Chemical Pathways of Peptide Degradation: IX. Metal-Catalyzed Oxidation of Histidine in Model Peptides

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Purpose. To elucidate the nature of the reactive oxygen species (i.e., superoxide anion radical, hydroxyl radical, and hydrogen peroxide) involved in the metal-catalyzed oxidation of histidine (His) in two model peptides.

Methods. The degradation of AcAla-His-ValNH$_2$ (Ala-peptide) and AcCysNH$_2$-S-S-AcCys-His-ValNH$_2$ (Cys-peptide) was investigated at pH 5.3 and 7.4 in an ascorbate/cupric chloride/oxygen (ascorbate/Cu(II)/O$_2$) system, both in the absence and presence of selective scavengers (i.e., catalase, superoxide dismutase, mannitol, sodium formate, isopropanol, and thiourea) of the reactive oxygen species. All reactions were monitored by HPLC. The major degradation products were characterized by electrospray mass spectrometry.

Results. The Cys-peptide was more stable than the Ala-peptide at pH 5.3 and 7.4. Both peptides displayed greater stability at pH 5.3 than at 7.4. At pH 5.3, 35 ± 0.7% of the Cys-peptide and 18 ± 1% of the Ala-peptide remained after 7 hours, whereas at pH 7.4, 16 ± 3% of the Cys-peptide and 4 ± 1% of the Ala-peptide remained. Catalase, thiourea, bicinchoninic acid, and ethylenediaminetetraacetate were effective at stabilizing both peptides toward oxidation, while superoxide dismutase, mannitol, isopropanol, and sodium formate were ineffective. The main degradation products of the Ala- and Cys-peptides at pH 7.4 appeared to be AcAla-2-oxo-His-ValNH$_2$ and AcCysNH$_2$-S-S-AcCys-2-oxo-His-ValNH$_2$, respectively.

Conclusions. Hydrogen peroxide, Cu(I), and superoxide anion radical were deduced to be intermediates involved in the oxidation of the Ala- and Cys-peptides. Hydrogen peroxide degradation to secondary reactive oxygen species may have led to the oxidation of the peptides. The degradation of hydrogen peroxide by a Fenton-type reaction was speculated to form a complexed form of hydroxyl radical that reacts with the peptide before diffusion into the bulk solution.

KEY WORDS: histidine; ascorbate; cupric chloride; metal-catalyzed oxidation; reactive oxygen species.

INTRODUCTION

Metal-catalyzed oxidation, in which a transition metal ion, reducing agent, and oxygen react to form reactive oxygen species (ROS), is known to occur *in vivo* and *in vitro* (1). *In vivo*, metals such as Fe(III) and Cu(II) can react with a reducing agent (e.g., flavoprotein, ascorbate, RSH) and oxygen to generate ROS such as hydroxyl radical (•OH), hydrogen peroxide (H$_2$0$_2$), and superoxide anion radical ($0_2^{\cdot-}$) which can ultimately cause damage to proteins (1,2). Oxidation of proteins in the body is believed to be the underlying mechanism of many disease states and even the aging process (2–4). For example, there is a growing body of evidence indicating that free radical damage to cellular function is involved with disease states such as atherosclerosis, arthritis, muscular dystrophy, cataractogenesis, pulmonary dysfunction, various neurological disorders and, very likely, cancer (1,5). The oxidation of histidine (His) to 2-oxo-His in proteins, which is the subject of this study, is believed to occur in mammalian cells during aging and oxidative stress (6,7).

Protein and peptide development in pharmaceutical processes such as synthesis, purification, storage of bulk drug, and storage of the dosage form can provide for conditions leading to metal-catalyzed oxidation reactions (8). An example of such a process is the utilization of immobilized metal affinity chromatography (IMAC) for protein separation and purification (9,10). IMAC takes advantage of the affinity of amino acids such as His and Cys for metal ligands [i.e., Cu(II), Fe(III)] as a basis for separation (11,12). It has been shown that IMAC using Cu(II) as the ligand can result in the metal-catalyzed oxidation of lactate dehydrogenase in the presence of reducing agents such as ascorbate and thiol agents (13). Therefore, the utilization of Cu(II) IMAC may result in damaged protein product when using crudes from cell lysates due to the presence of endogenous reducing agents that initiate metal-catalyzed oxidation reactions (13).

