# Chemical Pathways of Peptide Degradation. X: Effect of Metal-Catalyzed Oxidation on the Solution Structure of a Histidine-Containing Peptide Fragment of Human Relaxin

**Mehrnaz Khossravi[1] and Ronald T. Borchardt[1,2]**

*Received February 2, 2000; accepted March 24, 2000*

***Purpose.*** To elucidate the major degradation products of the metal-catalyzed oxidation of (cyclo S-S) AcCys-Ala-X-Val-Gly-CysNH$_2$ (X = His, cyclic-His peptide), which is a fragment of the protein relaxin, and the effect of this oxidation on its solution structure.

***Methods.*** The cyclic-His peptide and its potential oxidative degradation products, cyclic-Asp peptide (X = Asp) and cyclic-Asn peptide (X = Asn), were prepared by using solid phase peptide synthesis and purified by preparative HPLC. The degradation of the cyclic-His peptide was investigated at pH 5.3 and 7.4 in an ascorbate/cupric chloride/oxygen [ascorbate/Cu(II)/$O_2$] system in the absence or presence of catalase (CAT), superoxide dismutase (SOD), isopropanol, and thiourea. The oxidation of the cyclic-His peptide was also studied in the presence of hydrogen peroxide ($H_2O_2$). All reactions were monitored by reversed-phase HPLC. The main degradation product of the cyclic-His peptide formed at pH 7.4 in the presence of ascorbate/Cu(II)/$O_2$ was isolated by preparative HPLC and identified by $^1$H NMR and electrospray mass spectrometry. The complexation of Cu(II) with the cyclic-His peptide was determined with $^1$H NMR. The solution structure of the cyclic-His peptide in the presence and absence of Cu(II) at pH 5.3 and 7.4 and the solution structure of the main degradation product were determined using circular dichroism (CD).

***Results.*** CAT and thiourea were effective in stabilizing the cyclic-His peptide to oxidation by ascorbate/Cu(II)/$O_2$, while SOD and isopropanol were ineffective. Cyclic-Asp and cyclic-Asn peptides were not observed as degradation products of the cyclic-His peptide oxidized at pH 5.3 and 7.4 in an ascorbate/Cu(II)/$O_2$ system. The main degradation product formed at pH 7.4 was the cyclic 2-oxo-His peptide (X = 2-oxo-His). At pH 5.3, numerous degradation products were formed in low yields, including the cyclic 2-oxo-His peptide. The cyclic 2-oxo-His peptide appeared to have a different secondary structure than did the cyclic-His peptide as determined by CD. $^1$H NMR results indicate complexation between the cyclic-His peptide and Cu(II). CD results indicated that the solution structure of the cyclic-His peptide in the presence of Cu(II) at pH 5.3 was different than the solution structure observed at pH 7.4.

***Conclusions.*** $H_2O_2$ and superoxide anion radical ($O_2^{\bullet-}$) were deduced to be the intermediates involved in the ascorbate/Cu(II)/$O_2$-induced oxidation of cyclic-His peptide. $H_2O_2$ degradation by a Fenton-type reaction appears to form secondary reactive-oxygen species (i.e., hydroxyl radical generated within complex forms or metal-bound forms of hydroxyl radical) that react with the peptide before they diffuse into the bulk solution. CD results indicate that different complexes are formed between the cyclic-His peptide and Cu(II) at pH 5.3 and pH 7.4. These different complexes may favor the formation of different degradation products. The apparent structural differences between the cyclic-His peptide and the cyclic 2-oxo-His peptide indicate that conformation of the cyclic-His peptide was impacted by metal-catalyzed oxidation.

**KEY WORDS:** ascorbate; cupric chloride; histidine; metal-catalyzed oxidation.

## INTRODUCTION

Metal-catalyzed oxidation may occur when a peptide/protein is exposed to a transition metal ion [i.e., Fe(III), Cu(II)], a reducing agent (i.e., ascorbate), and oxygen (1). Under these conditions, reactive-oxygen species such as $O_2^{\bullet-}$, $^{\bullet}OH$, and $H_2O_2$, which may ultimately cause damage to peptides/proteins, are generated (1,2). A feature of metal-catalyzed oxidations is the site-specific nature of the reaction, i.e. specific amino acid residues located at the metal binding sites are generally altered (2). The amino acid residues that are most susceptible to metal-catalyzed oxidations are His, Arg, Lys, Pro, Met, and Cys (1,3). A factor influencing the susceptibility of these amino acid residues to metal-catalyzed oxidation is their ability to form complexes with metals such as Cu(II) or Fe(III). It is within these complexes that reactive-oxygen species are generated, and oxidation of specific amino acid residues occurs in what is referred to as a "caged" process (1,4).

Metal-catalyzed oxidation of the protein relaxin in an ascorbate/Cu(II)/$O_2$ system has previously been studied in our laboratory (5). The primary amino acids that underwent oxidation in relaxin under these conditions were shown to be His and Met residues (5). Of particular interest was the observation that oxidation under these conditions led to precipitation of the protein in a pH-dependent manner, i.e., at pH 5 soluble degradants were observed, whereas at pH 7.4 degradants that precipitated were observed (5). In another study of relaxin using $H_2O_2$ as the oxidant, Nguyen *et al.* (6) reported only the oxidation of Met residues and no precipitation of the oxidized protein. Based on these observations, Li *et al.* (5) hypothesized that the metal-catalyzed oxidation of His in relaxin leads to aggregation/precipitation of the protein.

Relaxin consists of an A chain and a B chain which are linked together by interchain disulfide bonds. Based on the X-ray crystal structure of relaxin (Brookhaven Protein Data Bank entry 6RLX, Eigenbrot *et al.*), the His A(12) exists in an extended loop that joins two $\propto$ helices (7). Büllesbach and Schwabe (8) have proposed that the structural integrity of this extended loop region of relaxin is important for maintenance of the overall higher-ordered structure of relaxin. Therefore, we hypothesized that oxidation of this His could lead to changes in the structure of this extended loop region, which could then lead to alterations in the higher-ordered structure of the two alpha helices, ultimately exposing hydrophobic regions of the protein leading to its aggregation and precipitation.

