Aggregation and Precipitation of Human Relaxin Induced by Metal-Catalyzed Oxidation[†,‡]

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ABSTRACT: The interactions of proteins with reactive oxygen species may result in covalent modifications of amino acid residues in proteins and possible alterations of protein conformation. In an attempt to elucidate the mechanisms of the metal-catalyzed oxidation of human relaxin, we employed ascorbic acid/transition metal ion [Cu(II) or Fe(III)]/O_2 as a model oxidizing system. Experimental results indicated selective oxidation of His and Met residues and rapid formation of aggregates (noncovalent, pH dependent) following the oxidation reaction. Amino acid analysis and LC/MS data following tryptic digestion demonstrated the oxidation of the His A(12) residue, which resulted in 2-oxohistidine and some other unidentified degradation products. The oxidation of both Met residues to Met-sulfoxides was also identified, and it was found that Met B(4) was more easily oxidized than Met B(25). The comparative kinetic studies of two Met-containing fragments of relaxin suggested that the preferred oxidation of Met B(4) is due to its close proximity to some metal-binding neighboring amino acid residues. These covalent alterations may lead to the modification of secondary and tertiary structure and increase the exposure of the hydrophobic surface of the protein which eventually induces aggregation of precipitation. The modification of relaxin by ascorbic acid/$CuCl_2$ solution could be totally inhibited by the presence of EDTA. In contrast, catalase and superoxide dismutase showed no effects on the oxidation process.

Various spontaneous processes lead to the covalent alteration of proteins and, in many cases, the loss of biological activity (Kosen, 1992). In recent years, attention has been focused on oxidation as one of the major degradation pathways in proteins. Much effort has been devoted to elucidating the mechanisms of protein oxidation since they are of great interest for the understanding of biological systems in connection with aging and the pathology of various disease states (Stadtman, 1992). In addition, the recent development of protein therapeutics through recombinant DNA technology has created a tremendous need for an understanding of the nature of protein oxidation and the development of strategies to stabilize protein formulations (Manning et al., 1989; Cleland et al., 1993).

Usually, the investigation of protein oxidation is complicated by the existence of different oxidation mechanisms under various conditions of oxidative stress (Stadtman, 1993). Among all amino acids, those containing a sulfur atom (Met and Cys) or an aromatic ring (His, Trp, Tyr, and Phe) are most susceptible to oxidative modifications. However, the selective damage of these labile amino acid residues and the underlying oxidation mechanisms would depend on the nature of the active oxygen species generated under various oxidation conditions. Two prominent factors which may dramatically accelerate spontaneous oxidation include light and the presence of trace metal ions. Our interest in metal-catalyzed activation of oxygen originates mainly from (i) the existence of metal ions in biological systems (Timothy & Steven, 1992) and (ii) the potential contaminating metals in reagents (e.g., buffers) used for processing and formulation of protein pharmaceuticals.

Relaxin (Rlx)[1] is a protein hormone which plays a major role in the reproductive biology of various species and has been developed as a therapeutic agent (Stults et al., 1990; Büllesbach & Schwabe, 1991). The primary structure of recombinant human Rlx is illustrated in Figure 1. Earlier studies indicated that oxidation was one of the primary degradation pathways for Rlx. It was reported that the oxidation of Met B(4) and Met B(25) was most significant when the Rlx molecule was exposed to light (Cipolla & Shire, 1991) or hydrogen peroxide (H_2O_2) (Nguyen et al., 1993). In the present study, we have attempted to characterize the metal-catalyzed oxidative modification of Rlx induced by ascorbic acid (AsA)/transition metal ions [Cu(II) or Fe(III)], a combination which has been used as a model system for the generation of active oxygen species (including H_2O_2, $O_2^{\bullet-}$, and $HO^{\bullet}$) in our previous studies (Li et al., 1993, 1995). It is demonstrated that, in addition to the oxidation of Met, the oxidation of His is also very pronounced under these conditions of metal-catalyzed oxidation. The resulting covalent alterations apparently cause structural modifications leading to subsequent aggregation of the protein.

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[‡] The X-ray crystal structure of relaxin is deposited in the Protein Data Bank under Accession Number 6RLX.

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[1] Abbreviations: ACN, acetonitrile; AsA, ascorbic acid; BSA, bovine serum albumin; CAT, catalase; DLS, dynamic light scattering; Rlx, human relaxin; SDS, sodium dodecyl sulfate; SEC, size-exclusion chromatography; SOD, superoxide dismutase; TFA, trifluoroacetic acid; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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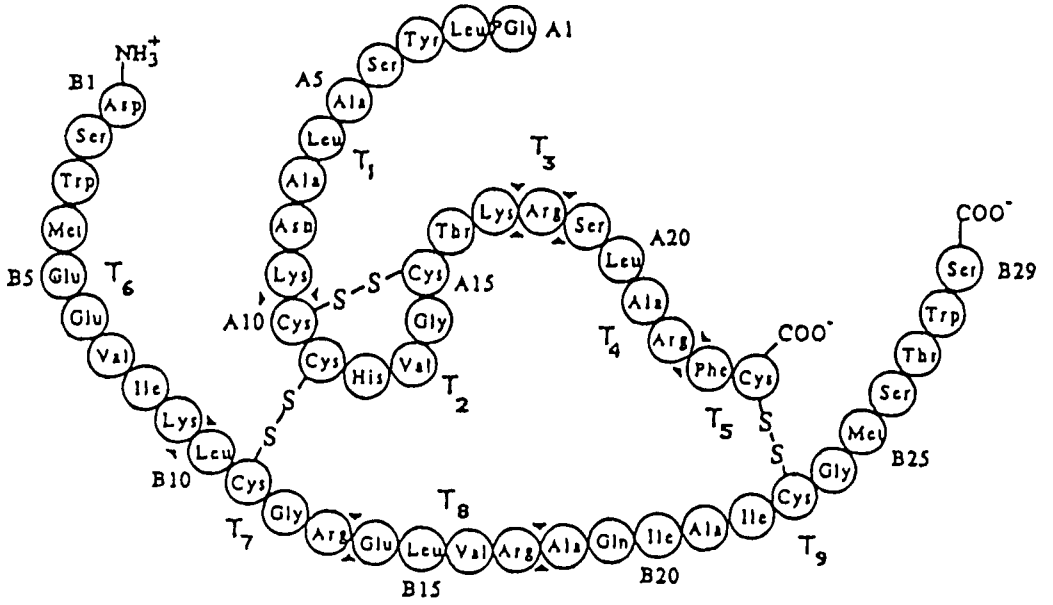

FIGURE 1: Representation of the primary structure of human relaxin. Arrows indicate potential trypsin cleavage sites and resulting peptide fragments.

MATERIALS AND METHODS

Materials

Recombinant human relaxin (Rlx) was produced by Genentech, Inc. (San Francisco, CA). The methods of synthesis and purification were described in previous reports (Stults et al., 1990; Canova-Davis et al., 1991). Two Met-containing fragments of Rlx, AcSer-Trp-Met-Glu-GluNH$_2$ and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$, were also synthesized and provided by Genentech, Inc. Ascorbic acid (AsA) and cupric chloride ($CuCl_2$) were supplied by Aldrich Chemical Co. (Milwaukee, WI). Buffers including Tris·HCl (Research Organics, Inc., Cleveland, OH), sodium acetate (Sigma Chemical Co., St. Louis, MO), and sodium phosphate (Fisher Scientific, Pitsburgh, PA) were all obtained as the analytical grade. Calcium chloride ($CaCl_2$) and acetonitrile (ACN, HPLC grade) were from Fisher. Trifluoroacetic acid (TFA, HPLC grade) was provided by Pierce Chemical Co. (Rockford, IL). BioRad (Richmond, CA) supplied the protein assay dye reagent concentrate. Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), and TPCK [L-1-(tosylamino)-2-phenylethyl chloromethyl ketone]-treated trypsin were purchased from Sigma.