Relaxin is another protein that undergoes metal-catalyzed oxidation (14). In an ascorbate/Cu(II)/O$_2$ system, both Met and His residues in relaxin underwent oxidation. The metal-catalyzed oxidation of relaxin led to aggregation and precipitation of the protein in a pH-dependent manner. At pH 5 there was formation of soluble aggregates, and at pH 7 precipitation of the protein took place (14). In another investigation of the oxidation of relaxin in a H$_2$0$_2$ system, it was found that the Met residues were oxidized (15). This oxidation did not induce conformational change or cause the loss of biological activity (15). Therefore, it was hypothesized that, in the metal-catalyzed oxidative system, the oxidation of the His residue was crucial in provoking the structural changes leading to the aggregation of the protein (14).

In an attempt to elucidate the nature of the ROS involved in the oxidation of His in relaxin, we have studied the ascorbate/Cu(II)/O$_2$ mediated oxidation of a model peptide AcCysNH$_2$-S-S-AcCys-His-ValNH$_2$ (Cys-peptide), which is the His-containing peptide fragment of relaxin. To determine whether the cystine (Cys-S-S-Cys) functional group on the N-terminal side of His affects the rate of oxidation or the nature of the oxidation products, a second model tripeptide, AcAla-His-ValNH$_2$ (Ala-peptide), was studied for comparison purposes.

MATERIALS AND METHODS

Materials

The model peptides AcAla-His-ValNH$_2$ and AcCysNH$_2$-S-S-AcCys-His-ValNH$_2$ were synthesized by the Biochemical Service Laboratory at The University of Kansas, Lawrence,

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ABBREVIATIONS: ROS, reactive oxygen species; TFA, trifluoroacetic acid; BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetate; CAT, catalase; SOD, superoxide dismutase; Cx, complex; IMAC, immobilized metal affinity chromatography; H$_2$O$_2$, hydrogen peroxide; •OH, hydroxyl radical; $O_2^{\cdot-}$, superoxide anion radical.

KS. Catalase (CAT) (bovine liver, thymol-free, 199,000 U/mg), superoxide dismutase (SOD) (bovine erythrocyte, 4,400 U/mg), ascorbic acid, $CuCl_2$, bicinchoninic acid (BCA), ethylenediaminetetraacetate (EDTA), sodium phosphate (monobasic and dibasic), D-mannitol, and H_2O_2 (30% W/W solution) were purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade), isopropanol, thiourea, and sodium formate were supplied by Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA, HPLC grade) was purchased from Acros (Springfield, NJ). The water used in all studies was from a Millipore MILLI-Q™ Water System. All reagents were obtained commercially as the analytical grade.

Reactions

Unless otherwise specified, the oxidation of the His-containing peptides by the ascorbate/Cu(II)/O_2 system was performed under the following conditions: 250µl solutions contained 0.273 mM peptide, 2 mM ascorbate, 50 µM $CuCl_2$, and 20 mM phosphate buffer at either pH 5.3 or 7.4. This solution was incubated at room temperature (25°C) in 250 µl inserts in 2 ml vials without the cap, which allowed for free exchange of atmospheric oxygen to the reaction mixture. The reagents were added in the following order: buffer, peptide, ascorbate, and $CuCl_2$. Stock solutions of ascorbate and $CuCl_2$ were prepared prior to each reaction. The involvement of reactive oxygen intermediates in the oxidation process was analyzed by the addition of CAT, SOD, and ·OH scavengers such as isopropanol, thiourea, sodium formate, and D-mannitol. All reactions were monitored by reversed-phase HPLC at room temperature (25°C).

HPLC Analysis

HPLC analysis was performed on a system consisting of a Shimadzu SCL-6B system controller, a Shimadzu SCL-6A pump, a Shimadzu SPD-6A UV spectrophotometric detector, a Perkin Elmer ISS-100 autoinjector and a C-R4A Chromatopac integrator. The analysis of the His-containing peptides was performed by HPLC using a Vydac 218TP C_{18} reverse phase column (4.6 × 250 mm) at room temperature (25°C). An isocratic system was utilized at a flow rate of 1 ml/min. The mobile phase was a mixture of acetonitrile/water (4/96, v/v) containing 0.1% TFA for the Ala-peptide where the Ala-peptide eluted at 10.3 min and the main degradation product determined to be AcAla-2-oxo-His-ValNH_2 eluted at 11.7 min. A mixture of acetonitrile/water (7/93, v/v) containing 0.1% TFA was utilized for the Cys-peptide where the Cys-peptide eluted at 8.9 rain and the main degradation product determined to be AcCysNH_2-S-S-AcCys-2-oxo-His-ValNH_2 eluted at 10.2 min. Detection of the His-containing peptides was achieved at 214 nm and was quantified by measuring peak areas referenced to standard curves generated with pure His-containing peptides.

