solutions containing 300 mM H_2O_2, 0.1 mg/ml G-CSF, 25 mM acetate (pH 4.5), and 0, 0.06, 0.6, 6, 60, 200 mM EDTA, respectively. Surprisingly, the rate of oxidation of Met^{122} decreased more and more with progressively increasing concentrations of EDTA (Fig. 7A). In further studies, similar phenomena were also observed (Fig. 8B) on Met^{127}, Met^{138} and Met^{122} (first phase). For example at 200 mM EDTA, the decrease in the rate of oxidation was 33% for Met^{122} (second phase), 26% for Met^{122} (first phase), 14% for Met^{127}, and 6.5% for Met^{138}. However, EDTA had no significant effect on the rate of oxidation of Met^1 (less than 1.5%) (Fig. 8A). The efficacy of EDTA on the rates of oxidation of methionine residues in G-CSF at pH 4.5 follows the order of $Met^{122} > Met^{127} > Met^{138} > Met^1$. This order is just the reverse of the order of the rates of oxidation of these four methionine residues induced by H_2O_2 (8,13). It seems that the

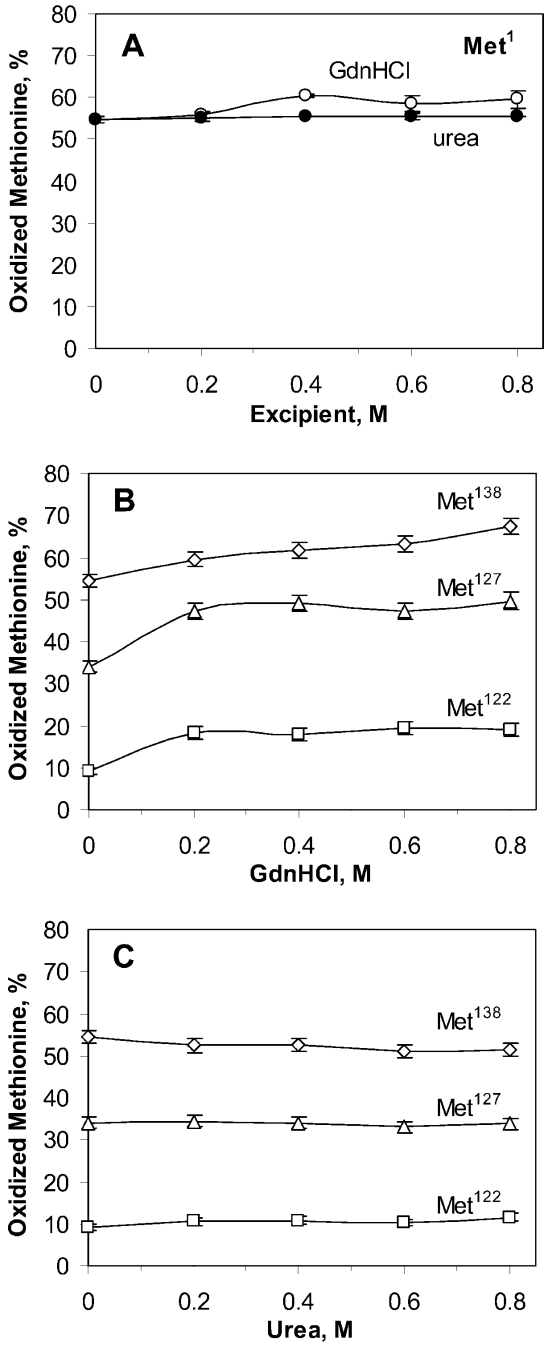

Fig. 5. Effect of GdnHCl and urea on the oxidation of individual methionine residues in G-CSF: (A) GdnHCl and urea to Met^1; (B) GdnHCl to $Met^{122,127,138}$; (C) urea to $Met^{122,127,138}$. Oxidation reactions were carried out with 60 mM H_2O_2, 0.3 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C for 0.5 h (Met^1) or 2 h ($Met^{122,127,138}$). Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

