Evidence for the Involvement of Histidine A(12) in the Aggregation and Precipitation of Human Relaxin Induced by Metal-Catalyzed Oxidation[†]

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ABSTRACT: The metal-catalyzed oxidation (ascorbate/cupric chloride/oxygen) of recombinant human relaxin (rhRlx, type II) was shown by Li et al. [Li, S., Nguyen, T. H., Schöneich, C., and Borchardt, R. T. (1995) *Biochemistry 34*, 5762−5772] to result in the chemical modification of His A(12), Met B(4), and Met B(25). Considering the fact that His A(12) exists in an extended loop that joins two α-helices in this protein, we hypothesized that oxidation of this specific amino acid leads to alterations in the secondary and tertiary structures of the protein, resulting in the pH-dependent aggregation/precipitation phenomena observed in our earlier studies (i.e., at pH >6.0 most of the degradants of rhRlx are insoluble). Evidence obtained in the current study that supports this hypothesis includes the following: (i) oxidation of rhRlx with hydrogen peroxide (H_2O_2), which leads only to modification of Met B(4) and Met B(25), does not result in the pH-dependent aggregation/precipitation of the protein; and (ii) metal-catalyzed oxidation of porcine relaxin (pRlx), which does not contain His at position A(12), leads to chemical degradation of the protein [e.g., Met A(2) is oxidized] but produces only slight pH-dependent aggregation/precipitation of the protein. In addition, experimental evidence is provided to show that the physical instability of rhRlx observed at pH >6.0 does not appear to be related to the pH-dependent solubility of a common protein degradant. Instead, it appears that several oxidation products of His A(12) are produced in a pH-dependent manner and that these oxidation products produce different effects on the physical stability of the protein. Evidence in support of this conclusion includes the observation that the soluble degradants of rhRlx showed reduced levels of His, reduced levels of the T_2-T_7 tryptic fragment that contained His A(12), and the presence of 2-oxo-His. Similarly, the precipitated degradants of rhRlx showed reduced levels of His but no 2-oxo-His. In addition, the soluble degradants, which contain 2-oxo-His, appear to exist as monomers having an average molecular weight similar to that of rhRlx. These results suggest that the metal-catalyzed oxidation of His A(12) leads to other, as yet unidentified oxidation products of His A(12) that affect the secondary/tertiary structure of the protein more significantly than does 2-oxo-His and ultimately lead to the physical instability of the protein observed at higher pH values.

A pathway of chemical degradation of therapeutic proteins during processing and storage is metal-catalyzed oxidation (*1*, *2*). Transition metal ions [e.g., Cu(II)] in the presence of a reducing agent (e.g., ascorbate) and O_2 react to generate a variety of reactive oxygen species [including H_2O_2,[1] superoxide anion radical ($O_2^{\bullet-}$), and hydroxyl radical (•OH)], which can ultimately cause chemical modification of specific amino acid residues (e.g., His, Arg, Lys, Pro, Met, Cys) in therapeutic proteins (*3*). A key factor influencing the susceptibility of amino acid residues to metal-catalyzed oxidation is their ability to complex with transition metals because it is within these complexes that reactive oxygen species are generated and oxidation occurs (*3*).

In addition to the chemical modification of specific amino acid residues (e.g., Met to Met sulfoxide), metal-catalyzed oxidations can also lead to the physical instability of therapeutic proteins. For example, Li et al. (*4*) reported that oxidation of recombinant human relaxin (rhRlx) with an ascorbate/Cu(II)/oxygen (ascorbate/Cu(II)/O_2) system not only resulted in the chemical modification of His A(12), Met B(4), and Met B(25) but also led to physical instability (aggregation/precipitation) of the protein at higher pH (>6.0). His A(12) exists in an extended loop that joins two α-helices in this protein (Figure 1) (*5*). Li et al. (*4*) hypothesized that oxidation of this specific amino acid lead to alteration in the secondary and tertiary structures of the protein, resulting in the pH-dependent physical instability. This hypothesis is supported by the proposal of Büllesbach and Schwabe (*6*) that the structural integrity of the His-containing extended loop region of rhRlx is important for the maintenance of the overall higher ordered structure of rhRlx. Büllesbach and Schwabe (*6*) observed that mutants of rhRlx which had amino

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DLS, dynamic light scattering; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; •OH, hydroxyl radical; OPA, *o*-phthaldialdehyde; pRlx, porcine relaxin; rhRlx, recombinant human relaxin; SEC, size-exclusion chromatography; $O_2^{\bullet-}$, superoxide anion radical; TFA, trifluoroacetic acid.

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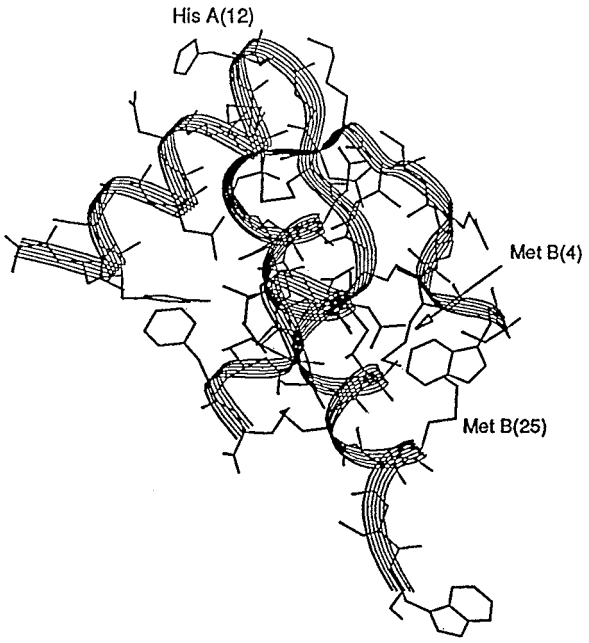

FIGURE 1: Representation of the three-dimensional structure of rhRlx. The X-ray crystal structure of the monomer of rhRlx was obtained from the Protein Data Bank [entry 6RLX; Eigenbrot et al. (*5*)]. The oxidation sites (His and Met) are distinguished.

acid residues in the His-containing loop region of rhRlx replaced by others had lost biological activity and appeared to display a change in the overall conformation of the protein.

In the present study, we have tested this hypothesis by investigating the physical stability of rhRlx when oxidized with H_2O_2 because this reactive oxygen species leads only to chemical modification of Met B(4) and Met B(25). In addition, we have examined the chemical and physical instability of porcine Rlx (pRlx), which does not contain His at position A(12) (*7*), in the presence of ascorbate/Cu(II)/O_2. The sequences of pRlx and rhRlx may be found in a manuscript by Shire et al. (*7*). Finally, experiments were designed to elucidate the nature of the degradation products arising from metal-catalyzed oxidation of His A(12) in rhRlx and to determine whether degradants may produce sufficient alterations in the secondary/tertiary structures of the protein to cause the pH-dependent physical instability observed by Li et al. (*4*).