To test this hypothesis, we synthesized a His-containing peptide fragment of the loop region of relaxin [i.e., (cyclo S-S) AcCys-Ala-His-Val-Gly-CysNH$_2$] in which the Cys A(11), present in relaxin to link the A chain to the B chain was replaced with an Ala. Earlier, our laboratory (9) has shown, using model

[1] Department of Pharmaceutical Chemistry; The University of Kansas, Lawrence, Kansas 66047.

[2] To whom correspondence should be addressed. (e-mail: rborchardt@ukans.edu)

**ABBREVIATIONS:** TFA, trifluoroacetic acid; BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetate; CAT, catalase; SOD, superoxide dismutase; $H_2O_2$, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet-}$, superoxide anion radical.



0724-8741/00/0700-0851$18.00/0 

peptides, that a cystine residue on the C-terminal side of the His can affect the rate of oxidation of the His by the ascorbate/$Cu(II)/O_2$ system, but this residue does not affect the nature of the reactive-oxygen species formed or the major His-degradation products. Experiments conducted with the cyclic-His peptide in the present study were designed: 1) to elucidate the structure(s) of major degradation product(s) generated by ascorbate/$Cu(II)/O_2$-induced oxidation; 2) to elucidate the nature of the reactive-oxygen species; 3) to determine the effect of pH on the metal-catalyzed oxidation of the model peptide; and 4) to determine solution conformation(s) of the degradant(s) and the cyclic-His peptide and to compare these to the structure of the loop region in relaxin.

## MATERIALS AND METHODS

### Materials

$N^{\alpha}$-9-fluorenylmethyloxycarbonyl (Fmoc)-amino acids, 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU), and DOD-resin (4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxymethyl-polystyrene resin) were obtained from BACHEM Bioscience Inc. (King of Prussia, PA). CAT (bovine liver, thymol-free, 199,000 U/mg), SOD (bovine erythrocyte, 4,400 U/mg), ascorbic acid, $CuCl_2$, bicinchoninic acid (BCA), ethylenediaminetetraacetate (EDTA), and $H_2O_2$ (30% w/w solution) were purchased from Sigma Chemical Company (St. Louis, MO). All other reagents were obtained commercially from Fisher Scientific (Pittsburgh, PA) or Acros (Springfield, NJ). The water used in all studies was from a Millipore MILLI-Q™ Water System.

### Synthesis and Purification of Peptides

The C- and N-terminally modified linear peptides, AcCys-Ala-X-Val-Gly-$CysNH_2$, (X = His, Asp, Asn), were synthesized using Fmoc-protected amino acids attached to DOD-resin (10). A manual protocol of peptide chain elongation was accomplished with HBTU and diisopropylethylamine (DIEA) as activating agents in the coupling steps (10 min) (11). Deprotection of the Fmoc-amino acids was accomplished with 20% piperidine in dimethylformamide (10 min). N-Terminal acetylation of the peptides was performed in the presence of acetic anhydride and dimethylaminopyridine in a 2:1 v/v ratio for 1 hour. The peptides were cleaved from the DOD-resin to obtain an amide on the C-terminal by incubation in 90% trifluoroacetic acid (TFA), 5% ethanedithiol, and 5% *p*-cresol for 2 hours at 35°C. Repetition of the cleavage process yielded additional quantities of the peptides. The crude peptides were precipitated by slowly adding the filtrate to cold peroxide-free diethyl ether while stirring. The precipitated crude peptides were dissolved in dilute acetic acid before lyophilization.

Purification of the crude peptides was achieved by preparative HPLC on a system consisting of a Beckman 420 system controller, Beckman 112 solvent delivery modules, a Kratos Analytical Spectroflow 783 UV spectrophotometric detector, a Pharmacia Biotech Frac-100 fraction collector and a Fisher Recordall Series 5000 recorder. Separation was accomplished with a Dynamax $C_{18}$ reversed-phase column (21.2 × 250 mm) packed with silica gel (300 Å pore size, 12 μm particle size) using a gradient system (flow rate = 5 ml/min) consisting of mobile phases A and B containing acetonitrile, water, and TFA mixed in the following proportions: 2/98/0.1 (v/v for mobile phase A) and 90/10/0.1 (V/V for mobile phase B). The time program gradient utilized was 2% B to 35% B over 60 min. The injection volume was 5 ml. Detection was achieved at 214 nm. Collected fractions were pooled and concentrated. The fractions containing linear peptides were identified by electrospray mass spectrometry and $^1$H NMR.

Cyclization of the linear peptides was accomplished by bubbling air through dilute peptide solutions containing 1.5 to $2 \times 10^{-4}$ M peptide in a 1:1 v/v mixture of 2,2,2-trifluoroethanol and phosphate buffer, 100 mM at pH 7.4. Dilute peptide solutions were utilized to promote intramolecular disulfide bond formation rather than intermolecular disulfide bond formation. The peptide solutions were concentrated in vacuo and lyophilized. Preparatory HPLC as described above was utilized for purification. The structures of (cyclo S-S) AcCys-Ala-His-Val-Gly-$CysNH_2$ (cyclic-His peptide), (cyclo S-S) AcCys-Ala-Asp-Val-Gly-$CysNH_2$ (cyclic-Asp peptide), and (cyclo S-S) AcCys-Ala-Asn-Val-Gly-$CysNH_2$ (cyclic-Asn peptide) were confirmed by electrospray mass spectrometry and $^1$H NMR.

### Oxidation Reactions

Unless otherwise specified, oxidation of the cyclic peptides was performed under the following conditions: 250 μl solutions contained 0.273 mM cyclic peptide, 2 mM ascorbate, 50 μM $CuCl_2$, and 20 mM phosphate buffer at either pH 5.3 or 7.4. These solutions were incubated at room temperature (25°C) in 250 μl inserts in 2 ml vials. The reagents were added in the following order: buffer, peptide, ascorbate, and $CuCl_2$. Prior to each reaction, fresh stock solutions of ascorbate and $CuCl_2$ were prepared. Reagents such as CAT (2000 U/ml), SOD (200 U/ml), and OH scavengers [isopropanol (8 mM) and thiourea (4 mM)] were used to assess the involvement of reactive-oxygen intermediates in the oxidation of the cyclic-His peptide. 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. Separation was accomplished with a Vydac 218TP $C_{18}$ reversed-phase column (4.6 × 250 mm) at room temperature (25°C). An isocratic system (flow rate = 1 ml/min) consisting of a mixture of acetonitrile/water (10/90, v/v) containing 0.1% TFA was employed. Detection of the cyclic peptides was achieved at 214 nm and was quantified by measuring peak areas referenced to standard curves generated with pure cyclic peptides.