Methods

Protein Solution Preparation. Rlx stock solutions were prepared in several different buffers for use in oxidation reactions. Rlx solutions initially reconstituted into citrate buffer solution were dialyzed into acetate or phosphate buffer (20 mM, pH 5) using Spectra/Por 3 membranes (MWCO 3500). The concentration of the Rlx solution was 1.00 mg/mL in acetate and 1.44 mg/mL in phosphate as determined by UV spectroscopy (HP 8451A diode array spectrophotometer) using an extinction coefficient of 2.5 mg^{-1} cm^{-1} mL at 280 nm.

Oxidation of Rlx by AsA/$CuCl_2$ Solution. All the oxidation reactions were carried out at room temperature (20−25 °C). The typical reaction mixtures contained 0.1 mg/mL Rlx, 2 mM AsA, and 0.02 mM $CuCl_2$ in both sodium phosphate and Tris·acetate (10 mM) buffer solutions at pH 5−8.

Comparative Kinetic Studies of the Oxidation of Two Model Peptides, AcSer-Trp-Met-Glu-GluNH$_2$ and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$, by AsA/$CuCl_2$. The N-terminal-acetylated and C-terminal-amidated peptides AcSer-Trp-Met-Glu-GluNH$_2$ (RlxP1) and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ (RlxP2) were designed to mimic the residues around Met B(4) and Met B(25), respectively. The oxidation kinetics were compared for the reactions of these two peptides in a mixture containing 0.05 mg/mL peptide, 2 mM AsA, and 0.02 mM $CuCl_2$ in 10 mM acetate buffer at pH 5.0.

Effects of Catalase (CAT), Superoxide Dismutase (SOD), and EDTA on the AsA/$CuCl_2$-Induced Oxidation of Rlx. The effect of CAT or SOD on the oxidation was examined by the addition of 2000 units/mL CAT or 2000 units/mL SOD to the reaction mixture containing 0.1 mg/mL Rlx and 2 mM AsA/0.02 mM $CuCl_2$ in phosphate buffer (10 mM) at pH 5. EDTA (0.1 mM; 5:1 EDTA:$CuCl_2$ ratio) was added to an identical reaction mixture to determine the effect of a chelating agent on the oxidation reaction. All reactions were monitored by RP-HPLC for 400 min at 25 °C.

Protein Assay. The soluble protein concentration following the oxidation was determined by the Bradford method (Bradford, 1976). It involved the addition of an acidic dye to the protein solution, and subsequent measurement at 595 nm with a Shimadzu UV-160 spectrophotometer. The comparison of the soluble protein to a standard curve (BSA) provides a relative measurement of the soluble protein concentration. Native Rlx and Rlx after oxidation by AsA/$CuCl_2$ at various pH values from pH 5 to 8 were centrifuged on a Fisher Micro-Centrifuge (Model 235 B) to remove the precipitate before the protein assay was conducted.

HPLC Analysis. RP (reversed phase)-HPLC was employed to monitor the oxidative degradation of Rlx. The chromatography was performed on a system consisting of a Shimadzu SCL-10A system controller, a Shimadzu LC-10AS pump, a SPD-10A UV spectrophotometric detector, a SIL-10A autoinjector, a sample cooler, and a C-R4A chromatopac

integrator. The system utilized a Vydac RP-C4 column (4.6 × 300 mm) which was equilibrated at 40 °C with a column heater (Jones Chromatography). Mobile phase A consisted of H_2O (0.1% TFA); mobile phase B was 9:1 (v/v) ACN/H_2O (0.08% TFA). A linear gradient started with 19% B and increased to 46% B over 30 min. The flow rate was 1 mL/min, and detection was achieved at 214 nm. The remaining protein and degradation products were quantified by measuring the respective peak areas (injection volumn: 10 μL). A Phenomenex Biosep-SEC-2000 column (7.8 × 300 mm) and a Biosep-SEC guard column (7.8 × 75 mm) were used for the SEC (size-exclusion chromatography)-HPLC analysis. The injection volume was 20 μL with a flow rate of 1 mL/min and detection at 214 nm. In order to analyze the Rlx aggregates formed in the reaction of AsA/$CuCl_2$ and to improve the resolution of the low molecular weight proteins (Montelaro, 1988), an aqueous mobile phase containing 0.5% SDS (pH 7.0) was employed without additional buffers.

For the analysis of the two model peptides RlxP1 and RlxP2, RP-HPLC was employed using a Nucleosil C18 (4.6 × 150 mm) column (Alltech) equilibrated at room temperature. The flow rate was 0.8 mL/min, and detection was at 214 nm. Mobile phases A and B were the same as those used for the RP-HPLC analysis of Rlx. The linear gradient was from 20 to 35% B over 20 min for RlxP1 and from 8 to 16% B over 20 min for RlxP2. The peptides and their Met-sulfoxide derivatives were well separated under such conditions. Both the remaining peptides and their corresponding Met-sulfoxides were quantified by comparing the peak areas to those of authentic standards.

Amino Acid Composition Analysis. The analysis of the amino acid composition of native Rlx and its oxidation products, i.e., a soluble protein at pH 5 and a precipitate at pH 8, was performed using a Beckman 6300 amino acid analyzer. The amino acids from the acid-hydrolyzed protein were derivatized with ninhydrin and detected at 400 and 570 nm. Quantitation was achieved by comparison of peak areas with external standard components. All data were the averages of triplet determinations.

CD Spectra Measurements. CD spectra of the native Rlx (0.2 mg/mL) and the oxidized Rlx (0.2 mg/mL) at pH 2 and 5 were measured with an AVIV circular dichroism spectropolarimeter (Model 60 DS). Cells of 2.000 and 0.010 cm were selected for the measurements of near-UV CD (250−400 nm) and far-UV CD (190−250 nm) spectra, respectively. Mean residue ellipticities ($\Delta\epsilon$) were calculated on the basis of average residue weights (MRW = 113 for Rlx). Estimates of secondary structure were obtained from data collected in the far-UV range by use of SELCON (self-consistent method) for data fitting (Sreerama & Woody, 1993).

DLS (Dynamic Light Scattering). DLS was employed to determine the size of the aggregates following oxidation of Rlx. The native Rlx solution (0.2 mg/mL) and the oxidized Rlx (0.2 mg/mL) at pH 5 (after 4 h reaction) were filtered through 0.45 μm Millipore filters. Each sample was placed in a 10 × 75 mm glass test tube that had been previously washed with deionized water from the Milli-Q filtration system. The solutions were then examined on the DLS instrument utilizing a 300 mW argon ion laser (Lexel, Inc., Model 95) operating at 488.0 nm attached to a goniometer sample holder and photomultiplier detector (Brookhaven Instruments Corp., Upton, NY, Models BI-200SM & BI-DS) positioned at 90° to the incident ion laser beam. The detector slit width was set at 200 μm and was placed at a right angle collection optical geometry. The integration time was 4 min with a sampling time of 0.5 μs to 1×10^5 μs with exponential spacing. Analysis was performed by a 386SX computer containing a BI-9000 Digital Correlator (Brookhaven) and running signal processing software (Brookhaven, BI-9000AT & BI-2030AT). Data were fit using a non-negative constrained regularized least squares from a calculated base line.

Tryptic Digestion. Rlx (0.4 mL) at a concentration of 0.5 mg/mL was digested with TPCK-treated trypsin in the presence of 0.2 mM calcium chloride at 30 °C for 4 h in 10 mM Tris·acetate buffer, pH 7.0. Two aliquots (10 μL each) of trypsin were added with an enzyme (trypsin)/substrate (Rlx) ratio of 1:100 (w/w) at the beginning and after 2 h of digestion, respectively. Due to the lower solubility of the Rlx oxidation product at pH 7.0, it was digested with an extra addition of trypsin aliquot over an extended digestion time (another 2 h). The precipitate of the oxidized Rlx at pH 8 was isolated and transferred into 10 mM Tris·acetate solution. The digestion followed the same procedure as that described earlier for the sample oxidized at pH 5. The tryptic peptides derived from the digestion of the precipitate eventually dissolved in the solution. The reactions were terminated by lowering the pH below 4 with 0.1 M HCl, and the solutions were kept frozen in the refrigerator for the following analysis.