EXPERIMENTAL PROCEDURES

Preparation of Protein Stock Solutions. rhRlx in citrate buffer, provided by Genentech, Inc. (South San Francisco, CA), was concentrated and washed extensively with doubly distilled deionized water by centrifugation (Beckman GS-6R) in Micron-3 microconcentrators (Amicon, Inc., Beverly, MA) at 8 °C. UV spectrophotometry (Hewlett-Packard Vectra XM Series 4) at 280 nm, using an extinction coefficient of 2.25 mg^{-1} cm^{-1} mL for rhRlx, was utilized to determine the concentration of the protein. pRlx purified from pregnant pig ovaries was provided in a lyophilized form by Professor Geoffrey Tregear (Howard Florey Institute of Experimental Physiology and Medicine, University of Melbourne, Parkville, Victoria, Australia). It was reconstituted in doubly distilled deionized water and underwent centrifugation in Micron-3 microconcentrators at 8 °C to remove any compounds less than 3000 in molecular weight.

Oxidation Conditions. Unless otherwise specified, the metal-catalyzed oxidation of rhRlx and pRlx was performed under the following conditions: solutions (250 μL) contained 0.033 mM protein, 2 mM ascorbate, 0.023 mM $CuCl_2$, and 10 mM phosphate buffer at pH values in the range of 5.0−7.8. The reagents were added in the following order: buffer, protein, ascorbate, and $CuCl_2$. The solutions were incubated at room temperature (25 °C) in 2 mL vials. Stock solutions of ascorbate and $CuCl_2$ were prepared prior to each reaction. The phosphate-buffered solutions were treated with Chelex 100 resin (5 g/100 mL, Bio-Rad Laboratories, Richmond, CA) to remove trace metal contaminants. Unless otherwise specified, the oxidation of rhRlx by H_2O_2 was performed under the following conditions: solutions (250 μL) contained 0.033 mM protein and 33 mM H_2O_2 in 10 mM phosphate buffer at pH values in the range of 5.0−7.8. The solutions were incubated at room temperature (25 °C) in 2 mL vials. The reagents were added in the following order: buffer, protein, and H_2O_2. Doubly distilled deionized water was utilized in making all solutions.

Chromatographic Analysis: Reversed-Phase (*RP*) *HPLC*. The rates and extent of degradation of rhRlx and pRlx were monitored by RP-HPLC on a Hewlett-Packard HP 1090 LC using a Vydac 214TP C_4 RP column (4.6 × 250 mm) at room temperature (25 °C). A gradient system (2% B to 30% B over 30 min) was utilized at a flow rate of 1 mL/min. Mobile phases A and B consisted of acetonitrile, water, and trifluoroacetic acid (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). Injection volumes were 10 μL, and detection of the proteins was achieved by measuring UV absorbance at 214 nm. Standard curves generated using pure rhRlx and pRlx were used for quantification.

Size-Exclusion Chromatography (*SEC*). Nondenaturing SEC, using a Pharmacia Biotech Superdex Peptide HR 10/30 column, was conducted on rhRlx and oxidized rhRlx to investigate protein aggregation. An isocratic system (20 mM phosphate buffer at pH 5.0 with 0.35 M NaCl) was utilized at a flow rate of 0.7 mL/min. The injection volumes were 70 μL, and detection was achieved by measuring UV absorbance at 214 nm.

Quantification of Soluble Protein. Soluble protein concentrations following oxidation of rhRlx and pRlx by ascorbate/Cu(II)/O_2 or H_2O_2 were determined by UV spectrophotometry (Hewlett-Packard Vectra XM Series 4). Oxidized and native proteins (rhRlx, pRlx) were centrifuged to remove precipitates before spectrophotometric analysis at 280 nm of the supernatant. An extinction coefficient of 2.25 mg^{-1} cm^{-1} mL was utilized to determine the final protein concentration (*7*).

Determination of the Impact of pH on the Solubility of Oxidized rhRlx. A sample of rhRlx was oxidized at pH 5.0 as described above, resulting in degradants that were completely soluble at this pH. The pH of this oxidized protein solution was then adjusted to higher pH values in the range of 5.4−7.4. These solutions were incubated at room temperature (25 °C) for 20 h, after which time the soluble protein content was determined in the manner described above.

Tryptic Digestion and LC/MS Characterization of the Tryptic Fragments. Tryptic digestion of rhRlx and soluble degradants of rhRlx was accomplished by incubation of the protein with a 1:50 w/w ratio of trypsin (Worthington

Biochemicals, Freehold, NJ) to rhRlx in 40 mM phosphate buffer (pH 7.2) at 37 °C. After 2 h, a second 1:50 (w/w) aliquot of trypsin:rhRlx was added. These samples were then incubated for another 4 h. The enzymatic reactions were terminated by lowering the pH below 4 with H_3PO_4, and the samples were stored at −70 °C. Separation of the tryptic fragments was achieved by RP-HPLC (Hewlett-Packard 1090 system) on a Nucleosil C18 (4.6 × 150 mm) column (Alltech) equilibrated at room temperature. A gradient system (2% B to 30% B over 54 min) was utilized at a flow rate of 1 mL/min. Mobile phases A and B consisted of 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). Absorbance was monitored at 214 and 280 nm. The molecular masses of the tryptic fragments of rhRlx and oxidized rhRlx separated by RP-HPLC were determined by electrospray ionization (LCQ Finnigan, Inc.). The molecular masses of the tryptic fragments were analyzed with the use of Bio-explore software (Biostructure, Illkirch-Graffenstaden, France).

NMR Analysis: pK_a Determination of the His A(12) Residue. rhRlx was separated from buffer components (citrate) by ultrafiltration using Micron-3 microconcentrators from Amicon, Inc. (Beverly, MA). The protein samples were twice lyophilized and reconstituted with 0.8 mL of 10 mM sodium phosphate buffer in D_2O (pD 7.2) to allow the complete exchange of the amide protons. The final concentration of rhRlx in the samples for NMR analysis was 1−2 mM. A Beckman Φ32 pH meter equipped with an Ingold combination electrode was used for pD measurements. The pD of the protein solution was adjusted to the desired pD values in the range of 2−9 with concentrated DCl and NaOD. The pD was measured before and after each NMR analysis. The second measurement was viewed as the more accurate value and was utilized in determination of the pK_a as described by Markley (*8*). NMR spectra were generated on a Varian INOVA 500 MHz instrument.

Complexation of rhRlx with Cu(II). Complexation of the imidazole protons of His A(12) of rhRlx with Cu(II) was investigated by NMR spectroscopy. rhRlx samples (2 mM) in the presence or absence of Cu(II) (1.34 mM) in 10 mM sodium phosphate buffer in D_2O at pD values ranging from 5.0 to 7.4 were analyzed.

NMR Analysis of Oxidized rhRlx. rhRlx was oxidized with ascorbate/Cu(II)/O_2 in 10 mM phosphate buffer at pH 6.4. The soluble fraction of oxidized rhRlx was separated from the precipitated fraction by centrifugation at 5000 rpm for 4 min. The soluble fraction of oxidized rhRlx was ultrafiltered using Micron-3 microconcentrators to remove oxidized ascorbate and Cu(II). The sample was then twice lyophilized and reconstituted with 0.8 mL of 10 mM sodium phosphate buffer in D_2O (pD 7.2) to permit for complete exchange of amide protons. The precipitated fraction of oxidized rhRlx was washed with doubly distilled deionized water. The sample was then lyophilized and reconstituted with 7 M deuterated urea in 10 mM phosphate buffer at pD 7.2 before analysis by NMR spectroscopy.