### Circular Dichroism (CD) Analysis

CD experiments were conducted on an AVIV CD-60DS. Samples contained 0.273 mM cyclic-His peptide in the presence or absence of 50 μM $CuCl_2$ in 20 mM phosphate buffer at pH 5.3 or 7.4. Data were collected in the range of 250 to 190 nm (far-UV) in steps of 1 nm and appropriate buffer blanks with or without $CuCl_2$ were subtracted as the background. A 0.1 cm

cell was used for generation of the far-UV CD spectra. The spectra were converted to mean residue weight ellipticities. Estimates of the secondary structure content were made by the convex constraint analysis method (CCA) (12).

## RESULTS

### Oxidation of Cyclic-His Peptide: Role of Cu(II), Cu(I), and Reactive-Oxygen Intermediates

The cyclic-His peptide was shown to be stable in the presence of $CuCl_2$ alone or ascorbate alone in 20 mM phosphate buffer at both pH 5.3 and 7.4 (Table I). In the presence of both ascorbate and $CuCl_2$, the cyclic-His peptide underwent extensive degradation at both pH 5.3 and 7.4 (Table I). It was more stable at pH 5.3 than at pH 7.4. The cyclic peptide displayed biphasic kinetics of degradation with a rapid phase (1–2 min) followed by a slower phase of degradation, resulting in a plateau after 10 hours where no further degradation was observed (data not shown). The plateau phase was determined to be due to the depletion of ascorbate during the oxidation process (data not shown).

The involvement of Cu(II) and Cu(I) in the oxidation of cyclic-His peptide was investigated by using the specific metal chelators EDTA and BCA. The inclusion of EDTA or BCA in the standard oxidation reaction mixture at pH 7.4 resulted in stabilization of the peptide towards oxidation (data not shown). The involvement of the reactive-oxygen intermediates $H_2O_2$, $O_2^{\bullet-}$, and $^{\bullet}OH$, which are generated in the ascorbate/Cu(II)/$O_2$ system, was investigated by observing the effects of CAT, SOD, and $^{\bullet}OH$ scavengers (i.e. thiourea, isopropanol) on the oxidation process. Table II summarizes the results and reaction conditions.

SOD, which catalyzes the dismutation of $O_2^{\bullet-}$ to oxygen and $H_2O_2$ (13), is commonly used to determine the role of freely diffusible $O_2^{\bullet-}$ in oxidation reactions. The addition of SOD to the metal-catalyzed oxidation reaction mixture had no impact on the degradation of cyclic-His peptide (Table II). This indicates that $O_2^{\bullet-}$ is not directly damaging to the peptide but, rather, may serve as a source for the generation of $H_2O_2$.

CAT, which catalyzes the decomposition of $H_2O_2$ to water and $O_2$ ($k > 10^7\ M^{-1}\ s^{-1}$) (14), is commonly used to determine the role of freely diffusible $H_2O_2$ in oxidation reactions. The addition of freshly prepared CAT to the metal-catalyzed oxidation reaction mixture containing cyclic-His peptide resulted in stabilization of the peptide toward oxidation (Table II). The stabilization of the cyclic-His peptide toward metal-catalyzed oxidation by CAT indicates that $H_2O_2$ is an intermediate in the oxidation process. The fact that the cyclic-His peptide does not undergo degradation in the presence of $H_2O_2$ alone (Table II) indicates that $H_2O_2$ itself is not the damaging species. Instead, it must degrade to form other reactive-oxygen species that are ultimately involved in the reaction with His. $H_2O_2$ is known to degrade by the Fenton-type reaction in the presence of Cu(I) to yield $^{\bullet}OH$.

The role of freely diffusible $^{\bullet}OH$ in the metal-catalyzed oxidation of the cyclic-His peptide was investigated using the following scavengers that react with $^{\bullet}OH$ at the indicated rates: isopropanol, $k = 1.9 \times 10^{-9}\ M^{-1}\ s^{-1}$; and thiourea $k = 3.9 \times 10^{-9}\ M^{-1}\ s^{-1}$ (15). Isopropanol did not have a significant protective effect on metal-catalyzed oxidation of cyclic-His peptide, whereas thiourea afforded some stabilization of the cyclic-His peptide (Table II). However, we believe that the stabilization produced by thiourea is not due to the scavenging of freely diffusible $^{\bullet}OH$. Kanawaza *et al.* (4) have proposed that thiourea protects papain from oxidation by trapping Cu(II), thus, preventing the formation of free radicals at the specific site of inactivation. Other investigators (16,17) have also reported that thiourea stabilizes molecules towards oxidation, whereas other $^{\bullet}OH$ scavengers were ineffective. The results indicate that freely diffusible $^{\bullet}OH$ is not damaging to the cyclic-His peptide.

**Table I.** Oxidation of the Cyclic-His Peptide in an Ascorbate/Cu(II)/$O_2$ System[a]

| Additions | Cyclic-His Peptide Remaining (%) | |
|---|---|---|
| | pH 5.3 | pH 7.4 |
| Ascorbate (2 mM) | 100 | 99 |
| $CuC1_2$ (50 μM) | 100 | 100 |
| Ascorbate (2 mM) $CuCl_2$(50 μM) | 48 | 33 |

[a] Reaction conditions: The reaction mixtures contained 0.273 mM peptide in 20 mM phosphate buffer at pH 5.3 or 7.4 with the additions indicated above. The incubations were carried out at room temperature (25°C) for 20 hours. The reactions were monitored by HPLC 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 than 4% for all experiments.