RESULTS

Oxidation of Ala- and Cys-Peptides by the Ascorbate/Cu(II)/O_2 System at pH 5.3 and 7.4

The Ala-peptide and Cys-peptide did not undergo degradation in solutions containing ascorbate or $CuCl_2$ alone up to 16 hours of incubation at pH 5.3 (Fig. 1, Table I) or pH 7.4 (Table I). However, both peptides underwent rapid degradation in the presence of ascorbate and $CuCl_2$ together at pH 5.3 (Fig. 1, Table I) and pH 7.4 (Table I). The peptides exhibited biphasic kinetics of degradation at both pH 5.3 and 7.4. Fig. 1 shows that at pH 5.3 a rapid initial phase (2–3 min) is followed by a slower phase of degradation, resulting in a plateau phase after 7 hours. No further degradation of the peptides occurred up to 16 hours of incubation. The incomplete degradation of the peptides was shown to result from depletion of ascorbate (data not shown). The data shown in Table I also indicate that the peptides are more stable at pH 5.3 than pH 7.4, and that the Cys-peptide is more stable than the Ala-peptide at both pH values studied.

The reaction mixtures containing ascorbate, $CuCl_2$, and the model peptides were analyzed by HPLC after 16 hours; in each case, a major degradation product and numerous minor degradation products were observed (data not shown). When these major degradation products from the Cys-peptide and Ala-peptide reaction mixtures at pH 7.4 were purified by HPLC, they were shown by electrospray mass spectrometry to have masses consistent with AcAla-2-oxo-His-ValNH_2 and

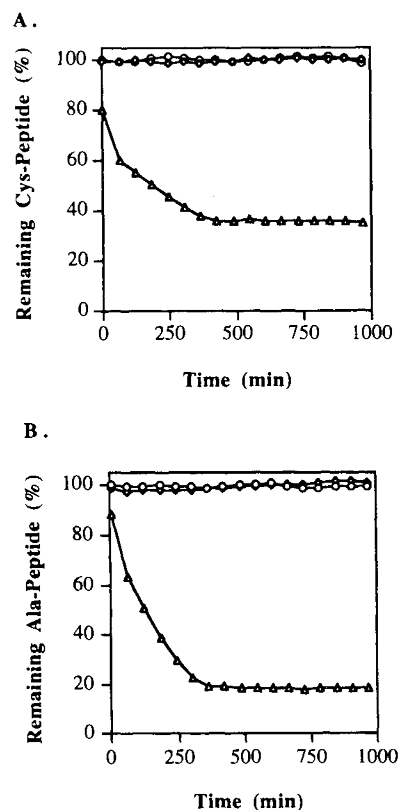


Fig. 1. Time course of degradation of the Ala- and Cys-peptides in an ascorbate/Cu(II)/O_2 system at pH 5.3. Reaction mixtures contained 0.273 mM peptide, 2 mM ascorbate, 50 µM $CuCl_2$, and 20 mM phosphate buffer at pH 5.3. Panel A: (○) Cys-peptide in the presence of ascorbate only; (◇) Cys-peptide in the presence of $CuCl_2$ only; (△) Cys-peptide in the presence of ascorbate and $CuCl_2$. Panel B: (○) Ala-peptide in the presence of ascorbate only; (◇) Ala-peptide in the presence of $CuCl_2$ only; (△) Ala-peptide in the presence of ascorbate and $CuCl_2$.

Table I. Oxidation of the Ala- and Cys-Peptides in an Ascorbate/ $Cu(II)/O_2$ System [a]

Additions	Ala-peptide remaining (%)		Cys-peptide remaining (%)	
	pH 5.3	pH 7.4	pH 5.3	pH 7.4
Ascorbate (2 mM)	100	99	100	100
$CuCl_2$ (50 μM)	100	100	100	100
Ascorbate (2 mM) $CuCl_2$ (50μM)	18	4	35	16
Ascorbate (2mM) $CuCl_2$ (50μM) EDTA (60 μM)	97	84	88	80

[a] Reaction conditions: The reaction mixtures contained 0.273 mM peptide in 20 mM phospate buffer at pH 5.3 or 7.4. The reactions were monitored by HPLC at room temperature (25°C) and % peptide remaining was determined once the reaction had reached completion, i.e. a plateau phase had been reached. All the data are averages of triplicates. Standard deviation is less then 4% for all experiments.

$AcCysNH_2$-S-S-AcCys-2-oxo-His-$ValNH_2$, respectively (data not shown).