Amino Acid Analysis. Samples of rhRlx were oxidized at pH values of 5.0, 6.4, and 7.4 under standard oxidation conditions as described above. In the pH 6.4 and 7.4 experiments, and the pH 5.0 reaction mixture, ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 0.1 mM EDTA. These samples were concentrated and washed with 0.1 mM EDTA by centrifugation in Micron-3 microconcentrators. UV spectra of the samples at 280 nm were generated. An extinction coefficient of 2.25 mg^{-1} cm^{-1} mL was utilized to determine the final protein concentrations. Approximately 1 nmol of each sample was hydrolyzed with 6 N HCl at 100 °C for 20 h. Postcolumn ninhydrin derivatization of amino acids from the acid-hydrolyzed protein permitted for detection by UV absorbance at 400 and 570 nm (*9*) using a Beckman 6300 amino acid analyzer. Quantification of amino acids was accomplished by comparison of peak areas with those of external standard amino acids. Data are averages of triplicate determinations of multiple experiments. The precipitates of rhRlx obtained from the experiments conducted at pH 6.4 and 7.4 were washed with 0.1 mM EDTA solution by centrifugation three times. Amino acid analyses of the precipitate were conducted as described above.

Analysis of 2-oxo-His in the oxidized samples of rhRlx was accomplished by manual precolumn *o*-phthaldialdehyde (OPA) derivatization using the methodology described by Lewisch and Levine (*10*). Samples of oxidized [ascorbate/Cu(II)/O_2] rhRlx before hydrolysis and derivatization for amino acid analysis using the methodology of Lewisch and Levine (*10*) were prepared in the same manner. Data are averages of triplicate determinations.

Analytical Ultracentrifugation. rhRlx samples were oxidized under standard metal-catalyzed oxidation conditions at pH values of 5.0, 6.4, and 6.6. rhRlx (0.033 mM) was also oxidized with H_2O_2 (33 mM) in phosphate buffer (10 mM) at pH values of 5.0, 6.4, and 6.6. Sodium chloride was added to the oxidized samples (H_2O_2 and metal-catalyzed oxidation conditions) to yield solutions containing 0.1 M sodium chloride. The average molecular weight of the proteins in the oxidized rhRlx samples was determined by sedimentation equilibrium studies using a Beckman XLA/1 ultracentrifuge. The oxidized samples were loaded into charcoal-filled Epon six-channel Yphantis cells. Phosphate buffer (10 mM) at the various pH values was loaded in the reference sectors. Experiments were conducted at 10 °C at two rotor speeds (40 000 and 44 000 rpm) for a period of 32 h (16 h at each rotor speed). Absorbance data at 280 nm as a function of radial position were collected every 0.001 cm. The partial specific volume of rhRlx was calculated by using values for individual amino acid residues and the additivity rule. The REEDIT PC program was used for editing the sedimentation equilibrium data. The edited data were analyzed as a single ideal species utilizing the nonlinear least-squares fitting program NONLIN to determine the average molecular weight (*11*). Dissociation constants were calculated for a monomer/dimer aggregation model for rhRlx and oxidized rhRlx.

RESULTS

Effect of pH on the Rate and Extent of Oxidation of rhRlx and pRlx by Ascorbate/Cu(II)/O_2. rhRlx and pRlx did not undergo degradation in the presence of ascorbate or $CuCl_2$ alone in media buffered in the range of pH 5.0−7.4 (data not shown). However, in the presence of both ascorbate and $CuCl_2$ in the same pH range, rhRlx (Figure 2, panel A) and pRlx (Figure 2, panel B) disappeared with time. In addition

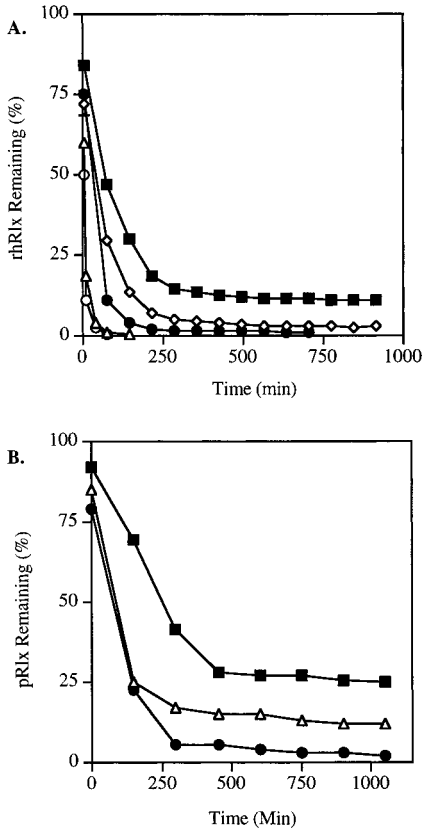

FIGURE 2: Effect of pH on the time course of degradation of rhRlx (panel A) and pRlx (panel B) in the ascorbate/Cu(II)/O_2 system. The reaction mixtures contained 0.033 mM protein, 2 mM ascorbate, 0.023 mM $CuCl_2$, and 10 mM phosphate buffer at the following pH values: panel A (■) pH 5, (◇) pH 5.8, (●) pH 6, (△) pH 6.4, and (○) pH 7.4; panel B (■) pH 5, (△) pH 6.4, and (●) pH 7.4. The amounts of rhRlx and pRlx remaining were determined by RP-HPLC as described in Experimental Procedures.

to affecting the rate of degradation (Figure 2), the pH of the metal-catalyzed oxidation reaction media also had an impact on the physical state (soluble vs precipitate) of the oxidized protein.

Effect of pH on the Amount of Soluble Protein Remaining after Oxidation of rhRlx and pRlx by Ascorbate/Cu(II)/O_2. rhRlx and pRlx did not undergo precipitation in the presence of ascorbate or $CuCl_2$ alone in media buffered in the range of pH 5.0−7.8 (data not shown). However, incubation of rhRlx with both ascorbate and $CuCl_2$ resulted in a pH-dependent precipitation phenomenon; i.e., more precipitation of the oxidized rhRlx was observed at higher pH values (Figure 3). This precipitation phenomenon was observed in both phosphate and Tris−acetate buffers. The precipitate of rhRlx could not be solubilized by lowering the pH. Incubation of pRlx with ascorbate and $CuCl_2$ also resulted in an increase in the amount of precipitated oxidized pRlx as the pH of the media was increased (Figure 3). However, the pH-dependent increase in the amount of the precipitated pRlx was significantly less than that observed for rhRlx (Figure 3).

The pH dependency of the precipitation phenomenon observed upon metal-catalyzed oxidation of rhRlx could result from lower solubility of oxidized rhRlx at higher media pH. To investigate this possibility, rhRlx was oxidized in the ascorbate/Cu(II)/O_2 system at pH 5.0, and then the pH of the solution was adjusted to higher values (5.4−7.8).

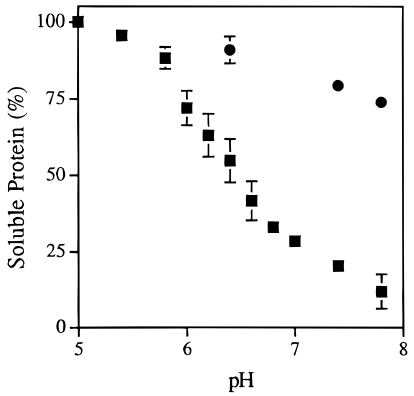

FIGURE 3: Effect of pH on the amount of soluble protein remaining following oxidation of rhRlx (■) and pRlx (●) in the ascorbate/Cu(II)/O_2 system. The reaction mixtures contained 0.033 mM protein, 2 mM ascorbate, 0.023 mM $CuCl_2$, and 10 mM phosphate buffer at the indicated pH values. The amounts of soluble protein remaining were determined by measuring the UV absorbance at 280 nm after centrifugation to remove precipitated protein as described in Experimental Procedures.