**Table II.** Effects of CAT, SOD, and •OH Scavengers on the Oxidation of the Cyclic-His Peptide in an Ascorbate/Cu(II)/$O_2$ System[a]

| Additions | % Peptide Remaining pH 7.4 |
|---|---|
| Ascorbate/$CuCl_2$ | 33 |
| Ascorbate/$CuCl_2$/CAT | 86 |
| Ascorbate/$CuCl_2$/Boiled CAT | 36 |
| Ascorbate/$CuCl_2$/SOD | 33 |
| $H_2O_2$ | 98 |
| Ascorbate/$CuCl_2$/Isopropanol | 31 |
| Ascorbate/$CuCl_2$/Thiourea | 60 |

[a] Reaction conditions: The reaction mixtures contained 0.273 mM peptide in 20 mM phosphate buffer at pH 7.4 and reagents as indicated above in the following concentrations: 2 mM ascorbate, 50 μM $CuCl_2$, 2000 U/ml CAT, 2000 U/ml boiled CAT, 200 U/ml SOD, 2 mM $H_2O_2$, 8 mM isopropanol, and 4 mM thiourea. The incubations were carried out at room temperature (25°C) for 20 hours. The reactions were monitored by HPLC 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 than 4% for all experiments.

### Characterization of the Main Degradation Product

When the oxidation of the cyclic-His peptide was conducted at pH 7.4, one major degradation product was observed (Fig. 1, chromatogram B). However, at pH 5.3, there did not appear to be a main degradation product but, rather, numerous degradation products were formed in low yield (Fig. 1, chromatogram A). It is important to note that when additional ascorbate

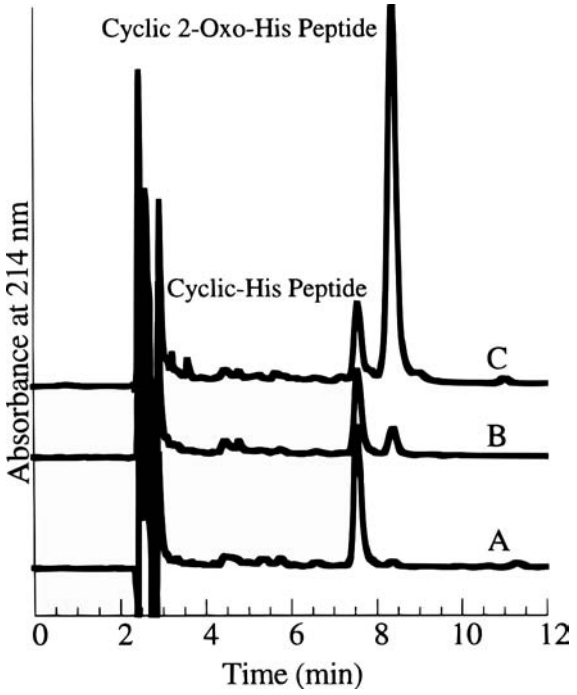


**Fig. 1.** Representative chromatograms obtained upon oxidation of cyclic-His peptide at pH 5.3 (A), pH 7.4 (B), and pH 7.4 after addition of 2-oxo-His cyclic peptide (C). Reaction mixtures initially contained 0.273 mM peptide, 2 mM ascorbate, 50 μM $CuCl_2$, and 20 mM phosphate buffer at pH 5.3 or pH 7.4 and were incubated at room temperature for 20 hours. HPLC analysis was performed with a Vydac 218TP $C_{18}$ reversed-phase column (4.6 × 250 mm). An isocratic system was utilized at a flow rate of 1 ml/min. The mobile phase was a mixture of acetonitrile/water (10/90, v/v) containing 0.1% TFA. Detection was achieved at 214 nm.

and Cu(II) were added to the oxidized cyclic-His peptide at pH 5.3, to achieve the same degree of degradation of the cyclic-His peptide observed at pH 7.4, the profile of the degradants was not changed (data not shown). The main degradation product observed at pH 7.4 was isolated and purified by preparative HPLC, and its structure was elucidated by electrospray mass spectrometry and $^1$H NMR. The mass of this oxidation product was 645 amu (16 amu higher then the parent peptide ion), which is consistent with the mass of the cyclic 2-oxo-His peptide. The $^1$H NMR spectra of this oxidation product revealed the absence of a resonance corresponding to the $C_2$ imidazole ring proton observed in the cyclic-His peptide. The $C_4$ imidazole ring proton resonance that appeared at 7.3 ppm for the cyclic-His peptide appeared at 6.3 ppm in the major degradation product. Similar $^1$H NMR spectra have been reported by other investigators for 2-oxo-His (18,19). Confirmation of the structure was accomplished by synthesizing the cyclic 2-oxo-His peptide and demonstrating that it coeluted with the major degradation product observed when the cyclic-His peptide was oxidized at pH 7.4 in an ascorbate/Cu(II)/$O_2$ system (Fig. 1).

Oxidation of His residues in peptides/proteins has been reported to yield Asp and Asn residues as degradation products (10,20). For example, Uchida *et al.* (21) have reported that 2-oxo-His undergoes degradation by a ring-opening mechanism to form Asp and Asn. To investigate if the cyclic-Asp peptide and/or the cyclic-Asn peptide were being formed as oxidative degradants of the cyclic-His peptide, these peptides were synthesized and their retention times compared to the degradation products observed upon oxidation of the cyclic-His peptide at pH 7.4. As shown in Fig. 2, the cyclic-Asp peptide and the cyclic-Asn peptide were not produced in significant amounts upon oxidation of the cyclic-His peptide at pH 7.4. The same results were obtained after oxidation of the cyclic-His peptide at pH 5.3 (data not shown). It is important to note that exposure of the cyclic-Asp peptide or the cyclic-Asn peptide to the ascorbate/Cu(II)/$O_2$ system did not result in significant degradation of these peptides (data not shown).

As noted above, there were differences observed in the degradation profile of the cyclic-His peptide upon oxidation in an ascorbate/Cu(II)/$O_2$ system at pH 5.3 vs pH 7.4, i.e., cyclic 2-oxo-His peptide was observed as a major degradant at pH 7.4 but at pH 5.3 numerous degradation products were formed in low yield including small amounts of the cyclic 2-oxo-His peptide. These differences may be attributed to the formation of different complexes between the cyclic-His peptide and Cu(II) at pH 5.3 vs pH 7.4. The different complexes may then lead to the formation of different degradation product profiles.