The Role of Cu(I) and Cu(II) in the Oxidation of the Ala- and Cys-Peptides in an Ascorbate/Cu(II)/O_2 System

The critical role of Cu(II) in the oxidation of these His-containing peptides is illustrated by the observation that inclusion of 60 μM EDTA in the ascorbate/Cu(II)/O_2 system stabilizes the peptides toward degradation (Table I). The role of Cu(I) in an ascorbate/Cu(II)/O_2 system was investigated by using BCA, which is a Cu(I) specific chelator. When $CuCl_2$ (50 μM) was added to solutions containing Ala-peptide (or Cys-peptide), 2 mM ascorbate, and 200 μM BCA in pH 7.4, 20 mM phosphate buffer, the solutions turned purple, which is indicative of BCA complexation with Cu(I) (13). When BCA was added to the ascorbate/Cu(II)/O_2 system, the oxidation of the His-containing peptides was completely inhibited (Fig. 2). This is indicative of the importance of Cu(I) as an intermediate in the oxidation process.

Determination of the Role of H_2O_2 and $O_2^{\cdot -}$ in the Oxidation of the Ala- and Cys-Peptides in an Ascorbate/Cu(II)/O_2 System

CAT is an enzyme commonly used to determine the role of freely diffusible H_2O_2 in oxidative reactions. CAT catalyzes the decomposition of H_2O_2 to water and oxygen ($k > 10^7$ $M^{-1}S^{-1}$) (16). When freshly prepared solutions of CAT (2000 U/ml) were added to the reaction mixture containing 2 mM ascorbate in pH 7.4, 20 mM phosphate buffer prior to the addition of 50 μM $CuCl_2$, the oxidation of the Ala- and Cys-peptides in an ascorbate/Cu(II)/O_2 was completely inhibited at both pH 5.3 and 7.4 (Table II). However, heat-inactivated CAT had no effect on the oxidation of the peptides, indicating that CAT was not competing with the peptides for the ROS (Table II). The stabilization of the peptides in the ascorbate/Cu(II)/O_2 system by native CAT indicates that freely diffusible H_2O_2 plays an important role in the oxidation of the His-containing peptides. However, when 1.3 mM H_2O_2 was added to the Ala- and Cys-peptides (0. 273 mM) in 20 mM phosphate buffer at pH 5.3 and 7.4, the peptides did not undergo any significant degradation over a 20-hour incubation period. This indicates that H_2O_2 alone is not damaging to the peptides. SOD is an enzyme commonly used to determine the role of freely diffusible $O_2^{\bullet -}$ in oxidative reactions. SOD catalyzes the dismutation of

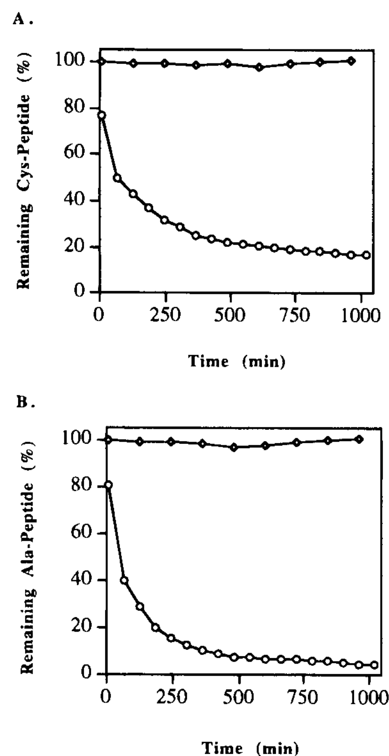


Fig. 2. Effect of BCA on the time course of degradation of the Ala- and Cys-peptides in an ascorbate/Cu(II)/O_2 system at pH 7.4. Reaction mixtures contained 0.273 mM peptide, 2 mM ascorbate, 50 μM $CuCl_2$, and 20 mM phosphate buffer (pH 7.4) in the absence or presence of 200 μM BCA. Panel A: (○) Cys-peptide in the presence of ascorbate and $CuCl_2$; (◇) Cys-peptide in the presence of ascorbate, $CuCl_2$, and BCA. Panel B: (○) Ala-peptide in the presence of ascorbate and $CuCl_2$; (◇) Ala-peptide in the presence of ascorbate, $CuCl_2$, and BCA.