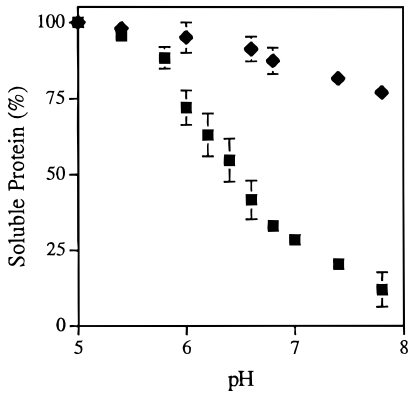

FIGURE 4: Effect of pH on the solubility of oxidized rhRlx. rhRlx was oxidized by the ascorbate/Cu(II)/O_2 system at pH 5.0 as described in the legend to Figure 3. The pH of the reaction medium was then adjusted to pH values ranging from 5.4 to 7.8 and the amount of soluble protein measured (◆). For comparison purposes, data are shown when the protein was oxidized at the indicated pH values (■). The amounts of protein remaining were determined by measuring the UV absorbance at 280 nm after centrifugation to remove precipitated protein as described in Experimental Procedures.

These solutions were then incubated at room temperature for 20 h. As shown in Figure 4, there was a slight decrease in the amount of soluble oxidized protein after adjustment to higher pH values. However, this decrease in the amount of soluble oxidized protein as a function of pH was significantly less than that observed when rhRlx was oxidized at the indicated pH values (Figure 4).

Effect of pH on the Amount of Soluble Protein Remaining after Oxidation of rhRlx by H_2O_2. Nguyen et al. (*12*) previously reported that the oxidation of the Met residues of rhRlx with H_2O_2 was fairly independent of pH over the pH range of 3−8. In their studies, they did not specifically address the issues of aggregation and precipitation. Therefore, we studied the oxidation of rhRlx by H_2O_2 at pH values in the range of 5.0−7.4. No precipitation of oxidized protein was observed at any of the pH values studied, even after the rhRlx was completely oxidized (data not shown).

Determination of Amino Acid Residues Modified after Oxidation of rhRlx by H_2O_2 and the Ascorbate/Cu(II)/O_2

Table 1: Amino Acid Composition Analysis of rhRlx Oxidized with H_2O_2 at the Indicated pH Values[a]

	composition (residues/mol)				
amino acid	theoretical values	native	oxidized rhRlx at pH 5.0	oxidized rhRlx at pH 6.4	oxidized rhRlx at pH 7.4
Asx	2	2.0	2.1	2.1	2.0
Thr	2	1.9	2.3	2.2	2.2
Ser	5	4.5	4.6	4.6	4.6
Glx	5	4.7	4.8	4.7	4.6
Gly	3	3.1	2.9	2.9	2.9
Ala	5	4.9	5.0	5.1	5.1
Cys	6	5.2	5.2	5.1	5.0
Val	3	2.6	2.6	2.7	2.7
Met	2	1.7	1.2	1.2	1.2
Ile	3	2.4	2.7	2.7	2.8
Leu	5	5.1	5.1	5.2	5.2
Tyr	1	1.1	1.0	1.0	1.0
Phe	1	1.1	1.1	1.1	1.1
His	1	1.0	1.0	1.0	1.0
Lys	3	2.9	3.1	3.1	3.1
Arg	4	3.9	3.6	3.5	3.5

[a] Conditions: reaction mixtures contained 0.033 mM rhRlx and 33 mM H_2O_2 in 10 mM phosphate buffer at the indicated pH values. The reaction mixtures were incubated at room temperature for a period of 24 h. The oxidized protein reaction mixtures underwent ultrafiltration to remove excess H_2O_2 before analysis.

Table 2: Amino Acid Composition Analysis of the Soluble Fraction of rhRlx Oxidized in an Ascorbate/Cu(II)/O_2 System[a]

	composition (residues/mol)				
amino acid	theoretical values	native	oxidized rhRlx at pH 5.0	oxidized rhRlx at pH 6.4	oxidized rhRlx at pH 7.4
Asx	2	2.0	2.0	2.0	1.9
Thr	2	1.9	2.3	2.3	2.4
Ser	5	4.5	4.6	4.8	5.0
Glx	5	4.7	4.8	4.7	4.6
Gly	3	3.1	3.2	3.4	3.4
Ala	5	4.9	5.2	5.2	5.1
Cys	6	5.2	5.2	4.9	5.0
Val	3	2.6	2.7	2.7	2.8
Met	2	1.7	1.1	1.0	1.2
Ile	3	2.4	2.8	2.9	2.8
Leu	5	5.1	5.1	5.2	5.2
Tyr	1	1.1	0.9	1.0	1.0
Phe	1	1.1	1.0	1.1	1.1
His	1	1.0	0.6	0.4	0.1
Lys	3	2.9	3.1	3.1	3.1
Arg	4	3.9	3.6	3.7	3.8

[a] Conditions: reaction mixtures contained 0.033 mM rhRlx, 0.023 mM $CuCl_2$, 2 mM ascorbate, and 10 mM phosphate buffer at the indicated pH values. The reaction mixtures were incubated at room temperature for a period of 24 h and then were centrifuged. The supernatant containing soluble oxidized protein underwent ultrafiltration to remove oxidized ascorbate and $CuCl_2$ before analysis.

System. To reconfirm observations made by Nguyen et al. (*12*) and Li et al. (*4*), amino acid analysis was used to implicate the primary amino acid residues (Met and/or His) impacted by the different oxidative systems. Amino acid analysis of the degradants produced by H_2O_2 oxidation of rhRlx at pH values of 5.0, 6.4, and 7.4 revealed that there was primarily a decrease in Met content; the amounts of other amino acid residues were not significantly altered (Table 1). The content of Trp was not determined due to its degradation during acid hydrolysis. It is important to note that the decrease in Met content was not quantitative due to reduction of Met sulfoxide to Met during the standard acid hydrolysis conditions. Nevertheless, a peak was observed to coelute with authentic Met sulfoxide in the hydrolyzed samples of oxidized rhRlx. Amino acid analysis indicated that the His residue remained intact after oxidation of rhRlx with H_2O_2 at pH values of 5.0, 6.4, and 7.4. This was not the case after metal-catalyzed oxidation of rhRlx.

The amino acid content of the soluble degradants produced by ascorbate/Cu(II)/O_2 oxidation of rhRlx at pH values of 5.0, 6.4, and 7.4 revealed increased losses in His as the pH increased (Table 2). Oxidations of rhRlx with the ascorbate/Cu(II)/O_2 system also resulted in a decrease in the Met content of the soluble degradants, but this loss appears to be pH independent. However, exact quantitation of this loss of Met is problematic because of the tendency of Met sulfoxide to be reduced to Met upon acid hydrolysis of the protein. It appears that other amino acid residues are not significantly altered upon metal-catalyzed oxidation of rhRlx. It should be noted that the content of Trp could not be determined because of its degradation during acid hydrolysis. It is important to note that the Trp residue is not susceptible to metal-catalyzed oxidation (*3*). It is susceptible to photooxidation (*2*). The photooxidation of Trp results in the formation of *N*-formylkynurenine and/or kinurenine that cause(s) a yellowish appearance of the solution and display(s) characteristic UV spectral scans (*2*). This phenomenon was not observed.