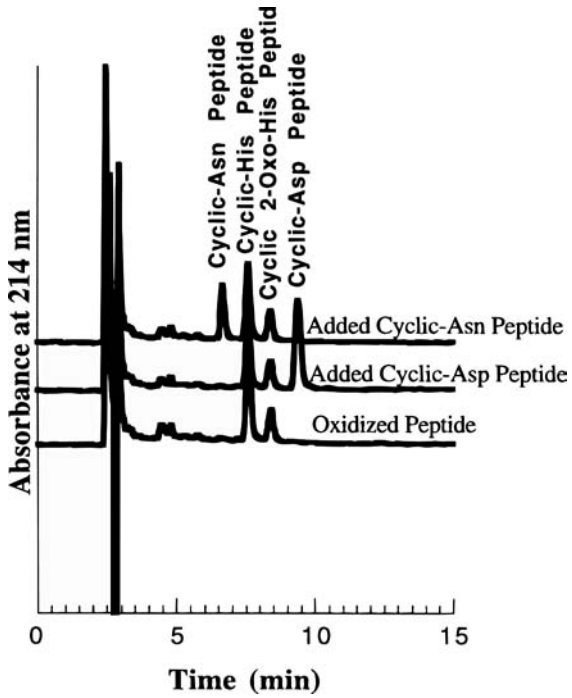


**Fig. 2.** Representative chromatograms obtained upon oxidation of cyclic-His peptide at pH 7.4 and after addition of cyclic-Asn peptide or cyclic-Asp peptide. Reaction mixtures initially contained 0.273 mM cyclic-His peptide, 2 mM ascorbate, 50 μM $CuCl_2$, and 20 mM phosphate buffer at pH 7.4 and were incubated at room temperature for 20 hours. HPLC analysis was then run on the oxidized cyclic-His peptide alone or in the presence of cyclic-Asp peptide or cyclic-Asn peptide. HPLC analysis was performed with a Vydac 218TP $C_{18}$ reversed-phase column (4.6 × 250 mm). An isocratic system was utilized at a flow rate of 1 ml/min. The mobile phase was a mixture of acetonitrile/water (10/90, v/v) containing 0.1% TFA. Detection was achieved at 214 nm.

### Effect of Presence or Absence of Cu(II) on the Solution Structure of the Cyclic-His Peptide at pH 5.3 and 7.4

$^1$H NMR was utilized to demonstrate the complexation of the His of the cyclic-His peptide with Cu(II). The resonances corresponding to the $C_2$ and $C_4$ imidazole ring protons were shown to selectively broaden in the presence of Cu(II) at both pH 5.3 and 7.4 (data not shown). This phenomenon has been observed by other researchers (22,23). The effect of complexation on the solution structure of the peptide was investigated by determining the CD spectra of the cyclic-His peptide (0.273 mM) in the presence or absence of 50 μM $CuCl_2$ in 20 mM phosphate buffer at pH 5.3 (Fig. 3A) and pH 7.4 (Fig. 3B). At pH 5.3, the secondary structure of the cyclic-His peptide in the presence or absence of Cu(II) did not appear to be significantly different (Fig. 3A). However, at pH 7.4 it is apparent that the presence of Cu(II) impacted the secondary structure of cyclic-His peptide (Fig. 3B). The CD spectrum indicates that the solution conformation of the cyclic-His peptide in the presence of Cu(II) at pH 5.3 is different from that at pH 7.4 (Fig. 3A & 3B).

### Comparison of the Solution Structure of Cyclic-His Peptide to That of Its Relaxin Counterpart

CD experiments in the far-UV range (190–250 nm) were conducted to determine the solution structure of the cyclic-His peptide at pH 7.4 (Fig. 3B). The CD spectral data were processed and deconvoluted to five components of secondary structure elements (α helix, β sheet, and β turns), which were assigned to component curves by comparison with those found in the literature (12). Deconvolution of the data for the cyclic-His peptide suggested that approximately 27% of the peptide existed in a type I β turn with the remainder being unordered structures. Two-dimensional (2D) NMR data on the cyclic-His peptide supported the CD data and indicated that this peptide existed in a type I β turn composed of the amino acids His-Val-Gly-Cys (data not shown). The backbone dihedral angle values corresponding to the same amino acid residues that formed a type I β turn in the cyclic-His peptide (His-Val-Gly-Cys) were obtained from the X-ray crystal structure of relaxin (Brookhaven Protein Data Bank entry 6RLX, Eigenbrot *et al.*) (7). The backbone dihedral angle values of these residues in relaxin did not correlate well with values observed in a typical type I β turn (data not shown). However, backbone dihedral angle values for the sequence Cys-Cys-His-Val found in this loop region of relaxin (obtained from the X-ray crystal structure, Brookhaven Protein Data Bank entry 6RLX, Eigenbrot *et al.*) (7) did correlate well with those found in a typical type I β turn (data not shown). Based on these data, it is apparent that a common feature of both the cyclic-His peptide and the His-containing loop region of relaxin is a type I β turn containing the His residue. The only differences are the locations of the His residue in these turns (i.e. residue 1 in the cyclic-His peptide and residue 3 in relaxin). Therefore, while the cyclic-His peptide is not a perfect model of the extended loop of relaxin, it exhibits similar higher-order structural features and, therefore, could be used to determine the impact of oxidation of the His on its solution structure.