Table II. Effects of CAT and SOD on the Oxidation of the Ala- and Cys-Peptides by the Ascorbate/Cu(II)/O_2 System[a]

Additions	Remaining Ala-peptide (%)		Remaining Cys-peptide (%)	
	pH 5.3	pH 7.4	pH 5.3	pH 7.4
Ascorbate/$CuCl_2$	18	4	35	16
Ascorbate/$CuCl_2$/CAT	100	100	100	95
Ascorbate/$CuCl_2$/Boiled CAT	17	7	38	15
Ascorbate/$CuCl_2$/SOD	14	8	36	17
H_2O_2	98	97	97	98

[a] Reaction conditions: The reaction mixtures contained Ala- or Cys-peptide (0.273 mM) in phosphate buffer (20 mM) at pH 5.3 or 7.4 and Ascorbate (2 mM)/$CuCl_2$ (50 μM) in the absences or presences of CAT (2000 U/ml), boiled CAT (2000 U/ml) or SOD (200 U/ml) or H_2O_2 (1.3 mM). The incubations were carried out at room temperature (25°C) for 20 hours. All the data are averages of triplicates. Standard deviation is less then 4%.

$O_2^{\bullet -}$ to O_2 and H_2O_2 (17). When freshly prepared solutions of SOD (200 U/ml) were added to the reaction mixture prior to the addition of $CuCl_2$, this enzyme had no effect on the degradation of the Ala- and Cys-peptides at pH 5.3 or 7.4 (Table II). These results indicate that freely diffusible $O_2^{\bullet -}$ is not directly involved in the oxidation of the peptides but may serve as a source for the generation of H_2O_2, which may then react with species in solution to yield other radicals that ultimately oxidize the peptides.

Oxidation of the Ala- and Cys-Peptides in a Cu(II)/H_2O_2 System

As described above, the His-containing peptides did not undergo oxidation in solutions containing $CuCl_2$ or H_2O_2 alone (Table I and Table II) However, both peptides underwent rapid degradation in 20 mM phosphate buffer, pH 7.4, containing H_2O_2 (2 mM) and $CuCl_2$ (50 μM). There were two interesting differences observed between the oxidation of the Ala- and Cys-peptides in the Cu(II)/H_2O_2 system (Fig. 3) versus the ascorbate/Cu(II)/O_2 system (Fig. 1): (i) in the Cu(II)/H_2O_2 system, the peptides were degraded completely, whereas the ascorbate/Cu(II)/O_2 system produced only partial degradation; and (ii) in the Cu(II)/H_2O_2 system, a different profile of degradation products was observed compared to the ascorbate/Cu(II)/O_2 system.

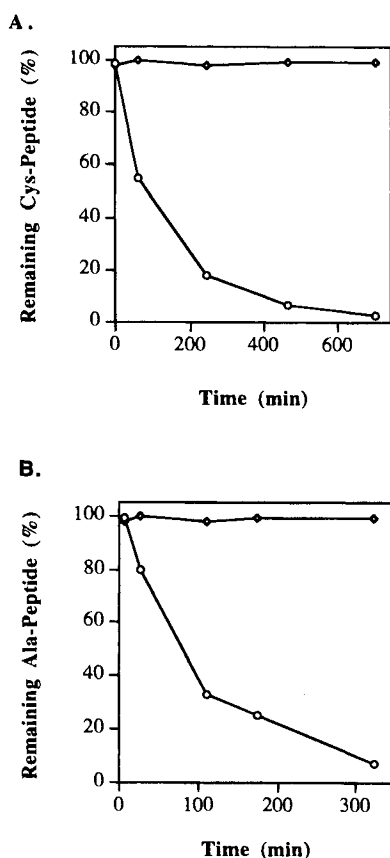


Fig. 3. Time course of degradation of the Ala- and Cys-peptides in a Cu(II)/H_2O_2 system at pH 7.4 in the presence or absence of BCA. Reaction mixtures contained 0.273 mM peptide, 2 mM H_2O_2, 50 μM $CuCl_2$, 20 mM phosphate buffer (pH 7.4) with or without 200 μM BCA. Panel A: (○) Cys-peptide in the presence of $CuCl_2$ and H_2O_2; (◇) Cys-peptide in the presence of $CuCl_2$, H_2O_2, and BCA. Panel B: (○) Ala-peptide in the presence of $CuCl_2$ and H_2O_2; (◇) Ala-peptide in the presence of $CuCl_2$, H_2O_2, and BCA.

The role of Cu(I) in a Cu(II)/hydrogen peroxide system was investigated by incubation of the Ala- and Cys-peptides with 50 μM $CuCl_2$, 2 mM H_2O_2 and 200 μM BCA in 20 mM phosphate buffer, pH 7.4. As stated previously, BCA is a Cu(I)-specific chelator. When $CuCl_2$ was added to the reaction mixture to initiate the reaction, the solution turned purple, indicating the complexation of Cu(I) with BCA (13). The Ala- and Cys-peptides were stable toward oxidation when BCA was included in the reaction mixtures (Fig. 3), which indicates that Cu(I) is generated in the Cu(II)/H_2O_2 system.