The amino acid content of the precipitated degradants produced by metal-catalyzed oxidation of rhRlx at pH 6.4 and 7.4 was also determined (data not shown). As observed with the soluble degradants of rhRlx, reductions in the content of His and Met were observed in the precipitated degradants (data not shown). To characterize the degradant(s) of the His and Met residues formed after metal-catalyzed oxidation of rhRlx, tryptic digests were prepared and characterized by LC/MS.

LC/MS Characterization of the Tryptic Peptides Generated from Oxidized rhRlx. Tryptic maps of rhRlx and the soluble degradants produced by ascorbate/Cu(II)/O_2 oxidation of rhRlx at pH values of 5.0, 6.4, and 7.4 were generated. Representative tryptic maps of rhRlx and oxidized rhRlx at pH 5.0 are shown in panels A and B of Figure 5, respectively. Theoretical and experimentally determined molecular masses for the tryptic peptides and oxidized tryptic peptides are displayed in Table 3. The His-containing peptide fragment (T_2-T_7) of rhRlx was significantly reduced in the protein oxidized at pH 5.0, 6.4, and 7.4. The structures of the degradants of His produced upon metal-catalyzed oxidation of rhRlx could not be characterized by this method. This may be due to the formation of numerous degradation products, all in relatively low yield, making structural characterization difficult. The inability to identify His degradants formed upon metal-catalyzed oxidation has posed difficulty to other researchers (*13*−*15*). The tryptic maps of rhRlx oxidized at pH 5.0, 6.4, and 7.4 showed two new major peaks with masses consistent with values expected for the incorporation of an oxygen into the Met-containing peptide fragments (T_6 and T_5-T_9). These data provide evidence that

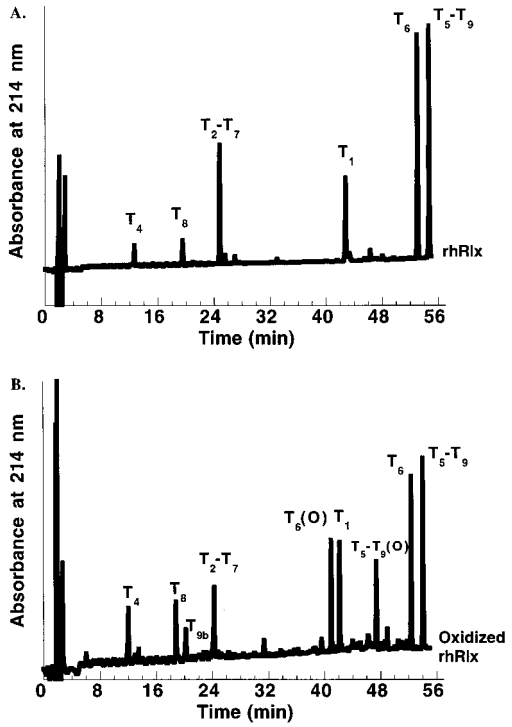

FIGURE 5: Representative RP-HPLC tryptic maps of rhRlx (panel A) and oxidized rhRlx (panel B) generated by oxidation of the protein in the ascorbate/Cu(II)/O_2 system at pH 5.0. Tryptic digestion of the proteins and LC/MS of the tryptic fragments were conducted as described in Experimental Procedures.

Table 3: Mass Spectral Analysis of Tryptic Peptides Generated from rhRlx and Oxidized rhRlx[a]

assignment	sequence chain and residue no.	theoretical masses	observed masses native	oxidized at pH 5.0
T_1	A1−9	990.55	990.4	990.3
T_2−T_7	A10−17, B10−13	1293.57	1293.4	1293.4
T_4	A19−22	446.27	446.3	446.3
T_5−T_9	A23−24, B18−29	1533.68	1533.3	1533.3
T_5−T_9 (O)	A23−24, B18−29	1549.68		1550.2
T_6	B1−9	1136.53	1136.3	1136.4
T_6 (O)	B1−9	1152.53		1152.3
T_8	B14−17	516.31	517.3	517.3
T_{9b}	B26−29	480.21	480.1	480.1

[a] Reaction mixtures contained 0.033 mM rhRlx, 0.023 mM $CuCl_2$, 2 mM ascorbate, and 10 mM phosphate buffer at pH 5.0.

metal-catalyzed oxidation of rhRlx results in the chemical modification of the Met B(4) and Met B(25) residues to Met sulfoxides. In an attempt to identify the degradant(s) of the His residue, other experimental techniques were employed. The precipitate of rhRlx was not analyzed by trypsin digest and LC/MS because of difficulty in solubilizing the precipitate of oxidized rhRlx under conditions which trypsin could be used for digest. The precipitate of oxidized rhRlx was analyzed by amino acid analysis.

Characterization of the Degradation Products of His A(12) in rhRlx Oxidized with the Ascorbate/Cu(II)/O_2 System. NMR spectroscopy of rhRlx showed that the imidazole C_2 and C_4 proton resonances of His A(12) shift as a function of pH due to the equilibria between the protonated and unprotonated forms of the imidazole ring. The shifts in resonances were

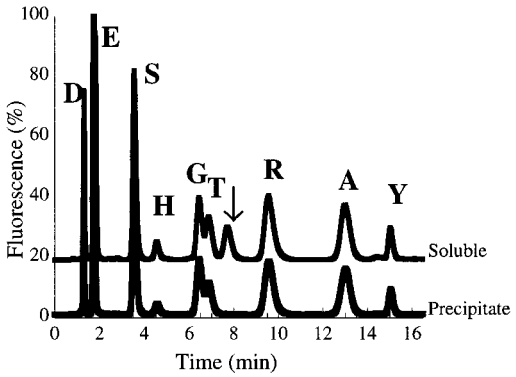

FIGURE 6: Representative chromatograms of the component amino acid residues after acid hydrolysis of the soluble and precipitated fractions of rhRlx oxidized by ascorbate/Cu(II)/O_2 in 10 mM phosphate buffer at pH 7.4. Sample hydrolysis and amino acid analysis were accomplished using the methodology described by Lewisch and Levine (*9*). Amino acid residues of hydrolysates were derivatized by manual precolumn OPA derivatization. OPA derivatives were detected by fluorescence at 445 nm after excitation at 230 nm.

used to identify the C_2 and C_4 imidazole protons and to determine the pK_a of His A(12). The pK_a of the His A(12) residue of rhRlx was determined to be approximately 6.3 (data not shown).

In comparing the NMR spectra of rhRlx to those of the soluble degradants of the protein generated by oxidation with the ascorbate/Cu(II)/O_2 system, it was apparent that the C_4 imidazole proton at approximately 7.8 ppm present in rhRlx was absent in the soluble degradants. The soluble degradants exhibited a new resonance at approximately 6.3 ppm (data not shown). These alterations in the NMR spectra of a His-containing protein have been observed by other researchers and have been attributed to the presence of 2-oxo-His (*15*, *16*).