RP-HPLC Analysis of the Tryptic Digest. An HP-1090L HPLC system was employed for the chromatography of the tryptic peptides. The tryptic fragments of Rlx and its oxidation products were analyzed on a Nucleosil C18 (4.6 × 150 mm) column (Alltech) equilibrated at room temperature. The initial equilibration buffer was 0.1% TFA in 3% ACN. A linear elution gradient was generated from 3 to 30% ACN over 54 min. The flow rate was held constant at 1 mL/min, and the absorbance was monitored at 214 and 280 nm.

LC/MS Spectra. The molecular weights of individual tryptic peptides were analyzed by LC/MS spectra. A Sciex API III triple-quadrupole instrument fitted with an ion spray (nebulized-assisted electrospray) was employed to collect mass spectral data. Molecular masses were calculated and analyzed by Hypermass software.

Amino-Terminal Sequencing of Tryptic Peptides. Tryptic peptides from HPLC were pooled and condensed under vacuum in a Savant Speed-Vac. The fractions were subjected to Edman degradation using an Applied Biosystems 447A protein sequencer with on-line phenylthiohydantoin detection, using an Applied Biosystems 120A analyzer.

Analysis of the Three-Dimensional Structure of Rlx. The locations of oxidation-labile residues in the three-dimensional structures of Rlx were evaluated on the basis of the X-ray crystal structure of Rlx obtained from the Protein Data Bank (Chemistry Department, Brookhaven National Laboratory, Upton, NY), with the assigned code 6RLX (Eigenbrot et al., 1991). Molecular modeling experiments were accomplished by using an IRIS Silicon Graphics (4D/25G) equipped with the Insight/Discover program from Biosym Technologies.

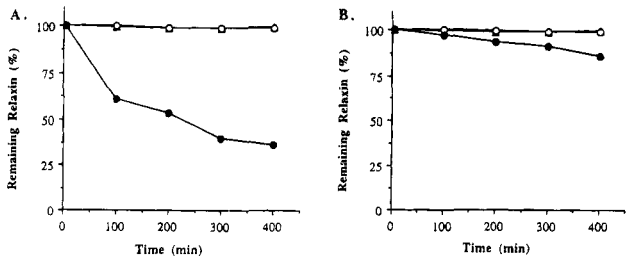

FIGURE 2: Oxidative modification profiles of relaxin (Rlx) in AsA/$CuCl_2$ and AsA/$FeCl_3$ solutions at pH 5. Reaction mixtures contained 0.1 mg/mL Rlx, 2 mM AsA, 0.02 mM $CuCl_2$ or $FeCl_3$, and 10 mM phosphate buffer. (A) (○) Rlx in the presence of AsA only; (▲) Rlx in the presence of $CuCl_2$ only; (●) Rlx in the presence of AsA/$CuCl_2$. (B) (○) Rlx in the presence of AsA only; (▲) Rlx in the presence of $FeCl_3$ only; (●) Rlx in the presence of AsA/$FeCl_3$.

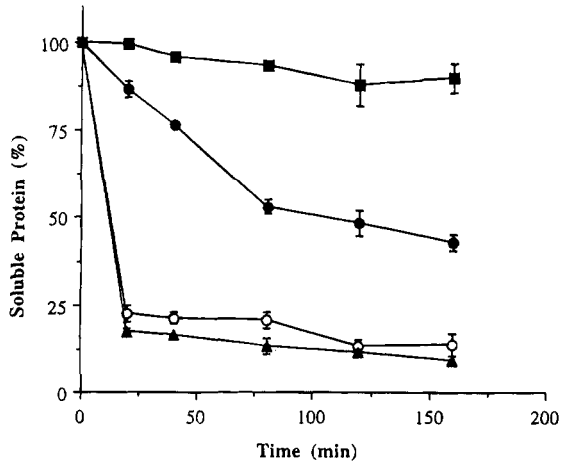

FIGURE 3: Effect of pH on the precipitation of protein following the oxidation of relaxin (Rlx). The reaction mixtures contained 0.2 mg/mL Rlx, 4 mM AsA, 0.2 mM $CuCl_2$, and 20 mM phosphate buffer at pH 5−8. (■) pH 5; (●) pH 6; (○) pH 7; (▲) pH 8. Soluble proteins were determined by Bradford assay. All data points are the average of two determinations.

RESULTS

Oxidation of Rlx by AsA/Transition Metal Systems. As shown in Figure 2, the modification of Rlx was rapid only in the presence of the combination of AsA and $CuCl_2$ or AsA and $FeCl_3$ solutions, whereas no modification was observed in solutions containing AsA, $CuCl_2$, or $FeCl_3$ alone over the same time period. It was observed that the oxidation was significantly faster in the AsA/$CuCl_2$ system than in the AsA/$FeCl_3$ system. The subsequent analysis and characterization of the degradation products focused mainly on AsA/$CuCl_2$-induced oxidation.

Effects of pH and Buffers on the Oxidative Modification of Rlx by the AsA/$CuCl_2$ System. Exposure of Rlx (0.2 mg/mL) to AsA (4 mM)/$CuCl_2$ (0.2 mM) in phosphate buffer (20 mM) resulted in a pH-dependent formation of precipitate (Figure 3). At higher pH values, Rlx solution became turbid within a minute after the addition of AsA and $CuCl_2$. The reaction product slowly precipitated after an induction period of approximately 10 h. The amount of residual soluble protein determined by the Bradford method indicated a significant loss through precipitation (>75−80% within 25 min) at pH 7 and 8. In contrast, less precipitation was observed at pH 5 and 6. When the oxidation reactions were carried out in acetate buffer in the same pH range, similar precipitation results were obtained (data not shown). Thus,

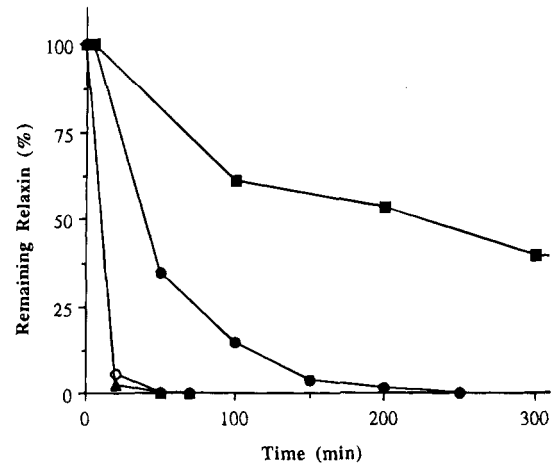

FIGURE 4: Effect of pH on the oxidation of relaxin (Rlx). The reaction mixtures contained 0.1 mg/mL Rlx, 2 mM AsA, 0.02 mM $CuCl_2$, and 10 mM phosphate buffer at pH 5−8. (■) pH 5; (●) pH 6; (○) pH 7; (▲) pH 8. The reactions were monitored by RP-HPLC. All data points are the average of two determinations, and the error bars are hidden inside of the symbols.

the AsA/$CuCl_2$-induced oxidation of Rlx was independent on these two buffer species.

Characterization of Rlx Precipitate Resulting from the Oxidation of Rlx at pH 7 and 8. As the precipitate accounted for the major degradant of Rlx at higher pH values, the nature (covalent or noncovalent) and the size of the precipitate were then characterized. SEC-HPLC was used for the analysis of the molecular weight of the precipitate, using an aqueous mobile phase consisting of 0.5% SDS. The precipitate resulting from the AsA/$CuCl_2$-induced oxidation of Rlx could be solubilized in 0.5% SDS, and SEC-HPLC analysis revealed a single peak with a retention time identical to that of the standard Rlx (data not shown). These results suggested that the precipitate was noncovalent in nature and that the size of the protein (after dissociation by SDS) remained unaltered.