The Role of •OH in the Oxidation of the Ala- and Cys-Peptides in an Ascorbate/Cu(II)/O_2 System

The role of freely diffusible •OH as a damaging species to the Ala- and Cys-peptides was investigated by the utilization of the following •OH scavengers that react with •OH with the indicated rate constants: mannitol, $k = 1.7 \times 10^{-9}\ M^{-1}\ s^{-1}$; sodium formate, $k = 3.5 \times 10^{-9}\ M^{-1}\ s^{-1}$; thiourea, $k = 3.9 \times 10^{-9}\ M^{-1}\ s^{-1}$; and isopropanol, $k = 1.9 \times 10^{-9}\ M^{-1}\ s^{-1}$ (18). Addition of these scavengers to the ascorbate/Cu(II)/O_2 system at concentrations that should have had a significant protective effect on oxidation of the His-containing peptides if freely diffusible •OH played a role, was investigated. Mannitol, sodium formate, and isopropanol had no impact on the oxidative process. Thiourea yielded some stabilization toward oxidation of the peptides (Table III). The overall results indicate that freely diffusible •OH is not directly responsible for the oxidation of the peptides.

DISCUSSION

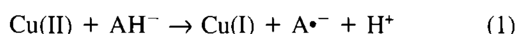
An interesting observation made in this study was that the Ala- and Cys-peptides did not undergo complete degradation in the ascorbate/Cu(II)/O_2 system. The incomplete degradation of the peptides appeared to be due to depletion of ascorbate during the oxidation process. An explanation for this phenomenon is obvious when one takes into consideration that ascorbate

Table III. Effect of •OH Scavengers on the Oxidation of the Ala- and Cys-Peptides in an Ascorbate/Cu(II)/O_2 System[a]

Additions	% Peptide remaining	
	Ala-peptide	Cys-peptide
None	4	16
Mannitol (8 mM)	7	21
Isopropanol (8 mM)	8	21
Sodium Formate (4 mM)	7	21
Thiourea (4 mM)	75	50

[a] Reaction conditions: The reaction mixtures contained Ala- or Cys-peptide (0.273 mM) and Ascorbate (2 mM)/$CuCl_2$ (50 μM) in 20 mM phosphate buffer, pH 7.4. The reactions were monitored by HPLC at room temperature (25°C) and % peptide remaining was determined once the reaction had reached completion, i.e., a plateau phase had been reached. All the data are averages of triplicates. Standard deviation is less then 4% for all experiments.

can be both an antioxidant and a prooxidant. As a prooxidant, ascorbate reacts with metal and O_2 to form ROS and, in the process, is oxidized to dehydroascorbic acid. The following reactions represent a possible route of ascorbate oxidation in the presence of Cu(II) where AH^-, $A\bullet^-$, and A denote ascorbate, ascorbyl radical anion, and dehydroascorbic acid, respectively.



$$\text{Cu(II)} + \text{AH}^- \rightarrow \text{Cu(I)} + \text{A}\bullet^- + \text{H}^+ \quad (1)$$

$$\text{Cu(II)} + \text{A}\bullet^- \leftrightarrow \text{Cu(I)} + \text{A} \quad (2)$$

The species formed in the reactions described above can then react with O_2 to form ROS including $O_2\bullet^-$, •OH, and H_2O_2 (19,20). However, ascorbate can also be an antioxidant, in which case it scavenges the ROS (21). Depletion of ascorbate in these processes leads to the plateau phase of reaction.