The degradants in the precipitate of oxidized rhRlx were also analyzed by NMR. To conduct these studies, it was necessary to solubilize the protein with deuterated urea. In comparing the NMR spectra of rhRlx to those of the degradants present in the precipitate of oxidized rhRlx, it was apparent that the signals for both of the imidazole protons had disappeared. Unfortunately, it was not possible to determine if a new signal for an imidazole proton at approximately 6.3 ppm was present in the oxidized protein because of the presence of the signal for the protons in urea (data not shown). Therefore, NMR analysis of the degradants in the precipitate of oxidized rhRlx showed that the His A(12) residue had degraded but confirmation of the presence of 2-oxo-His was not possible using this technique.

Determination of the Amino Acid Composition of the Degradants in the Soluble and Precipitated Fractions of rhRlx Oxidized by Ascorbate/Cu(II)/O_2. Amino acid analyses of the soluble and precipitated fractions of rhRlx oxidized at pH values of 5.0, 6.4, and 7.4 were conducted using specific methods developed by Lewisch and Levine (*10*) for detection of 2-oxo-His. The chromatograms of amino acids present in the soluble degradants of rhRlx oxidized at pH 5.0, 6.4, and 7.4 displayed a new peak between Thr (T) and Arg (R) (Figure 6). A similar peak was not observed in the amino acid pool generated by hydrolysis of the precipitated degradants of rhRlx oxidized at pH 6.4 and 7.4 (Figure 6).

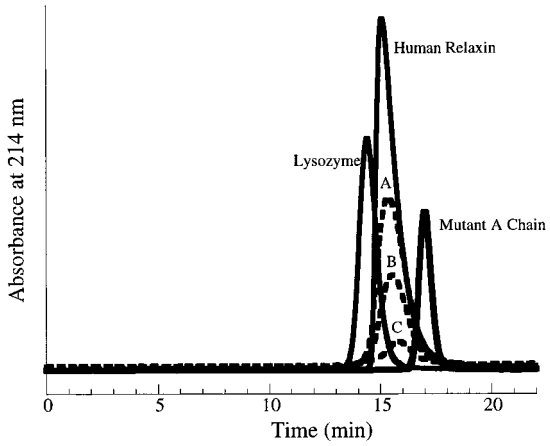

FIGURE 7: Representative nondenaturing SEC−HPLC chromatograms of rhRlx (MW 5963), the mutant A chain fragment of rhRlx [MW 2590, Cys A(11), and Cys A(24) replaced with Ala], lysozyme (MW 14 388), and rhRlx oxidized by ascorbate/Cu(II)/O_2 at pH 5.0 (A), 6.4 (B), and 7.4 (C).

The extra peak observed in the amino acid pool generated by hydrolysis of the soluble degradants of rhRlx elutes with a retention time similar to that reported by Lewisch and Levine (*10*) for 2-oxo-His. The content of the new peak also behaved like 2-oxo-His in that it was unstable under basic conditions; i.e., when the pH of the hydrolysate was increased to 10 for approximately 30 min before derivatization and injection onto the column, the extra peak was no longer detectable (data not shown). Similar behavior for 2-oxo-His was observed by Lewisch and Levine (*10*). Furthermore, it was observed that if 40 mM DTT was not included during the hydrolysis of the soluble degradants with 6 N HCl, the amount of the new peak observed in the amino acid pool was reduced, which is another characteristic of 2-oxo-His observed by Lewisch and Levine (*10*).

It is interesting to note that the amount of 2-oxo-His present in the soluble degradants of rhRlx oxidized with ascorbate/Cu(II)/O_2 appears to increase with increases in the pH of the reaction media (data not shown). Similar observations were recently reported by our laboratory using a His-containing peptide fragment of rhRlx (personal communications). These results suggest that 2-oxo-His is one of the degradation products of His A(12) in rhRlx induced by metal-catalyzed oxidation and that 2-oxo-His appears in the soluble degradants of this protein but not in the precipitated degradants.

Characterization of the Aggregation Tendency of the Soluble Degradants of rhRlx Generated by Ascorbate/Cu(II)/O_2 or H_2O_2-Induced Oxidation. Nondenaturing SEC with UV detection at 214 nm was utilized to determine aggregate formation upon oxidation of rhRlx in a metal-catalyzed oxidation system or a H_2O_2 oxidation system at pH values in the range of 5.0−7.4. The soluble degradants of rhRlx oxidized by ascorbate/Cu(II)/O_2 at various pH values (5.0−7.4) eluted with the same retention time as rhRlx (MW 5963) (Figure 7). In Figure 7, the elution profile of lysozyme (MW 14388) is provided as a marker of where a dimer of rhRlx would be expected to elute. The elution profile of a mutant A chain [MW 2590, Cys A(11) and Cys A(24) replaced with Ala] of rhRLx is also provided in Figure 7 as a marker for the expected elution of fragmented rhRlx. These results indicate that there was no significant aggregate formation (covalent or noncovalent) or fragmentation into the component A and B chains induced by metal-catalyzed oxidation of rhRlx. Similar results were obtained when rhRlx was oxidized by H_2O_2 at various pH values in the range of 5.0−7.4 (data not shown). It is important to note that the aggregation tendency of oxidized rhRlx in an ascorbate/Cu(II)/O_2 system was further analyzed with SEC using a multiangle laser light scattering detector (more sensitive than UV spectrophotometry at 214 nm) for analysis. The results indicated that there was an extremely small population (1−3%) of larger aggregate formed and the predominant species was monomer. Detection of noncovalent aggregates that have relatively weak association constants with nondenaturing SEC may be problematic due to the effect of dilution upon HPLC analysis. For example, rhRlx (0.1 mg/mL) was shown by ultracentrifugation studies to naturally exist in a monomer/dimer equilibrium that is noncovalent in nature (*7*). The dimer of rhRlx was detectable at a concentration of 2 mg/mL by nondenaturing SEC but was not detectable at lower concentrations (i.e., 0.4 mg/mL) (*17*).

Because of the limitation of SEC to detect noncovalent aggregates, the aggregation tendencies of the soluble degradants of oxidized rhRlx were determined by ultracentrifugation. Dissociation constants were calculated for the monomer/dimer equilibrium of rhRlx and the soluble degradants of rhRlx oxidized with the ascorbate/Cu(II)/O_2 system at pH values in the range 5.0−6.6. The dissociation constants of rhRlx and the soluble degradants of oxidized rhRlx generated at different pH values were not significantly different from each other (data not shown). These results indicate that the monomer/dimer equilibrium of rhRlx was not significantly impacted by metal-catalyzed oxidation. Furthermore, these results indicate that the predominant population of the soluble degradants of this protein does not exist as larger aggregates. Similar results were obtained for rhRlx oxidized by H_2O_2 at pH values in the range of 5.0−6.6 (data not shown).

DISCUSSION

Li et al. (*4*) hypothesized that metal-catalyzed oxidation of His A(12) in rhRlx leads to alterations in the secondary and tertiary structures of the protein (as observed by CD studies) that result in the physical instability (precipitation) observed at higher pH (>6.0). To test this hypothesis, we describe here the results of experiments involving the oxidation of rhRlx with H_2O_2 and the oxidation of pRlx with the ascorbate/Cu(II)/O_2 system.