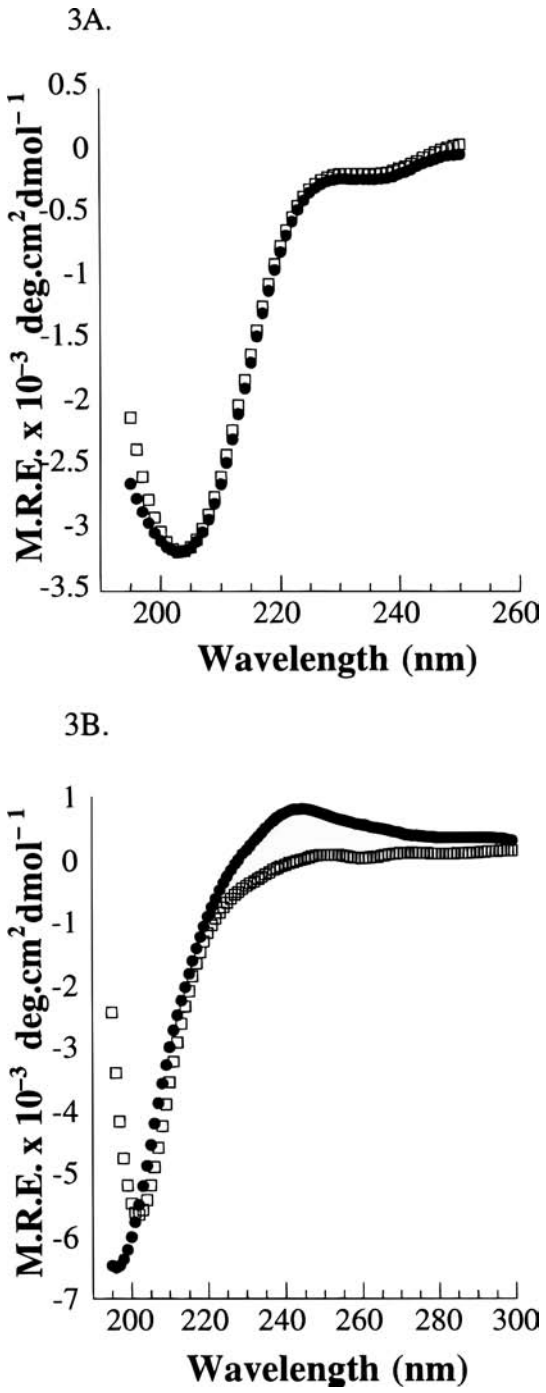


**Fig. 3.** (A) CD spectra of cyclic-His peptide in the presence or absence of Cu(II) at pH 5.3. Conditions: (●) 0.273 mM cyclic-His peptide and 50 μM $CuCl_2$ in 20 mM phosphate buffer at pH 5.3. (□) 0.273 mM cyclic-His peptide in 20 mM phosphate buffer at pH 5.3. (B) CD spectra of cyclic-His peptide in the presence or absence of Cu(II) at pH 7.4. Conditions: (●) 0.273 mM cyclic-His peptide and 50 μM $CuCl_2$ in 20 mM phosphate buffer at pH 7.4. (□) 0.273 mM cyclic-His peptide in 20 mM phosphate buffer at pH 7.4.

### Effect of Oxidation on the Solution Structure of the Cyclic-His Peptide

CD experiments in the far-UV range (190–250 nm) were conducted to compare the solution structure of the cyclic-His peptide to that of the cyclic 2-oxo-His peptide (Fig. 4). The CD spectrum for cyclic 2-oxo-His peptide was processed and deconvoluted in the same manner as noted above for the cyclic-His peptide. CD results for the cyclic 2-oxo-His peptide suggested that it existed in approximately 51% type II β turn, 9% type I β turn, and the remainder as unordered structures. As noted before, deconvolution of the data for the cyclic-His peptide suggested that approximately 27% of the peptide existed in a type I β turn with the remainder being unordered structures. These results indicate that the solution structure of the cyclic-His peptide is significantly different than that of the cyclic 2-oxo-His peptide. It is important to note that variations in CD spectra of the type described above may result because of different side chains of amino acid residues, i.e., aromatic residue vs nonaromatic residue (12). The differences in the CD spectra between the cyclic-His peptide and the cyclic 2-oxo-His peptide are probably not due to the inherent structural differences between His and 2-oxo-His because His is not an aromatic amino acid (24). Furthermore, the cyclic-His peptide and the cyclic 2-oxo-His peptide displayed similar UV spectrophotometry absorption spectra in the range of 190–350 nm (data not shown). Therefore, the differences observed in the amide far-UV CD spectra shown in Fig. 4 are probably due to differences in the backbone region of the peptides.

## DISCUSSION

The results obtained from the experiments designed to characterize the role of Cu(II), Cu(I), and reactive-oxygen intermediates ($H_2O_2$, $O_2^{\bullet -}$) suggest that the reaction scheme proposed previously by Khossravi and Borchardt (9) for the metal-catalyzed oxidation of linear His-containing peptides is applicable to this cyclic His-containing peptide. The following equations show how Cu(I), $H_2O_2$, and $O_2^{\bullet -}$, which were shown in this study to be the intermediates involved in the oxidation of the cyclic-His peptide, are formed. In the equations shown below, Cx represents a complex that may include water, ascorbate, peptide, Cu(II) and/or phosphate. The pertinent reacting species of the complex are illustrated individually. First, Cu(I) may be generated by reduction of Cu(II) by ascorbate (Eq. 1). Ascorbate and ascorbyl radical anion are denoted by $AH^-$ and $A^{\bullet -}$.

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

The Cu(I) can then react with oxygen to ultimately form $H_2O_2$ (Eqs. 2–4).

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

$$CxCu(II)(O_2^{\bullet -}) + CxCu(I) \rightarrow CxCu(II)(O_2^{-2})CxCu(II) \quad (3)$$

$$CxCu(II)(O_2^{-2})CxCu(II) + 2H^+ \rightarrow 2CxCu(II) + H_2O_2 \quad (4)$$

$H_2O_2$ can ultimately degrade by a Fenton-type reaction, which can generate $^{\bullet}OH$ within the complex forms (Eq. 5) or a metal-bound form of $^{\bullet}OH$ (Eq. 6) that react with the cyclic-His peptide before diffusion into the bulk solution.

$$CxCu(I) + H_2O_2 \rightarrow CxCu(II)(^{\bullet}OH)(HO^-) \quad (6)$$

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

It is important to note that, while the experimental data reported in this manuscript strongly suggest the involvement of $H_2O_2$ and $O_2^{\bullet -}$ as intermediates in the oxidation and complexed forms of $^{\circ}OH$ as the actual oxidant, the results are not definitive.