As shown in Table I, both His-containing peptides displayed greater stability at pH 5.3 than at pH 7.4 in the ascorbate/Cu(II)/O_2 system. This pH-dependent stability could result from the difference in metal-binding capacity of the protonated versus unprotonated His imidazole ring. His oxidation is a site-specific process in which Cu(II) forms a complex with the imidazole ring of His and other species in solution (22,23). It is within this complex that metal-catalyzed oxidation occurs, leading to site-specific damage (24,25). Based on the reported pKa values for the imidazole ring of the Gly-His peptide (pKa = 6.76) and Ala-His peptide (pKa = 6.77) (26), it is not unreasonable to assume that the imidazole rings in the His-containing peptides used in this study have pKa values in the range of 6.5–7.0. Therefore, the His-containing peptides will have a higher fraction of the unprotonated form at pH 7.4 than at pH 5.3. It is well documented (26,27) that the unprotonated imidazole ring binds metals more effectively than the protonated form. Another factor that may influence the stability of the peptides at different pH values is the ionization state of ascorbic acid ($pKa_1 = 4.17$) ($pKa_2 = 11.57$). At a higher pH (e.g. pH 7.4), ascorbic acid exists as the anion ascorbate. Ascorbate is more efficient than ascorbic acid at reducing Cu(II) to Cu(I) and, therefore, promotes oxidation (21). However, ascorbate increasingly functions as an antioxidant at higher pH values and in turn scavenges the ROS (21). This delicate balance is yet another factor that might determine the extent and rate of oxidation of substrates like the His-containing peptides. It is interesting to note that in an investigation of the metal-catalyzed oxidation of glutamine synthetase, the highest yield of oxidation was observed at pH 7.5 (28).

Based on the experimental results with BCA (Fig. 2), Cu(I) appears to play an important role in the oxidation of the peptides in the ascorbate/Cu(II)/O_2 system. As described above (Eq. 1 and 2), Cu(I) may be generated via the reduction of Cu(II) by ascorbate. Cu(I) may then undergo further reaction to generate ROS. Free aqueous Cu(I) is reported to be very unstable (29). Therefore, Cu(I) is most likely complexed with the species in solution.

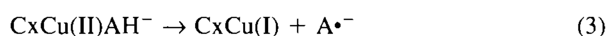
In an attempt to elucidate the nature of the ROS, we studied the effects of CAT, SOD, and •OH scavengers on the oxidation of the model peptides. The results with CAT (Table II) suggested that freely diffusible H_2O_2 was an important intermediate in the oxidation of these peptides. However, the His-containing peptides were stable toward oxidation when incubated with H_2O_2 alone (Table II), which indicates that H_2O_2 itself was not the damaging species. In contrast, SOD had no significant effect on the oxidation of the model peptides (Table II). This suggests that diffusible $O_2\bullet^-$ was not the direct damaging species but rather served as a source for the generation of H_2O_2, which could then react further in solution to form other ROS that would oxidize the peptides.

A possible pathway of H_2O_2 degradation was investigated by incubating the peptides with $CuCl_2$ and H_2O_2, which led to the degradation of the peptides. It is important to note that in some cases degradation of peptides (i.e., Gly-Gly-His-Gly, Asp-Ala-His-Gly, Gly-His-His-Gly, and Gly-His-Lys) (30) and proteins (i.e., acetylcholine esterase) (31) is not observed in a Cu(II)/H_2O_2 system, although it is observed in an ascorbate/Cu(II)/O_2 system. This phenomenon has been rationalized by Ueda *et al.* (30), who proposed that the redox potential of the Cu(II) ion changes upon complexation to the peptide, leading to the inability of H_2O_2 to reduce the complexed Cu(II) to generate Cu(I). This was not the case with the Ala- and Cys-peptides. Cu(I) was generated, which may have then reacted with H_2O_2 to yield ROS damaging to the peptides (28). However, there were some distinct differences in the oxidation of the peptides in the ascorbate/Cu(II)/O_2 and Cu(II)/H_2O_2 systems. When exposed to the Cu(II)/H_2O_2 system, the model peptides underwent rapid and complete oxidation with no plateau phase observed (Fig. 3). However, in the ascorbate/Cu(II)/O_2 system, the peptides underwent incomplete degradation, resulting in a plateau phase. Furthermore, the degradant profile observed for the peptides was dependent on the oxidative system used. These results are consistent with literature reports that show that the Cu(II)/H_2O_2 system results in oxidation of His to Asp (32–34) while the ascorbate/Cu(II)/O_2 results in oxidation of His to 2-oxo-His (35–37). The differences in the degradation profiles observed with the Cu(II)/H_2O_2 and ascorbate/Cu(II)/O_2 systems may be due to the formation of different complexes and thus different degradants. In an ascorbate/Cu(II)/O_2 system it has been proposed that ascorbate, Cu(II), and the peptide are involved in the formation of a complex in which bound Cu(II) is reduced by ascorbate to generate site-specific ROS (24,25). The geometry and redox potential of this complex may play an important role in determining the nature of the ROS and thus the degradation products that are formed. Although the Cu(II)/H_2O_2 route of degradation can occur with the Ala- and Cys-peptides, it is obviously not the predominant pathway of degradation in the ascorbate/Cu(II)/O_2 system.