The experiments involving the oxidation of rhRlx with H_2O_2 were conducted because Nguyen et al. (*12*) have reported that H_2O_2 oxidizes only Met B(4) and Met B(25) to their corresponding sulfoxides in rhRlx and the reaction was fairly independent of pH over the pH range of 3−8. If our hypothesis about the role of His A(12) in the physical instability of this protein is correct, oxidation of rhRlx with H_2O_2 should lead not to physical instability of the protein but simply to chemical modification of the Met residues. The results observed here are consistent with this hypothesis; i.e., H_2O_2 oxidation of rhRlx in the pH range 5.0−7.8 led to chemical modification of Met B(4) and Met B(25), and the oxidized degradants showed no evidence of physical instability (i.e., precipitation).

The experiments involving the oxidation of pRlx with the ascorbate/Cu(II)/O_2 system were conducted because this protein lacks His in the A(12) position and does not contain any other His residue (*7*). If our hypothesis about the role of His A(12) in the physical instability of rhRlx is correct, metal-catalyzed oxidation of pRlx should lead to chemical modification of its Met residue at A(2) but not to physical instability of the protein. It should be noted that pRlx also lacks the Met B(4) and B(25) that are found in rhRlx. However, on the basis of the experimental results described above for H_2O_2 oxidation of rhRlx, it is apparent that these Met residues are not involved in the physical instability of this protein. The other amino acid differences between pRlx and rhRlx were not deemed as important because of the highly specific mechanism of metal-catalyzed oxidation reactions requiring amino acid residues to complex with transition metal ions. As noted before, these susceptible amino acid residues are His, Arg, Met, Lys, Pro, and Cys (*3*). In comparing the sequence of rhRlx to that of pRlx, the Arg, Lys, and Cys residues were preserved. rhRlx and pRlx do not have a Pro residue. It is important to note that pRlx has a secondary structure similar to that of rhRlx (*7*). When pRlx was oxidized with the ascorbate/Cu(II)/O_2 system, it underwent rapid chemical degradation similar to that observed with rhRlx. However, the oxidative degradants generated from pRlx were much more soluble than those generated from rhRlx over the pH range 6.0−7.8. Again, these results with pRlx are consistent with the hypothesis that oxidation of His A(12) is crucial to the physical instability of rhRlx.

Several mechanisms/observations may be possible to explain the pH-dependent physical instability of oxidized rhRlx, including the following: (i) a common oxidative degradant of rhRlx is generated by the ascorbate/Cu(II)/O_2 system at all pH values studied, but the solubility of this degradant decreases with increasing pH; (ii) the intermediate soluble aggregates of this oxidative degradant increase in size as the pH of the medium is increased; and (iii) different pathways of degradation of His A(12) predominate at different pH values, and the oxidative products of this amino acid that are generated at higher pH cause more significant alterations in the secondary/tertiary structures of the proteins leading to the pH-dependent physical instability (e.g., precipitation).

The solubility of the oxidative degradants of rhRlx is a possible explanation for the pH-dependent physical instability of this protein because the residue that is chemically modified is His, a basic amino acid. The possible oxidative products of His are either neutral, i.e., 2-oxo-His (*16*, *18*) and Asn (*19*, *20*), or acidic, i.e., Asp (*20*, *21*). Metal-catalyzed oxidation of proteins has been reported to lower the p*I* of the protein and to make it more hydrophobic (*22*, *23*). The p*I* of rhRlx has been calculated as 9.8 (*24*) and experimentally determined by isoelectric focusing to be 9.1 (*25*). Therefore, if the p*I*(s) of common degradant(s) arising from metal-catalyzed oxidation of rhRlx was (were) significantly less than that of rhRlx itself, it is conceivable that they would exhibit solubility differences in the pH range (5.0−7.8) used in our studies. In an attempt to experimentally test the possibility that common oxidative degradants that exhibited pH-dependent solubility were being formed, rhRlx was oxidized with the ascorbate/Cu(II)/O_2 system at pH 5.0 to generate only soluble oxidative degradants. The pH of aliquots of this solution of soluble oxidative degradants was adjusted to 5.4−7.8. After the samples were allowed to equilibrate, the amounts of soluble vs precipitated protein were determined. While the amount of soluble protein decreased slightly with increasing pH (i.e., pH 5.0, 100% soluble, vs pH 7.8, 77% soluble; Figure 4), the changes were not as dramatic as those observed when the protein was oxidized at these same pH values (i.e., pH 5.0, 100% soluble, vs pH 7.8, 12% soluble; Figure 4). If the same degradants were formed over the pH range 5.0−7.8, both experiments should have shown a similar pH−solubility relationship.

Another possible explanation for the pH-dependent physical instability of the oxidized rhRlx is the formation of soluble intermediate aggregates that increase in size with increasing pH. Li et al. (*4*) have determined with dynamic light scattering (DLS) that rhRlx oxidized with the ascorbate/Cu(II)/O_2 system at pH 5.0 forms some large soluble aggregates (mean diameter = 181 nm). A shortcoming of DLS is that this technique does not quantitate the fraction of species that actually exists as these aggregates. Therefore, a very small population of large aggregates may scatter sufficient light to lead one to believe that this is the predominant species in solution. Therefore, we examined more closely the aggregation tendency of rhRlx oxidized by ascorbate/Cu(II)/O_2 using nondenaturing SEC and equilibrium ultracentrifugation. As noted before, no aggregates of oxidized (ascorbate/Cu(II)/O_2 or H_2O_2 at pH values of 5.0−7.4) soluble rhRlx were detected by nondenaturing SEC with UV detection at 214 nm. The multiangle laser light scattering detector is much more sensitive than UV spectrophotometry at 214 nm. Using nondenaturing SEC with on-line multiangle laser light scattering detection, we could detect a minor population of large aggregated species in the soluble fraction of rhRlx oxidized with the ascorbate/Cu(II)/O_2 system, but the major average species existed as a monomer similar in nature to rhRlx. A closer look at the aggregation tendency of oxidized soluble rhRlx was accomplished with equilibrium ultracentrifugation studies, which are not influenced by dilution of the samples as is nondenaturing SEC. Equilibrium ultracentrifugation studies of rhRlx and rhRlx oxidized in the ascorbate/Cu(II)/O_2 system (or H_2O_2) at pH 5.0−6.6 confirmed that the predominant species were the monomers with some dimer in an equilibrium. It was apparent that the aggregation tendency of the remaining soluble fraction of oxidized rhRlx at the different pH values is similar to native rhRlx as indicated by nondenaturing SEC.