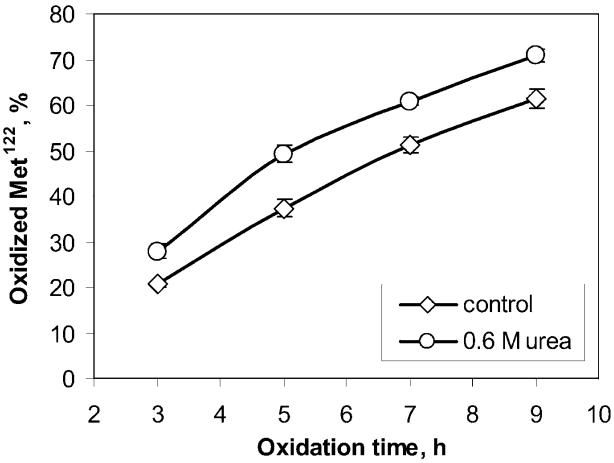

Fig. 6. Effect of urea on the oxidation of Met^{122} in G-CSF. Oxidation reactions were carried out with 300 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C. Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

more exposed the methionine residue is, the more difficult it is to be blocked.

The effect of citrate on the rates of oxidation of individual methionine residues in G-CSF is different from that of EDTA. As shown in Fig. 7B, the oxidation of Met^{122} (second phase) decreases by 11% when the concentration of citrate is changed from 0 to 0.6 mM. Increasing the concentration of citrate up to 200 mM increases the rate of oxidation of Met^{122} (second phase) by up to 40%. Similar trends on the rate of oxidation of $Met^{1,127,138}$ and Met^{122} (first phase) were also observed (Fig. 9). When the concentration of citrate was changed from 0 to 0.6 mM, the rate of oxidation was decreased by 1.2% for Met^1, 16% for Met^{122} (first phase), 5.8% for Met^{127}, and 1.7% for Met^{138}. Increasing the concentration of citrate to 200 mM increased the rate of oxidation by 10% for Met^1, 35% for Met^{122} (first phase), 14% for Met^{127}, and 5.8% for Met^{138}.

A full explanation of why EDTA and citrate affect H_2O_2-induced methionine oxidation of G-CSF does not yet exist. In general, EDTA and citrate are recognized as chelating agents. However, they are also salts that will be ionized above certain pHs. As chelating agents, EDTA and citrate may form complexes with transition metal ions, inhibiting the metal catalyzed, site-specific oxidation. As salts or ions, they may bind to protein sites to form protein-electrolyte complexes. According to studies on other salts (2), polyions (21), and small-molecular-weight multi-ions (22), the effect of salts/ions on protein stability is complex: it may stabilize, destabilize, or have no effect on protein stability, depending on the type and concentration of salt, the nature of ionic interactions, and the effects of charged residues in proteins. The net effect of EDTA or citrate on protein oxidation is a combination or balance between its action as a metal scavenger and its specific ion binding to a protein. At low concentrations, the dominant effect of EDTA and citrate is that of metal scavengers. Therefore, EDTA and citrate were observed to decrease the rates of oxidation of methionine residues in G-CSF. At high concentrations, the metal scavenger effect is saturated and the specific ion binding effect becomes important. That low concentrations of EDTA or citrate decrease the rates of oxidation of methionine residues in G-CSF also implies 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 (23,24).