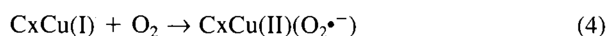
The reaction of Cu(I) with H_2O_2 can yield •OH by a Fenton-type reaction pathway. The role of freely diffusible •OH in the ascorbate/Cu(II)/O_2 system was investigated at pH 7.4 with the utilization of •OH scavengers. Mannitol, sodium formate, and isopropanol had no effect on the oxidation of the peptide. Thiourea yielded some stabilization of the peptides (Table III). However, the stabilization yielded by thiourea in this case was not thought to be due to the scavenging of freely diffusible •OH. This assumption is based on the observation by Kanazawa *et al.* (25) that thiourea protects papain from oxidation by trapping of Cu(II) and thus prevents the formation of free radicals at the specific site of inactivation. It is interesting to note that other investigators (14,38) have also reported that thiourea stabilizes molecules towards oxidation, whereas other •OH scavengers were ineffective.

While the results with •OH scavengers suggest that freely diffusible •OH is not responsible for the degradation of the Ala- and Cys-peptides, this does not rule out •OH as a damaging

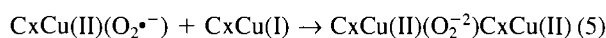
ROS. Instead, •OH may be generated in a site-specific manner and react immediately with the imidazole ring of His before diffusion into the bulk solution (1). It is apparent that both the His-containing peptides undergo a similar reaction pathway in the ascorbate/Cu(II)/O_2 system, in which H_2O_2 is generated and degraded by Cu(I) to yield the damaging ROS. As noted before, His-oxidation is a site-specific process where the imidazole ring of the His forms a complex with Cu(II) and other species in solution (22,23). ROS are generated within this complex, leading to site-specific degradation of the His-containing peptides (24,25). The reaction pathway resulting in formation of H_2O_2 is proposed in the following scheme (Eq. 3–6), in which a complex (Cx) is formed that may include water, ascorbate, peptide, phosphate, and Cu(II), within which bound Cu(II) is reduced by ascorbate. In this series of equations, ascorbate and ascorbyl radical anion are denoted by AH^- and $A\bullet^-$. The pertinent reacting species of the complex are illustrated individually.



$$\mathrm{CxCu(II)AH^-} \rightarrow \mathrm{CxCu(I)} + \mathrm{A\bullet^-} \quad (3)$$



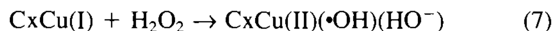
$$\mathrm{CxCu(I)} + \mathrm{O_2} \rightarrow \mathrm{CxCu(II)(O_2\bullet^-)} \quad (4)$$



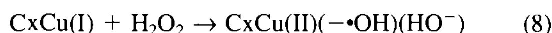
$$\mathrm{CxCu(II)(O_2\bullet^-)} + \mathrm{CxCu(I)} \rightarrow \mathrm{CxCu(II)(O_2^{-2})CxCu(II)} \quad (5)$$

$$\mathrm{CxCu(II)(O_2^{-2})CxCu(II)} + 2\mathrm{H^+} \rightarrow 2\mathrm{CxCu(II)} + \mathrm{H_2O_2} \quad (6)$$

The H_2O_2 generated may then undergo further reaction with CxCu(I) via a Fenton-type reaction pathway. In this reaction, •OH (Eq. 7) or an equivalent bound form of •OH (Eq. 8) may be generated in a site-specific manner, resulting in preferential oxidation of the His residue.



$$\mathrm{CxCu(I)} + \mathrm{H_2O_2} \rightarrow \mathrm{CxCu(II)(\bullet OH)(HO^-)} \quad (7)$$



$$\mathrm{CxCu(I)} + \mathrm{H_2O_2} \rightarrow \mathrm{CxCu(II)(-\bullet OH)(HO^-)} \quad (8)$$

A complexed form of •OH is hypothesized to be the damaging species that results in the formation of 2-oxo-His. In this regard, the formation of 2-oxo-His has been proposed to be due to a complexed and/or free form of hydroxyl radical in the metal-catalyzed oxidation of human growth hormone (36) and Cu(I) superoxide dismutase (7). It is important to note that the definite presence of the •OH has yet to be determined. Therefore, at this point in time, only speculation has been possible.

In conclusion, it appears that both the Ala- and Cys-peptides undergo a similar reaction pathway of degradation in which Cu(I) and H_2O_2 are important intermediates involved in the oxidation of the His residue to 2-oxo-His. The ultimate ROS responsible for damage to the peptides is speculated to be a complexed form of •OH generated via a Fenton-type reaction.

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