Metal-catalyzed oxidation of peptides and proteins has been reported to result in the conversion of His residues to Asp, Asn, and/or 2-oxo-His residues as degradation products (10,19,20). As shown in this study, metal-catalyzed oxidation of the cyclic-His peptide at pH 5.3 and pH 7.4 did not result in detectable levels of the cyclic-Asp or the cyclic-Asn peptides. The major degradation product formed at pH 7.4 was shown to be the cyclic 2-oxo-His peptide. At pH 5.3, numerous degradants were formed in low yield, including the cyclic 2-oxo-His peptide. The different degradant profiles observed at pHs 5.3 and 7.4 may result from different complexes or different populations of complexes at different pHs. These ideas are elaborated on in the following discussion.

As indicated by $^1H$ NMR, the imidazole ring of the cyclic-His peptide appears to be involved in the formation of complexes with Cu(II). The differences in the CD spectra of the cyclic-His peptide in the presence of Cu(II) at pH 5.3 vs pH 7.4 (Fig. 3A & 3B) support the hypothesis that different metal-ligand complexes have different solution structures depending on pH. Other researchers have observed changes in CD spectra that have been attributed to different coordination modes of binding for metal-ligand complexes, affecting the conformation of the peptide (25,26). For example, bleomycin was shown to form three different complexes with Cu(II) depending on pH. Each complex exhibited a different CD spectrum (27).

Since metal-catalyzed oxidation is a site-specific process, the coordination-ligand mode of a particular metal-ligand complex may dictate the profile of degradant(s) that are formed.

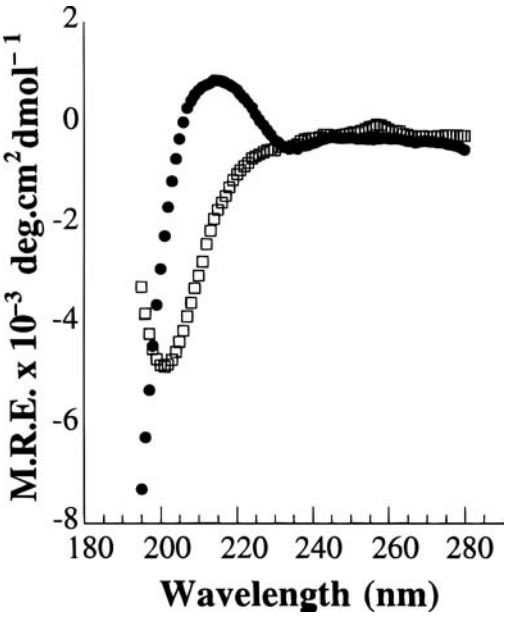


**Fig. 4.** CD spectra of cyclic-His peptide and 2-oxo-His cyclic peptide. Conditions: (●) 0.273 mM 2-oxo-His cyclic peptide in 20 mM phosphate buffer at pH 7.4. (□) 0.273 mM cyclic-His peptide in 20 mM phosphate buffer at pH 7.4.

At pH 7.4, the imidazole ring of the His residue in the Cu(II) [or Cu(I)]-cyclic-His peptide complex must be oriented in such a way as to favor attack at the $C_2$ position of the imidazole ring and the generation of the 2-oxo-His degradant. Other metal-ligand complexes with different ligand-coordination modes of binding probably exist and favor generation of the other degradation products.

Not only can there be different metal-ligand complexes at a given pH, but researchers have also reported that the distribution of various complexes may vary as a function of pH (25,26). Therefore, we propose that the Cu(II) [or Cu(I)]-cyclic-His peptide complex giving rise to the 2-oxo-His degradant may represent a smaller fraction of the total population of complexes at pH 5.3 vs pH 7.4; thus, providing an explanation for the formation of a larger amount of cyclic 2-oxo-His peptide at pH 7.4 vs pH 5.3.

As noted earlier, the cyclic-His peptide was designed as a model of the His-containing extended loop region of relaxin. We hoped to use this cyclic peptide to generate evidence to support our hypothesis that oxidation of the His residue in this loop region of relaxin leads to changes in the secondary structure of the protein, ultimately leading to aggregation and precipitation. Unfortunately, from a structural perspective, the cyclic-His peptide proved not to be a perfect model of the extended loop region of relaxin. Imperfections in this model peptide reside in the exact type of turn structures it exhibits in solution compared to what is found in the extended loop of relaxin. Our CD and $^1$H NMR results of the cyclic-His peptide and our analysis of the X-ray crystal structure of relaxin suggest that the cyclic-His peptide and the His-containing extended loop of relaxin both exhibit type I β turns. However, the differences reside in the exact location of His in these turns. In the model peptide, His is in position 1 of the turn, whereas His is in position 3 of the turn in the extended loop region of relaxin.

Despite these differences, the results forthcoming from our studies of the oxidation of this model cyclic-His peptide have been valuable because they have shown that modification of the His residue can result in alterations in the secondary structure of the peptide. Specifically, we showed by CD spectroscopy that the solution structures of the cyclic 2-oxo-His peptide, one of the possible degradants formed from metal-catalyzed oxidation, appear to be quite different from the solution structures of the cyclic-His peptide (Fig. 4). These results suggest that other oxidation products of the His residue in this cyclic peptide (and in the extended loop of relaxin) are likely to produce similar changes in the molecule's secondary structures.

In more extensive studies of the metal-catalyzed oxidation of relaxin, our laboratory (28) recently observed that 2-oxo-His was present in the soluble degradants formed after ascorbate/Cu(II)/$O_2$ oxidation of relaxin. In contrast, much lower levels of 2-oxo-His were detected in precipitated degradants. These observations would suggest that conversion of the His residue in the extended loop region of relaxin to 2-oxo-His does not produce sufficient (or appropriate) changes in the protein's higher-ordered structure to cause aggregation and precipitation of the protein. However, like the observations made here with the cyclic-His peptide, relaxin is converted in these metal-catalyzed reactions to a plethora of other oxidation products. One or more of these other oxidation products of the His residue in the extended loop could lead to the types of changes in the protein's higher-ordered structure that lead to aggregation and precipitation.

In summary, we have shown in these studies that a model cyclic-His peptide exhibits solution structures similar, but not identical to those observed in the extended loop of relaxin. It has been shown that oxidation of this cyclic-His peptide leads to 2-oxo-His as the major degradation product and that this modified cyclic peptide has different structures in solution than the parent model peptide. However, our recent observation (28) that 2-oxo-His containing degradants of relaxin are soluble suggests that the formation of this specific degradant of His does not lead to the aggregation and precipitation of the protein. Current studies support the hypothesis that other, unidentified oxidative degradants of the His residue in the extended loop of relaxin are ultimately responsible for the physical instability of this protein (28).