Another possible explanation for physical insolubility of the oxidized rhRlx at higher pH is the pH-dependent generation of different oxidative products from His A(12), which have different effects on the physical stability of the protein. Metal-catalyzed oxidation of His in proteins has been reported to yield various products, including Asp, Asn, and 2-oxo-His (*18*−*20*). On the basis of amino acid analysis of both the soluble and precipitated degradants of rhRlx generated by oxidation with ascorbate/Cu(II)/O_2, it appears that little or no conversion of His A(12) to Asn or Asp occurs. These results are consistent with observations recently reported by our laboratory on a His-containing peptide fragment of rhRlx, (cyclo S−S) Ac-Cys-Ala-His-Val-Gly-Cys-NH_2 (personal communications). The soluble degradants generated from ascorbate/Cu(II)/O_2 oxidation of this peptide

fragment as well as from rhRlx were shown to not contain Asp or Asn but to contain 2-oxo-His by NMR and amino acid analysis. However, although a loss of His was observed in the precipitated fraction of oxidized (ascorbate/Cu(II)/O_2) rhRlx generated at pH 6.4 and 7.4, no 2-oxo-His was detected by amino acid analysis. These results suggest that the precipitated degradants of oxidized (ascorbate/Cu(II)/O_2) rhRlx contain some yet unidentified oxidative product(s) of His. Other researchers (*13*−*15*, *18*, *19*) have encountered similar difficulty in accounting for all of the oxidative degradants of this amino acid. On the basis of these results, we can conclude that formation of 2-oxo-His does not appear to produce sufficient alteration in the secondary and tertiary structures of the protein to result in precipitation. Instead, other as yet unidentified oxidative products arising from His A(12) that are formed in larger quantities at higher pH are responsible for the physical instability of the protein.

It is important to note that the degradants formed upon metal-catalyzed oxidation of rhRlx (i.e., one degradant pathway leading to soluble oxidized protein and another to the precipitate of oxidized protein) appear to be formed concurrently. In other words, it does not appear that a soluble degradant is formed, which then undergoes further chemical modification to yield an insoluble degradant. This hypothesis is supported by the observation that addition of more ascorbate and Cu(II) to the soluble portion of oxidized rhRlx at pH 5.0, 6.4, and 7.4 does not result in additional precipitation of protein.

Oxidative modification of His in various proteins, including vanadium bromoperoxidase (*14*), bovine serum albumin (*15*), and *Escherichia coli* glutamine synthetase (*19*, *26*), has been reported to produce alterations in the overall conformations of the proteins and/or their biological activity. Recent studies from our laboratory, using a His-containing peptide fragment of rhRlx (cyclic-His peptide), have provided insight into the way in which chemical modification of His A(12) in this protein could ultimately result in changes in the protein's secondary/tertiary structures. This cyclic-His peptide was designed as a model of the extended loop in rhRlx (personal communications), which contains His A(12). Büllesbach and Schwabe (*6*) have proposed that the structural integrity of this His-containing extended loop region of rhRlx is important for maintenance of the overall higher ordered structure of rhRlx. This statement by Büllesbach and Schwabe (*6*) was based on characteristics of mutants of rhRlx which had amino acid residues including the His in the extended loop of rhRlx replaced by others. The mutants displayed less helical structure than native rhRlx and were biologically inactive. Since the biological activity of rhRlx is imparted by the Arg residues located in the B chain of rhRlx, it appeared that the modification of residues in the extended loop region of the A chain of rhRlx leads to alterations in the overall conformation of the protein to result in a loss of biological activity (*6*). This loop in rhRlx is located between two α-helices on the A chain (Figure 1) (*6*). Metal-catalyzed oxidation of the cyclic-His peptide was shown to result in the formation of the cyclic 2-oxo-His peptide as a major degradant and numerous other degradants in low yield (personal communications). The cyclic 2-oxo-His peptide was shown by CD to have a conformation different from that of the cyclic-His peptide (personal communications). These results suggest that modification of His A(12) in rhRlx to the 2-oxo-His residue (or other oxidative products) could result in alteration in the conformation of the extended loop region of the protein, ultimately impacting its overall higher ordered conformation. Consistent with this hypothesis was the observation made by Li et al. (*4*) that the helical content of the soluble oxidized (ascorbate/Cu(II)/O_2) rhRlx was less than that of rhRlx itself as determined by CD studies (*4*). It appears that modification of the His A(12) of rhRlx to the 2-oxo-His residue does perturb the higher ordered structure of the protein but not significantly enough to cause precipitation of the protein because 2-oxo-His is found only in the soluble fraction and not in the precipitated fraction of this protein. It is possible that there exist other oxidative products of His that disrupt the conformation of the extended loop region of rhRlx more significantly, resulting in the ultimate precipitation of the protein.

To gain insight into the possible origin of the pH-dependent formation of different oxidation products of His, it is important to understand that metal-catalyzed oxidation is a site-specific process in which complexes are formed between the side chains of susceptible amino acid residues (i.e., imidazole of His), the transition metal ion [i.e., Cu(II)], and other species (i.e., ascorbate, buffer) in solution. It is within these complexes that reactive oxygen species are generated, which eventually produce damage to the protein (*3*, *27*). This significant site-specific feature of metal-catalyzed oxidation reactions is the reason that, generally, at most 1 or 2 amino acid residues in a given protein are modified by these reactions (*28*−*30*). The site specificity of the oxidation of His A(12) in rhRlx probably arises from the selective formation of complexes between the imidazole of His with Cu(II) and possibly other species in solution. Formation of these complexes was suggested in this study by NMR. It is important to realize that the geometry and ligand coordination mode of these types of complexes are dependent on pH, and the relative distribution of these complexes is, therefore, variable (*31*, *32*). In fact, we have shown in earlier work using the cyclic-His peptide mimetic of the extended loop of rhRlx that different populations of complexes exist between the His residue and Cu(II) at pH 5.3 vs pH 7.4 (personal communications). Due to the highly site-specific nature of metal-catalyzed oxidation reactions in which the generated reactive oxygen species react with the functional group (i.e., imidazole ring of His) in very close proximity before diffusion into the bulk solution, the geometries of these different complex forms could reasonably give rise to different degradation products. The varying population of these complex forms, as a function of pH, may also dictate the amounts of degradants formed. This may be the reason that the 2-oxo-His degradant of His A(12) in rhRlx appeared to be formed to a greater extent as the pH of the metal-catalyzed oxidation reaction was increased. A similar phenomenon was observed upon metal-catalyzed oxidation of the cyclic-His peptide (personal communications). Other researchers have observed that the increase (or decrease) in a particular complex form of His as a function of pH often resembles a titration curve (*33*, *34*). It is interesting to note that a plot of the percent soluble oxidized rhRlx remaining vs pH also resembles a titration curve (Figure 3). There may be a correlation between the pH-dependent formation of a particular His/Cu(II) complex and its related degradants

(unidentified) that results in precipitation of rhRlx oxidized by ascorbate/Cu(II)/O_2. The pH dependency of complex formation for His/Cu(II) complexes and the precipitation phenomenon observed for oxidized rhRlx are other important factors in support of the involvement of His A(12) in the precipitation phenomenon observed upon metal-catalyzed oxidation of rhRlx.

In conclusion, there appear to be at least two independent degradant pathways of His A(12) when rhRlx is oxidized with ascorbate/Cu(II)/O_2. The rates of these pathways appear to be dependent on pH, probably because of the site-specific nature of metal-catalyzed oxidation reactions, which are dependent on complex formation [i.e., His/Cu(II)] and also on pH. One degradant pathway leads to soluble oxidized protein and the other to insoluble oxidized protein. The alteration of the His residue in the loop region of rhRlx to 2-oxo-His may result in changes in the overall conformation of the protein, but these changes do not appear to be sufficient to result in physical instability; thus, degradants arising from this pathway were soluble. In contrast, degradation of this His via other as yet unidentified pathways apparently leads to alterations in the secondary structure of the loop region of rhRlx in a manner that causes more dramatic changes, resulting in precipitation.

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