Analysis and Identification of the Oxidation Products of Rlx at pH 5. Modification of Rlx by AsA/$CuCl_2$-containing solutions at pH 5 resulted in a rapid loss of Rlx as determined by RP-HPLC (Figure 4), although with significantly less formation of precipitate (Figure 3). However, except for minor amounts of Met-sulfoxide formation, no other degradation products were detected by RP-HPLC (Figure 5A). In contrast to the rapid disappearance of Rlx as determined by RP-HPLC analysis, Rlx eluted at approximately the same retention time, and the absorbance of the peak did not decrease during the reaction as determined by SEC-HPLC in the presence of 0.5% SDS in the mobile phase (Figure 5B). This suggests that the size of the Rlx molecule upon dissociation by SDS did not change. In an effort to identify possible hydrophobic degradation products or soluble aggregates, the RP-C4 column temperature was raised from 40 to 60 °C, paralleled by an increase of the ACN concentration in the gradient. No additional degradation products could be observed from the chromatograms under such analytical conditions. It is apparent from this result and the following DLS analysis that the formation of noncovalent aggregate accounted for the disappearance of the Rlx peak on RP-HPLC at pH 5.

Determination of the Size of the Aggregate by DLS. SDS−PAGE is a common technique for determining the size of

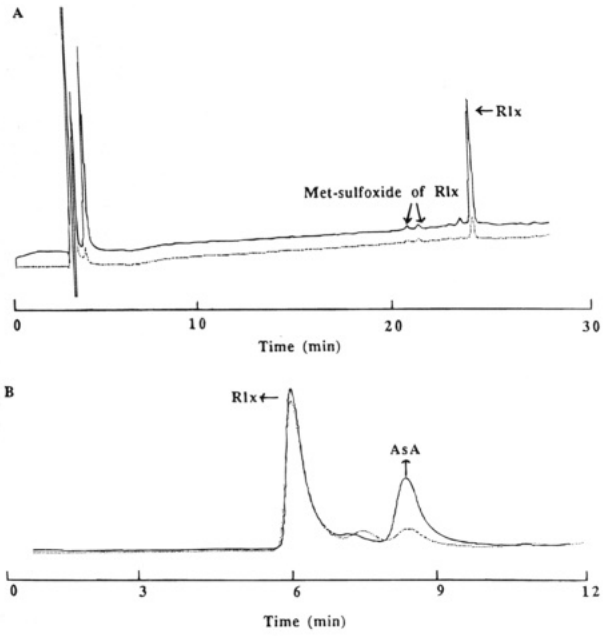

FIGURE 5: HPLC chromatograms of the oxidation of relaxin in AsA/$CuCl_2$ solutions. The oxidation conditions were the same as those described in Figure 4. (A) RP-HPLC chromatograms. Solid line, 1 h reaction; dotted line, 7 h reaction. (B) SEC-HPLC chromatograms. Solid line, 1 h reaction; dotted line, 24 h reaction.

the protein. This method, however, can give only the apparent size of protein monomer or covalently linked monomers, which may not represent the size of the active protein. Furthermore, because of the low molecular weight of Rlx (MW 5963), SDS−PAGE was not effective in determining the molecular weight of either native Rlx or its aggregates (data not shown). Therefore, DLS was applied to characterize the formation of soluble aggregate obtained from the oxidation of Rlx at pH 5. DLS, using powerful laser light sources, can record changes of a few nanometers in protein molecular size. It has been applied in the determination of protein size (2−1000 nm) upon denaturation (Nicoli & Benedek, 1976) or aggregation in solutions (Cohen et al., 1975). As the diameter of the native Rlx monomer is less than 2 nm, the size could not be determined accurately by DLS. Nevertheless, due to the relative similarities between the structures of insulin and Rlx, we assume that the Rlx monomer has an approximate radius of ~12.7 Å, similar to the insulin monomer (Bohidar & Geissler, 1984). The average diameter of the Rlx aggregate induced by oxidation at pH 5 was 181 nm as determined by DLS (Figure 6A). The particle size decreased to 146 nm diameter upon the addition of 0.1% SDS (Figure 6B), and became too small to measure (<2 nm) upon further addition of 0.5% SDS (data not shown). The size of the aggregate also decreased as the pH of the solution was adjusted to 2 (75 nm) (Figure 6C). In addition to the quantitative determination of the size of soluble aggregate, this result also confirmed the noncovalent nature of the aggregate analyzed by SEC-HPLC.

Determination of the Secondary and Tertiary Structure following Oxidation of Rlx. Efforts to obtain a CD spectrum of oxidized Rlx at pH 5 were hindered by light scattering from the aggregates. Thus, the pH values of both native Rlx and oxidized Rlx (pH 5) solutions were lowered to 2 for the measurements. At lower pH, less scattering was observed in the CD spectrum, which was consistent with the decreased size of the aggregate at lower pH determined by DLS. The comparisons of the far-UV CD (190−250 nm) and near-UV CD (250−400 nm) spectra of the native Rlx and its oxidized form were recorded and are shown in Figure 7. Modifications of the ordered secondary structures can be evaluated from far-UV CD (190−250 nm) (Figure 7A). It is evident from the analysis that the fractions of α-helix and β-sheet of native Rlx are not modified significantly upon pH change (Table 1), and the secondary structure of Rlx at pH 2 can be used as a reasonable reference for the conformational alterations upon the oxidation of Rlx. It appeared that metal-catalyzed oxidation of Rlx induced a partial conversion of α-helix to β-sheet (Table 1). The occurrence of secondary structure change can also be identified by the difference in the intensity of these two CD spectra. CD spectra at the near-UV CD range (250−400 nm) tend to be more sensitive to the tertiary structure. The near-UV CD spectra of proteins are generally associated with the aromatic side chains, e.g., Trp (288−292 nm) and Tyr (277 and 283 nm), as well as the disulfide bond. As shown in Figure 7B, the spectrum of oxidized Rlx differs significantly from native Rlx in the region of 250−320 nm. The Trp band of native Rlx at pH 2 appears as a broad shoulder centered around 295 nm, similar to that observed at pH 5 (Shire et al., 1991). This signal decreased considerably in the oxidized Rlx. Furthermore, the negative dichroic band of the Tyr residue at ~277 nm also shows a clear decrease in the intensity of the CD signal. The observed difference in the near-UV region indicates partial denaturation of the

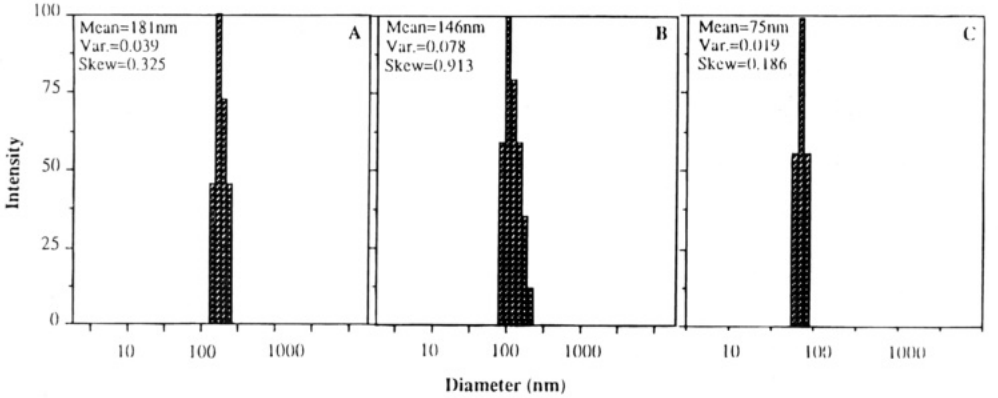

FIGURE 6: Measurements of the size of the relaxin aggregate following oxidation in AsA/$CuCl_2$ solutions at pH 5. The oxidation conditions were the same as those described in Figure 3, except using acetate instead of phosphate buffer. The analysis was achieved by dynamic light scattering (DLS). (A) Aggregate of oxidized relaxin (pH 5). (B) Aggregate upon addition of 0.1% SDS. (C) Aggregate upon addition of HCl to pH 2.