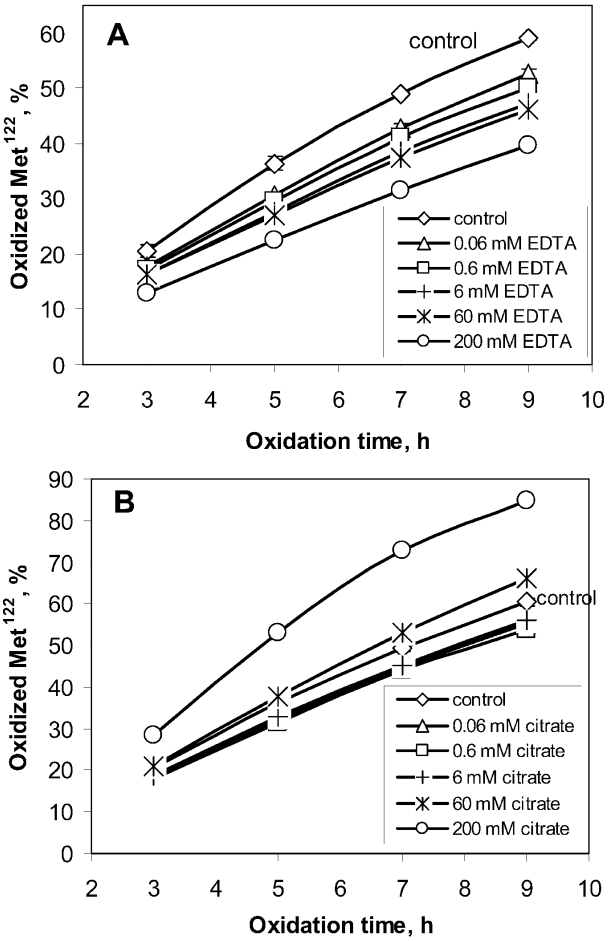

Fig. 7. Effect of EDTA and citrate on the oxidation of Met^{122} in G-CSF: (A) EDTA; (B) citrate. Oxidation reactions were carried out with 300 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C. Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

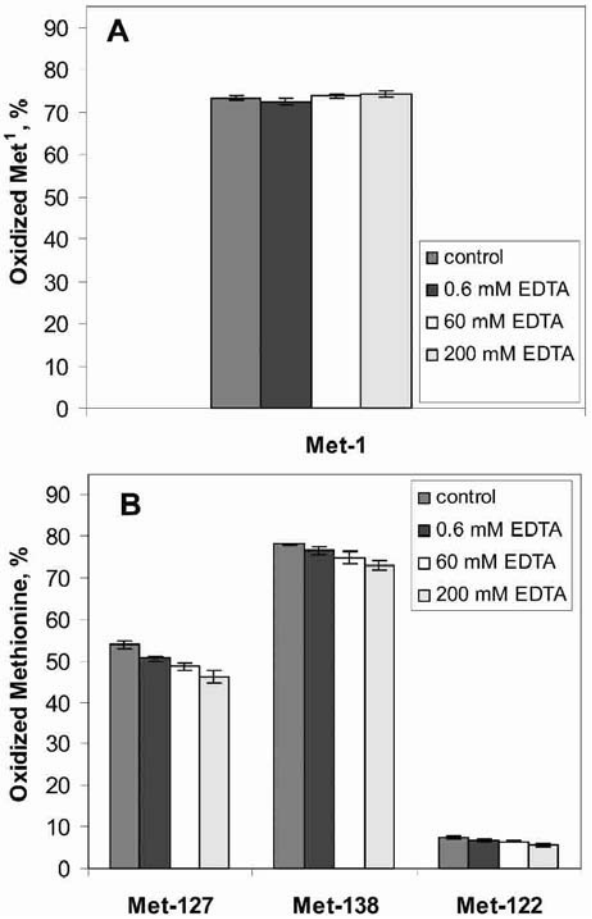

Fig. 8. Effect of EDTA on the oxidation of individual methionine residues in G-CSF: (A) Met^1; (B) $Met^{122,127,138}$. Oxidation reactions were carried out with 60 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C for 0.8 h (Met^1) or 4 h ($Met^{122,127,138}$). Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