## ACKNOWLEDGMENTS

The authors would like to thank Dr. Christian Schöneich for his advice and Drs. David Vander Velde and Martha Morton for their assistance in product identification by NMR. We would further like to thank Dr. Jois D. S. Seetharama for his help with the CD experiments. This work was supported by the United States Public Health Service (GM08359).

## REFERENCES

1. E. R. Stadtman. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Ann. Rev. Biochem.* **62**:797–821 (1993).
2. E. R. Stadtman. Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. *Free Rad. Biol. Med.* **9**:315–325 (1990).
3. A. Amici, R. L. Levine, L. Tsai, and E. R. Stadtman. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J. Biol. Chem.* **264**:3341–3346 (1989).
4. H. Kanazawa, S. Fujimoto, and A. Ohara. Effect of radical scavengers on the inactivation of papain by ascorbic acid in the presence of cupric ions. *Biol. Pharm. Bull.* **17**:476–481 (1994).
5. S. Li, T. H. Nguyen, C. Schöneich, and R. T. Borchardt. Aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. *Biochemistry* **34**:5762–5772 (1995).
6. T. H. Nguyen, J. Burnier, and W. Meng. The kinetics of relaxin oxidation by hydrogen peroxide. *Pharm. Res.* **10**:1563–1571 (1993).
7. C. Eigenbrot, M. Randal, C. Quan, J. Burnier, L. O'Connell, E. Rinderknecht, and A. A. Kossiakoff. X-ray structure of human relaxin at 1.5 Å. Comparison of insulin and implications for receptor binding determinants. *J. Mol. Biol.* **221**:15–21 (1991).
8. E. E. Büllesbach and C. Schwabe. Structural contribution of the A-chain loop in relaxin. *Int. J. Peptide Protein Res.* **46**:238–243 (1995).
9. M. Khossravi and R. T. Borchardt. Chemical pathways of peptide degradation: IX. Metal-catalyzed oxidation of histidine in model peptides. *Pharm. Res.* **15**:1096–1102 (1998).
10. R. T. Dean, S. P. Wolff, and M. A. McElligott. Histidine and proline are important sites of free radical damage to proteins. *Free Rad. Res. Commun.* **7**:97–103 (1989).
11. M. Schnölzer, P. Alewood, A. Jones, D. Alewood, and S. B. H. Kent. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Peptide Protein Res.* **40**:180–193 (1992).
12. A. Perczel and M. Hollosi. *Circular Dichroism and the Conformational Analysis of Biomolecules*, Plenum Press, New York, 1996.
13. G. R. Schonbaum and B. Chance. *Catalase, The Enzymes, Vol. XIII*, Academic Press, New York, 1976.

14. I. Fridovich. Superoxide dismutases. *J. Biol. Chem.* **264**:7761–7764 (1989).
15. G. V. Buxton, C. L. Greenstock, W. P. Helman, and A. B. Ross. Critical review of rate constants for reactions of hydrated electrons, hydrogen atoms and hydroxyl radicals in aqueous solution. *J. Phys. Chem. Ref. Data* **17**:513–886 (1988).
16. S. Li, C. Schöneich, and R. T. Borchardt. Chemical pathways of peptide degradation. VIII. Oxidation of methionine in small model peptides by prooxidant/transition metal ion systems: Influence of selective scavengers for reactive oxygen intermediates. *Pharm. Res.* **12**:348–355 (1995).
17. D. A. Rowley and B. Halliwell. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: A physiologically significant reaction? *Arch. Biochem. Biophys.* **225**:279–284 (1983).
18. K. Uchida and S. Kawakishi. Selective oxidation of imidazole ring in histidine residues by the ascorbic acid-copper ion system. *Biochem. Biophys. Res. Commun.* **138**:659–665 (1986).
19. F. Zhao, J. Yang, and C. Schöneich. Effects of polyaminocarboxylate metal chelators on iron-thiolate induced oxidation of methionine- and histidine-containing peptides. *Pharm. Res.* **13**:931–938 (1996).
20. R. L. Levine. Oxidative modification of glutamine synthetase II. Characterization of the ascorbate system. *J. Biol. Chem.* **258**:11828–11833 (1983).
21. K. Uchida and S. Kawakishi. Ascorbate-mediated specific oxidation of the imidazole ring in a histidine derivative. *Bioorg. Chem.* **17**:330–343 (1989).
22. D. Muller, B. D. Révérend, and B. Sarkar. Studies of copper(II) binding to glycylglycyl-L-tyrosine-N-methyl amide, a peptide mimicking the $NH_2$-terminal copper(II)-binding site of dog serum albumin by analytical potentiometry, spectrophotometry, CD, and NMR spectroscopy. *J. Inorg. Biochem.* **21**:215–226 (1984).
23. D. P. Naughton, J. Knappitt, K. Fairburn, K. Gaffney, D. R. Blake, and M. Grootveld. Detection and investigation of the molecular nature of low-molecular-mass copper ions in isolated rheumatoid knee-joint synovial fluid. *FEBS Letters* **361**:167–172 (1995).
24. T. E. Creighton. *Proteins: Structures and Molecular Properties*, W. H. Freeman and Company, New York, 1993.
25. T. P. A. Kruck and B. Sarkar. Structure of the species in the copper (II)-L-histidine system. *Can. J. Chem.* **51**:3563–3571 (1973).
26. L. Casella and M. Gullotti. Coordination modes of histidine. 4. Coordination structures in the copper(II)-L-histidine (1:2) system. *J. Inorg. Biochem.* **18**:19–31 (1983).
27. J. P. Albertini and A. Garnier-Suillerot. Formation of copper-bleomycin complexes: Evidence of a three-step process. *J. Inorg. Biochem.* **25**:15–24 (1985).
28. M. Khossravi, S. J. Shire, and R. T. Borchardt. Evidence for the involvement of histidine A(12) in the aggregation and precipitation of human relaxin induced by metal-catalyzed oxidation. *Biochemistry* **39**:5876–5885 (2000).