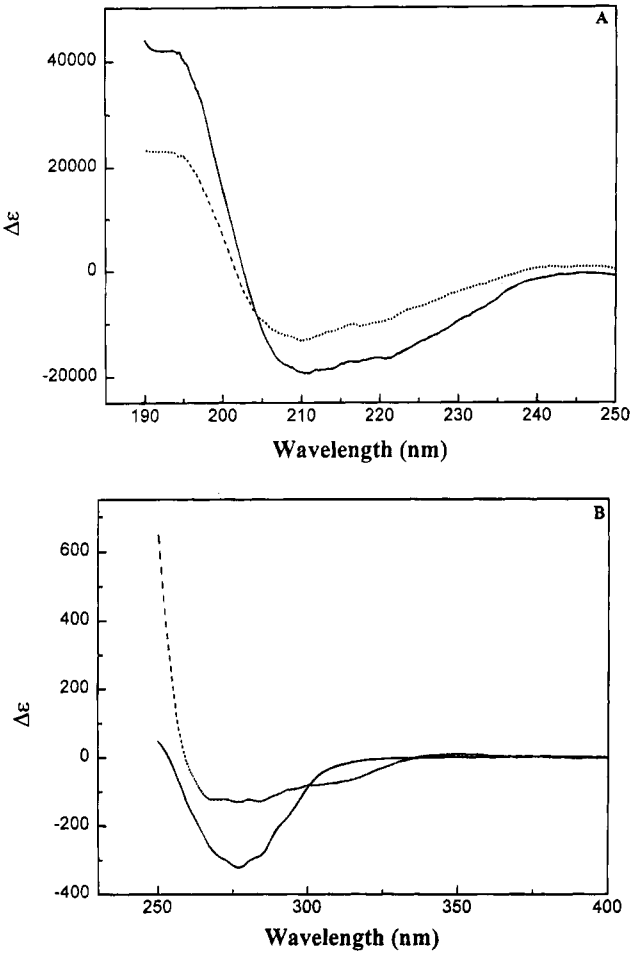

FIGURE 7: CD spectra of native relaxin and oxidized relaxin, pH 2. (A) Far-UV CD spectra (190−250 nm). (B) Near-UV CD spectra (250−400 nm). Solid line, native relaxin; dotted line, oxidized relaxin. Buffer blanks were subtracted from the protein spectra. Mean residue ellipticities were converted by using 113 as the mean residue weight for relaxin. The secondary structure estimated by SELCON is presented in Table 1.

Table 1: Secondary Structure Estimations for Human Relaxin (Rlx) and Its Oxidized Form from Data Collected in Far-UV CD

	native Rlx[a]	oxidized Rlx
	pH 2	
α-helix (%)	59	36
β-sheet (%)	25	40
β-turn (%)	6	7
others (%)	9	18
	pH 5	
α-helix (%)	51	
β-sheet (%)	26	
β-turn (%)	9	
others (%)	15	

[a] All the data are calculated by the method of SELCON.

protein upon oxidation. Consequently, the loss of tertiary structure of the oxidized Rlx could be the major cause for the subsequent formation of aggregate.

Effect of pH on the Formation of Precipitate. No precipitation was observed upon exposure of Rlx to Cu(II) solution alone at pH 5−8. Therefore, the identified precipitate must have originated from Rlx oxidation. In order to examine whether the precipitate of the oxidized Rlx induced by AsA/$CuCl_2$ at higher pH is formed in a pH-dependent process, and reversible with the soluble degradant at pH 5,

Table 2: Amino Acid Composition Analysis of Human Relaxin and Its Oxidized Forms[a]

	composition (residues/mol)[b]			
amino acid	theoretical values	native	oxidized (pH 5)	oxidized (pH 8)
Asx	2	2.0	1.8	1.4
Glx	5	4.9	5.1	5.1
Ser	5	4.4	4.6	4.5
Gly	3	3.0	3.2	3.3
His	1	1.0	0.5	0.6
Arg	4	3.9	3.7	4.1
Thr	2	1.9	2.0	2.0
Ala	5	5.0	5.2	5.2
Tyr	1	1.0	1.0	1.0
Val	3	2.8	2.6	2.7
Met	2	2.0	1.7	1.7
Cys	6	4.1	4.9	5.6
Ileu	3	2.5	2.7	2.7
Leu	5	5.0	5.0	5.0
Phe	1	1.0	1.0	1.1
Lys	3	3.3	2.8	2.8

[a] The oxidation reaction of Rlx at pH 5 contained a reaction mixture of 0.2 mg/mL Rlx, 4 mM AsA/0.2 mM $CuCl_2$, and 20 mM phosphate buffer. The Rlx precipitate from the oxidation at pH 8 was under the same conditions and was isolated by centrifugation. [b] All the data are the average of triplet determinations.

we changed the pH of reaction mixtures after 3 h reaction time. The pH of the reaction was adjusted from 8 to 5 by the addition of HCl, and the pH 5 mixture was adjusted to 8 by the addition of NaOH. The solution became slightly cloudy instantaneously as the pH was changed from 5 to 8, and significant precipitation could be seen after 24 h. Analysis of the soluble protein by the protein assay indicated that the precipitate accounted for approximately 88% of the total protein. In contrast, as the pH was adjusted from 8 to 5, the precipitate did not redissolve in the solution (~1% as determined by the protein assay).

Analysis of Amino Acid Composition of the Oxidized Rlx at pH 5 and 8. Amino acid analysis revealed a significant loss of His: 50% at pH 5 and 40% at pH 8 upon the oxidation of Rlx by the AsA/$CuCl_2$ system (Table 2). A small amount of reduction in Met content was also observed. However, since Met-sulfoxide is readily reduced under conditions used for acid hydrolysis (Brot & Weissbach, 1983), the accurate determination of Met content required further experiments. LC/MS data of tryptic peptide maps shown later clearly indicated the formation of Met-sulfoxides from both Met residues in Rlx. In addition, we observed a 15% decrease in Lys content and a minor loss of Arg only at pH 5. The content of Trp was not determined, since it was degraded during acid hydrolysis.

Analysis and Identification of Tryptic Peptides of the Oxidized Rlx Derived from Different pH Values. The tryptic maps of the degradation products of Rlx induced by AsA/$CuCl_2$ at pH 5 and 8 were similar to each other but different from the tryptic map of native Rlx (Figure 8). Theoretical tryptic fragments of Rlx are listed in Table 3. The identification of each tryptic peptide was achieved by LC/MS and amino acid sequencing. The assignments of the individual tryptic peptides and plausible oxidation products were based on the theoretical mass corresponding to these fragments and sequencing data. Every tryptic fragment of the native Rlx agreed with the expected mass (Figure 8A) and was confirmed by peptide sequencing. For oxidized Rlx,