G-CSF has an isoelectric point (pI) of 6.2 (25), and its residues that may be charged include four Asp (pKa = 3.9), nine Glu (pKa = 4.07), five His (pKa = 6.04), four Lys (pKa = 10.79), and five Arg (pKa = 12.48) (26). EDTA has four carboxylic acid groups (pKa's = 0.032, 1.0, 2.0, 2.66) and two amine groups (pKa's = 6.16, 10.26), while citric acid has three acidic sites (pKa's = 3.13, 4.77, 6.40) (27). At pH 4.5, the major forms of EDTA are $(EDTA)^{4-}$ (98% of total), a multi-ion, while the major forms of citrate are $(citrate)^-$ and $(citrate)^{2-}$. As a multi-ion, $(EDTA)^{4-}$ may by chance have the appropriate geometry to cross-link unpaired charges on the surface of native G-CSF and "lock" or "staple" G-CSF to its native state. In addition, the bound EDTA may block a certain portion of the G-CSF surface. Such effects could be studied directly using molecular simulations (13,14,28). The crosslinking interaction and/or blocking would be expected to decrease the rates of oxidation of methionine residues, especially Met^{122}, because Met^{122} is buried and can be blocked easily. It seems that $(citrate)^-$ and $(citrate)^{2-}$ bind such that oxidizable sites in G-CSF are more exposed. Additional work in this area is still needed in order to understand the detailed mechanisms of EDTA and citrate effects. Molecular simulations could be of tremendous help in this regard (13,14,28).

CONCLUSIONS

The current work reveals that different preferential excluding or binding excipients have different effects on the oxidation of methionine residues in G-CSF. Preferential excluding excipients such as sorbitol, sucrose and trehalose do not reduce the rates of oxidation of methionine residues in G-CSF. Even at low concentrations, the preferential binding excipients urea and GdnHCl do not reduce the rates of oxidation of methionine residues in G-CSF either. The chelating agents EDTA and citrate have different effects on G-CSF oxidation. At low concentrations, EDTA and citrate can both slightly decrease the rates of oxidation of methionine residues in G-CSF. The effect is probably due to their action as metal chelators so that they form complexes with transition metal ions and inhibit the metal catalyzed site-specific oxidation. At high concentrations, citrate increases and EDTA decreases the rates of oxidation of methionine residues in G-CSF. The mechanism may be due to specific ion binding effects. These

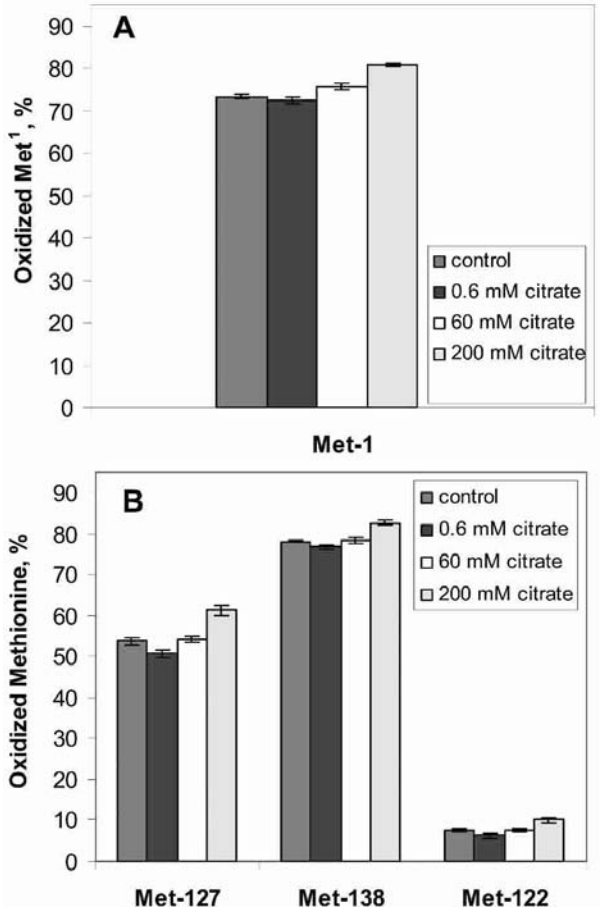

Fig. 9. Effect of citrate on the oxidation of individual methionine residues in G-CSF: (A) Met^1; (B) $Met^{122,127,138}$. Oxidation reactions were carried out with 60 mM H_2O_2, 0.1 mg/ml G-CSF in 25 mM acetate (pH 4.5) at 25°C for 0.8 h (Met^1) or 4 h ($Met^{122,127,138}$). Values are the mean of duplicate experiments, and the vertical bars represent the range spanned by the highest and lowest values.

effects could be studied using all atom molecular simulations, a subject of on-going research in our group.

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