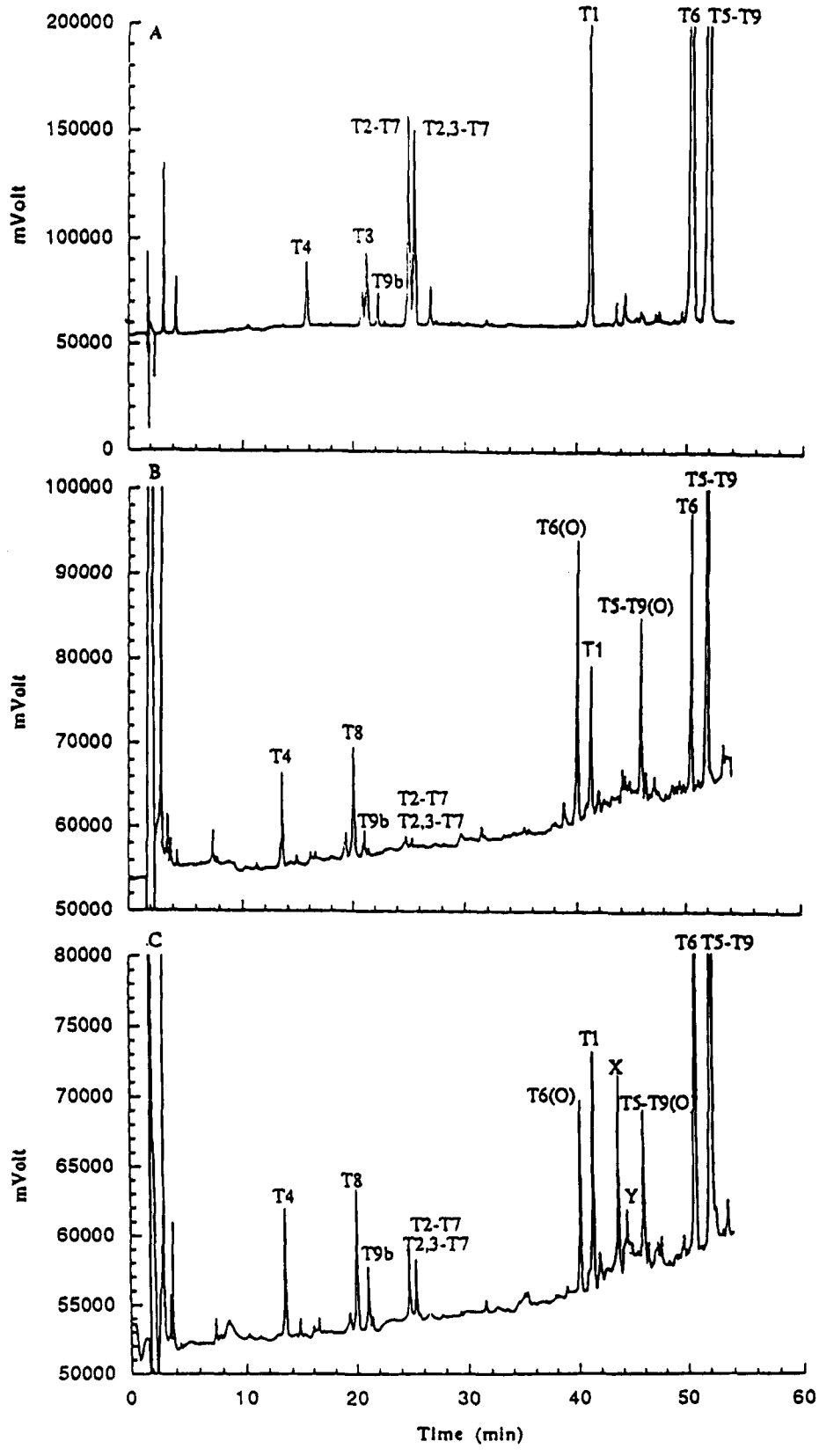

FIGURE 8: Tryptic maps of relaxin samples monitored by RP-HPLC at 214 nm. (A) Native relaxin. (B) Oxidized relaxin induced by $AsA/CuCl_2$ in acetate buffer solution at pH 5. (C) Oxidized relaxin induced by $AsA/CuCl_2$ in Tris·acetate buffer solution at pH 8.

significant modifications were observed for the fragments T_1 and T_2-T_7 (containing His). At pH 5, the T_2-T_7 peptide almost totally disappeared, with the resulting mixture possibly eluting as a number of small peaks at retention times between 40 and 60 min (Figure 8B). The latter could not be characterized by LC/MS. Some remaining T_2-T_7 could still be observed in the tryptic map of Rlx oxidized at pH 8 (Figure 8C). Two major peaks were detected that were related to the incomplete digestion of the precipitate formed at pH 8. Peak x with a mass of 2423.4 daltons was assigned as $T_1-T_2-T_7$ (Table 4). This contributed to the apparent loss of fragment T_1 for the oxidized Rlx which was observed

Table 3: Theoretical Tryptic Fragments of Human Relaxin

Fragment	Sequence
T_1 ([a]A1-9)	[b]pGlu-Leu-Tyr-Ser-Ala-Leu-Ala-Asn-Lys
T_2-T_7 (A10-17, B10-13)	Cys-Cys-His-Val-Gly-Cys-Thr-Lys Leu-Cys-Gly-Arg
T_3 (A18)	Arg
T_4 (A19-22)	Ser-Leu-Ala-Arg
T_5-T_9 (A23-24, B18-29)	Phe-Cys Ala-Gln-Ile-Ala-Ile-Cys-Gly-Met-Ser-Thr-Trp-Ser
T_6 (B1-9)	Asp-Ser-Trp-Met-Glu-Glu-Val-Ile-Lys
T_8 (B14-17)	Glu-Leu-Val-Arg

[a] Protein chain and residue number. [b] pGlu: pyroglutamic acid.

Table 4: Analysis of LC/MS Spectra of Tryptic Peptides from Relaxin and Its Oxidized Forms[a]

assignment	theoretical masses	observed native	oxidized (pH 5)	oxidized (pH 8)
T_2–T_7	1293.57	1293.6	1293.6	1293.6
x[b]				2423.4
y[c]				2439.4
$T_{2,3}$–T_7	1449.67	1449.8	1449.8	1449.8
T_6	1136.53	1136.6	1136.6	1136.6
T_6(O)[d]	1152.53		1152.6	1152.6
T_6–T_9	1533.68	1533.7	1533.7	1533.7
T_5–T_9(O)[e]	1549.68		1550.4	1550.4

[a] The oxidation conditions of Rlx were the same as described in Table 2. Only the modified tryptic peptides are presented in this table. [b] T_1–T_2–T_7. [c] T_1–T_2–T_7 His(O). [d] T_6(O) = T_6 Met-sulfoxide. [e] T_5–T_9(O) = T_5–T_9 Met-sulfoxide.

in the tryptic maps (Figure 8). The mass of peak y (2439.4 daltons) was 16 units higher than that of peak x, corresponding to the addition of one oxygen to this fragment. An examination of the primary sequence of the fragment suggested that it could be 2-oxohistidine, which was reported to be one of the oxidation products of His (Uchida & Kawakishi, 1986, 1994). T_6 and T_5–T_9 (both containing Met) were partially converted to fragments with a 16 mass unit increase in molecular weight, consistent with the oxidation of Met to Met-sulfoxides (Table 4). Peptide sequencing also confirmed the primary structure of these two Met-sulfoxide-containing fragments, which corresponded to T_6 (Asp-Ser-Trp-Met-Glu-Glu-Val-Ile-Lys) and T_5–T_9 [Ala-Gln-Ile-Ala-Ile-Cys(-Cys-Phe)-Gly-Met-Ser-Thr-Trp-Ser]. Most interestingly, it appeared that Met-sulfoxide formation was more pronounced for T_6 than for T_5–T_9 (Figure 8B,C), particularly at pH 5, as the amount of conversion from T_6 to T_6(O) was much more than that of T_5–T_9.

Effects of CAT, SOD, and EDTA on the Oxidation of Rlx. Neither CAT nor SOD showed any effect on the oxidation of Rlx induced by AsA/$CuCl_2$, suggesting that free diffusible H_2O_2 and $O_2^{\bullet-}$ were not responsible for the oxidation (Table 5). Sufficient activity of CAT remained during the reaction time (data not shown). In contrast, the addition of the chelating agent EDTA almost completely inhibited the Rlx oxidation induced by AsA/$CuCl_2$.

Comparison of the Oxidation Kinetics of RlxP1 and RlxP2 in the AsA/$CuCl_2$ System. Both RlxP1 and RlxP2 underwent rapid modification by the AsA/$CuCl_2$ system. As shown in Figure 9, the degradation of RlxP1 and the formation of its corresponding Met-sulfoxide were much faster and occurred to a greater extent than those of RlxP2. It was also observed that Met-sulfoxide was not the only degradation product induced by AsA/$CuCl_2$ for these two model peptides. The yield of Met-sulfoxide was 50% for RlxP1 but only 16% for RlxP2.

Table 5: Effects of Catalase (CAT), Superoxide Dismutase (SOD), and EDTA on the Oxidation of Relaxin (Rlx) Induced by AsA/$CuCl_2$[a]

addition	remaining Rlx (%)
control	36 ± 1
+CAT (2000 units/mL)	40 ± 1
+SOD (2000 units/mL)	35 ± 3
+EDTA (0.1 mM)	98 ± 1

[a] The reaction mixtures contained 0.1 mg/mL Rlx, 2 mM AsA, and 0.02 mM $CuCl_2$ in phosphate buffer (10 mM) at pH 5, with the addition of CAT, SOD, and EDTA, respectively. All the reactions were monitored for 400 min by RP-HPLC.

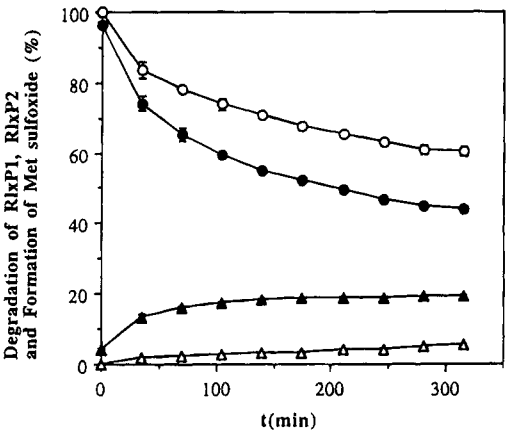

FIGURE 9: Oxidative modification of two model peptides, AcSer-Trp-Met-Glu-GluNH$_2$ (RlxP1) and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ (RlxP2), by the AsA/$CuCl_2$ system. The reaction mixtures contained 0.05 mg/mL Rlx peptides, 2 mM AsA, 0.02 mM $CuCl_2$, and 10 mM acetate buffer at pH 5. (●) RlxP1; (○) RlxP2; (▲) RlxP1 Met-sulfoxide; (△) RlxP2 Met-sulfoxide. The reactions were monitored by RP-HPLC. All data points are the average of two independent determinations.

DISCUSSION

The oxidation of peptides and proteins can be induced by prooxidants (e.g., AsA) in the presence of trace amounts of transition metal ions. The combination of AsA and transition metal ions [Cu(II) or Fe(III)] generates various active oxygen species and has been used as a model oxidizing system for the mechanistic study of peptide (Li et al., 1993) and protein oxidation (Levine, 1983). It is shown in this study that Rlx undergoes rapid oxidation and subsequent aggregation in the AsA/$CuCl_2$/O_2 system. The lack of effects of CAT and SOD on the oxidation of Rlx is in accord with a site-specific mechanism (Gutteridge & Wilkins, 1983; Stadtman, 1990), suggesting that the active oxygen species are formed on or near the metal binding sites of the protein and react predominantly with amino acid residues of close proximity. It has been known that Cu(II) tends to form high-affinity complexes with peptides and proteins (Peisach et al., 1966; Pickart et al., 1980). Therefore, the chelating agent EDTA inhibited the oxidation of Rlx by removing Cu(II) from the protein, forming redox-inactive complexes instead.

The potential modification sites of relaxin through the metal-catalyzed mechanism were investigated. We demonstrated that His was the residue in Rlx most susceptible to metal-catalyzed oxidation. This observation is in agreement with previous reports, indicating that His is one of the preferred targets in proteins under conditions of site-specific metal-catalyzed oxidation (Stadtman, 1993). One of the oxidation products of His was identified as 2-oxohistidine (Uchida & Kawakishi, 1986, 1994). No asparagine/aspartate (Farber & Levine, 1986) could be found as the product of His from our experimental data. The identification of the oxidation products of His was further complicated by the close proximity of Lys to His in the same tryptic fragment, which might also be oxidized under such conditions (Stadtman, 1993). However, on the basis of the amino acid analysis (Table 2), the modification of Lys was less pronounced compared to that of His. Despite the fact that Met has not been reported to be a predominant target in protein oxidation by metal-catalyzed mechanisms, we identified a significant amount of oxidation of both Met residues to Met-sulfoxides in the Rlx molecule. Furthermore, we observed that the oxidation of Met B(4) occurred to a greater extent than that of Met B(25). As reported in previous studies, the simple oxidation of Met to Met-sulfoxide by both light and H_2O_2 cannot induce the conformational changes or cause the loss of biological activity (Cipolla & Shire, 1991; Nguyen et al., 1993). Thus, it seemed that the oxidative modification of His in Rlx was crucial for provoking the changes in secondary and tertiary structure and the formation of aggregates. There is no evidence that the aromatic amino acid residues in Rlx (e.g., Trp or Tyr) are oxidized under such conditions, which is in line with earlier suggestions that aromatic residues are less easily oxidized under metal-catalyzed oxidation conditions (Stadtman, 1990). Hydrolytic cleavage of N-terminal Asp B(1) is known to be another covalent degradation pathway of Rlx (Nguyen et al., 1993), which is favored at lower pH (Nguyen et al., unpublished results). Under the metal-catalyzed oxidation conditions, we also observed a significant loss of Asx as shown in amino acid analysis (Table 2). It cannot be concluded, however, if the reduction of the Asx content at higher pH was the consequence of some structural modification following the oxidation, which rendered the protein more susceptible to hydrolysis.

Earlier studies indicated that the Rlx molecule underwent degradation upon exposure to intense light. The major oxidation products were Met-sulfoxides, and it appeared that Met B(25) was more easily oxidized than Met B(4) (Cipolla & Shire, 1991). Nevertheless, a number of other degradation products were also observed, but were not yet identified. They could be related to the oxidation of His, Trp, or Tyr, as they are known to be very reactive toward photosensitized oxidation (Matheson & Lee, 1979). The oxidation of Met B(4) and Met B(25) was also prominent when the Rlx molecule was exposed to H_2O_2 (Nguyen et al., 1993). Again, it was reported that Met B(25) was oxidized at a significantly faster rate than was Met B(4), consistent with the results of the light-induced oxidation of Rlx. In contrast to both photooxidation and H_2O_2-induced oxidation of Rlx, the metal-catalyzed oxidation (induced by AsA/$CuCl_2$) resulted in a preferred modification at Met B(4) rather than at Met B(25). This difference may be interpreted by the consideration that metal-catalyzed oxidation is a site-specific mechanism. As we compare the spatial locations of Met B(4) and Met B(25) in the three-dimensional crystal structure of Rlx (Figure 10), it is evident that Met B(4) is surrounded by more residues with ionic side chains, i.e., Asp and Glu. Such amino acids are potential metal-binding sites that may facilitate copper ion binding at these locations together with the binding of protein amide backbone, resulting in an enhanced oxidation of Met B(4). We note that the actual redox-state of the catalytic form of copper ion cannot be specified. Fenton-like chemistry would suggest that Cu(I) may interact with O_2 or H_2O_2 to form superoxide and hydroxyl radicals (or their metal-bound analogs), respectively. In the reduced form, Cu(I) is expected to have poor affinity to Asp or Glu residues (Peisach et al., 1966). However, the kinetics of Cu(I) formation and the subsequent oxidation inside the framework of protein ligands as well as the ligand dynamics of the release of Cu(I) from its previous binding sites have to be known in order to argue against the site-specificity of the mechanism. If the release of Cu(I) were slower than the oxidation of Cu(I) to Cu(II), the reaction mechanism would still be site-specific on an average time scale. A recent study on the kinetics of ligand reorientation upon the oxidation of tetrahedral $Cu^{I}(TTCN)_2$ (TTCN = 1,4,7-trithiacyclononane) to octahedral $Cu^{II}(TTCN)_2$ (Hungerbühler et al., unpublished results) revealed that an initial oxidation of the central metal ion was followed by a relatively slow reorientation on the millisecond time scale. In addition to Asp and Glu, the structure of Rlx reveals a close proximity of Met B(4) to Lys A(17), which is another potential metal-binding site. The local structure around Met B(25) indicates that there are no adjacent amino acids with charged side chains. Thus, Met B(25) is less readily attacked by oxygen radicals in a site-specific reaction. This hypoth-

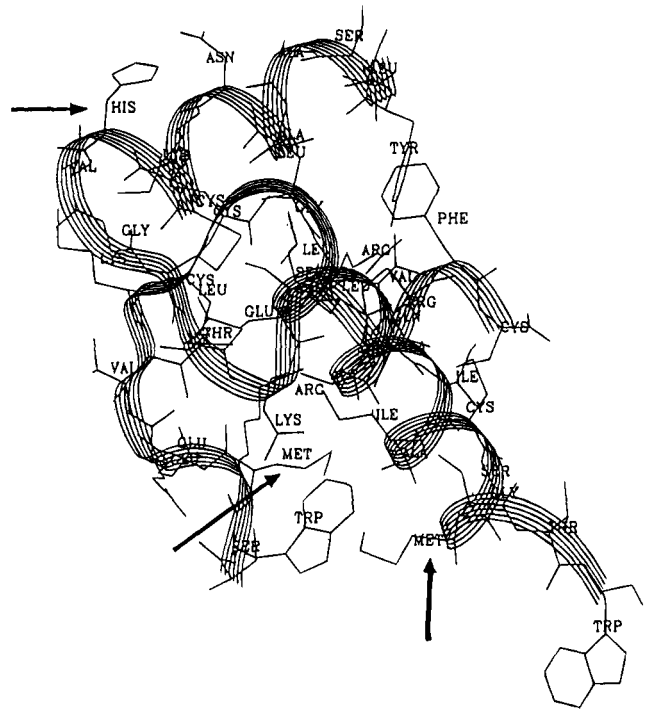

FIGURE 10: Location of the oxidation sites in the three-dimensional structure of human relaxin. The monomer structure of relaxin is modified from the X-ray crystal structure of relaxin at 1.5 Å resolution obtained from the Protein Data Bank with the assigned code 6RLX [references to Eigenbrot et al. (1991)]. It contains three major helices as shown by ribbon bands. The positions of His and Met residues are directed by arrows.

esis is strongly supported by our results on the metal-catalyzed oxidation of the two Met-containing fragments which mimic the residues around Met B(4) (AcSer-Trp-Met-Glu-GluNH$_2$) and Met B(25) (AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$). The preferred oxidation of Met B(4) to its Met-sulfoxide could be contributed by some binding of Cu(II) to the ionic side chains of the neighboring residues.

It is important to recognize that the labile amino acid residues in a protein may have different susceptibility under different oxidation conditions. Oxidation induced either by light (possible generation of $^1O_2$) or by H_2O_2 has been shown to be dependent on the accessibility of the labile amino acid residues to such exogenous oxidizing agents. In general, the residues which are located in the buried positions within a protein are less susceptible to oxidation under such conditions (Shechter et al., 1975; Maier et al., 1989; Pearlman & Bewley, 1993). The X-ray structure of Rlx shows that both Met residues are located on the surface of the Rlx molecule and exposed to the solvent (Eigenbrot et al., 1991). The different reactivities of the two Met residues toward H_2O_2 were suggested to be caused by steric hindrance (Nguyen et al., 1993). Our results seem to suggest that the site-specific metal-catalyzed oxidation may be less sensitive to the solvent exposure of the amino acid residues. High reactivity would be expected as long as the labile amino acid residues are on or in the vicinity of metal-binding sites. Apparently, the susceptibility of His to oxidation originates not only from its intrinsic reactivity but also from the strong metal-binding capability of its imidazole ring. For Rlx, His is the residue most susceptible to oxidation, even though it is located in a partially buried position (~57% exposed to surface) (Eigenbrot, personal communications). These findings may help to rationalize the behavior of some other proteins, e.g., the fact that Met 222 of subtilisin is the Met residue most susceptible to oxidation, despite the fact that it occupies a partially buried position (Wells et al., 1987).

Physicochemical properties of a protein can be significantly altered upon covalent modifications. Previous studies have shown that metal-catalyzed oxidation may result in either fragmentation (Marx & Chevion, 1985; Kim et al., 1986; Uchida & Kawakishi, 1988) or aggregation of the proteins, as demonstrated in this study. The available data for Rlx suggest that modification of amino acid residues (especially His) leads to alterations in secondary and tertiary structure, and may increase the exposure of the hydrophobic surface area which results in consequent noncovalent aggregation. The conformation change upon covalent modification of the protein is not surprising as we consider the net charge and polarity alterations on the protein, which may perturb the delicate balance of various forces responsible for maintaining the native structure. The partial denaturation and loss of tertiary structure upon oxidation of Rlx were revealed by near-UV CD spectra. In addition, the analysis indicates that the secondary structure of native Rlx obtained from aqueous solution at pH 2 does not differ considerably from that at pH 5. Therefore, the observed difference of the native Rlx and the oxidized form in the far-UV region at pH 2 can be attributed to the secondary structure change upon oxidation. The low solubility and increased size of the aggregate at higher pH might also be related to the p*I* value of the Rlx degradant. The theoretical p*I* of native Rlx is 9.8 as calculated by the method of Shire (1983) and is estimated to be 9.1 by isoelectric focusing (IEF) (Canova-Davis et al., 1991). The p*I* value of oxidized Rlx could be altered slightly due to the modifications of certain amino acids, but may still be close to that of native Rlx. Therefore, as the pH of the Rlx degradant approaches its p*I* value, it tends to be less soluble. It needs to be mentioned that the nature of the aggregate induced by metal-catalyzed oxidation is different from that of the covalently cross-linked aggregate provoked by radiolysis (Davies, 1987; Davies et al., 1987; Davies & Delsignore, 1987). The identification of the distinct nature of the protein aggregate is important for elucidating the nature of the oxygen species formed during the oxidative process and, thereby, gaining insight into the mechanisms of the reactions involved.

Strategies to prevent oxidation of proteins in pharmaceutical formulations must be developed based on the underlying mechanisms which lead to protein modification. The non-site-specific mechanism would occur in the case of an initial presence of oxidants in the protein formulation system. The oxidants, e.g., peroxide, might be derived from various unexpected sources including excipients (Cleland et al., 1993). Under such oxidation conditions, an appropriate choice of antioxidants or oxygen radical scavengers would be effective in inhibiting the oxidative modification of the proteins. However, due to the unavoidable contamination of trace metal ions in reagents used for the processing and formulation of protein pharmaceuticals, the site-specific mechanism can always be a potential degradation pathway. In this case, the addition of antioxidants may accelerate rather than inhibit the oxidation process. Chelating agents, as shown in this study, proved to be an effective method of stabilizing the protein. Nevertheless, it must be stressed that not all chelating agents are able to inhibit the metal-catalyzed oxidation, particularly if iron instead of copper is the catalytic species. Under certain circumstances, chelates may even accelerate the oxidation processes, as shown in previous studies on EDTA−Fe(III)-induced peptide oxidation (Li et al., 1993). In line with this study, EDTA−Fe(III) was suggested to facilitate the generation of HO$^\bullet$ radicals and enhance the oxidation reactions (Flitter et al., 1983).

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