Pharmaceutical Research, Vol. 10, No. 11, 1993

Research Article

The Kinetics of Relaxin Oxidation by Hydrogen Peroxide

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Received January 8, 1993; accepted May 5, 1993

In this study, hydrogen peroxide was used to study the oxidation of rhRlx under various conditions. Oxidation of rhRlx occurred at both of the two methionines on the B chain, Met B(4) and Met B(25), as expected from the three-dimensional structure of the molecule, which shows that these two residues are located on the surface of the molecule and exposed to solvent. The reaction produced three different oxidized forms of rhRlx containing either Met B(4) sulfoxide, Met B(25) sulfoxide, or both residues oxidized. The corresponding sulfone was not formed under these conditions. The oxidation at the two methionines proceeded independently from each other but Met B(25) was oxidized at a significantly faster rate than Met B(4). The fact that the rate of oxidation at Met B(25) was identical to the rate of oxidation of free methionine and that of two model peptides mimicking the residues around Met B(4) and Met B(25) suggests that the lower reactivity at Met B(4) was due to steric hindrance, and at least in this case, neighboring groups do not influence the oxidation kinetics of methionine residues. The reaction was independent of pH, ionic strength, and buffer concentration in the range studied. The enthalpy of activation for the reaction was approximately 10–14 kcal mol^{-1}, with an entropy of activation of the order of -30 cal K^{-1} mol^{-1}. These data are consistent with previously published mechanisms for organic sulfide oxidation by alkyl hydroperoxides.

KEY WORDS: recombinant human relaxin; relaxin oxidation; protein oxidation; oxidation by hydrogen peroxide; oxidation kinetics.

INTRODUCTION

Relaxin is a protein hormone known primarily for its role in the metabolism of smooth muscle and connective tissue during pregnancy and parturition (1–4). Intramuscular injection of porcine relaxin to dairy heifers caused an increase in pelvic area and cervical dilatation and a reduction in calving intervals (5–7). In a limited clinical trial porcine relaxin administered intracervically induced ripening of the cervix and onset of labor (8). Its presence in the male seminal fluid suggests that it is also produced by the prostate gland and may have a role in sperm motility and oocyte implantation (9,10).

The primary structure of human relaxin was elucidated following the identification of the human gene responsible for the expression of the molecule (11,12). Recently, human relaxin was synthesized both by total chemical synthesis (13) and by recombinant DNA technology (14–16). Recombinant human relaxin (rhRlx) consists of two nonidentical polypeptide chains linked by two disulfide bridges (Fig. 1). The A chain, which begins with a pyroglutamic acid, is 24 amino acids in length and contains one intrachain disulfide bond between Cys A(10) and Cyst A(15). The B chain is a 29-amino acid polypeptide. It is linked to the A chain by Cys A(11)–Cys B(11) and Cys A(24)–Cys B(23) disulfide bridges. The molecular weights of the reduced A chain, the reduced B chain, and the intact molecule based on amino acid composition are 2656, 3313, and 5963, respectively.

Preliminary formulation development work indicated that the two primary degradation pathways for rhRlx in solution were cleavage of the Asp B(1) residue and oxidation of Met B(4) and Met B(25) (13,17–19). Cleavage of Asp B(1) was minimized by selecting a formulation pH above 4.0 and storing the formulation at a low temperature. The oxidation of Met B(4) and Met B(25) was more pronounced when the formulation was exposed to light or when rhRlx was formulated in a cellulose-based gel for topical administration (17–19). In this study hydrogen peroxide was used to examine selectively the oxidation of rhRlx under various conditions. The degradation products were isolated and identified. The kinetics of the reaction were determined as a function of pH, temperature, and ionic strength.

MATERIALS AND METHODS

Recombinant DNA-Derived Human Relaxin. The synthesis and purification of rhRlx were described previously (14–16). In summary, the A chain and B chain of human relaxin were expressed in *Escherichia coli* and purified to greater than 90% purity by reversed-phase high-performance liquid chromatography (RP-HPLC). The polypeptide chains were combined to form rhRlx, which was subsequently separated from the reactants following a series of chromatographic steps. The final product contains more than 95% pure rhRlx as determined by RP-HPLC.

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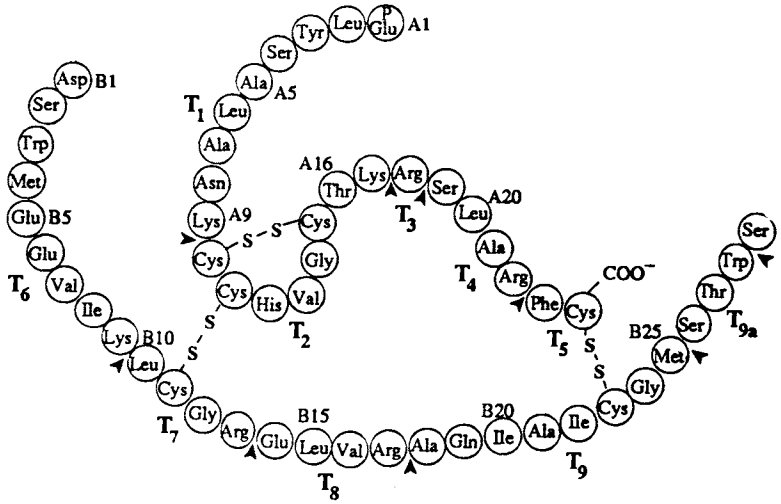


Fig. 1. Primary structure of recombinant human relaxin. Arrowheads indicate potential trypsin cleavage sites and resulting peptide fragments.

Synthesis of AcSer-Trp-Met-Glu-GluNH$_2$ and Ac-CysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ Peptides. The peptides were synthesized via solid-phase methodology (20) utilizing *N*-butoxycarbonyl (t-boc) chemistry. The peptide containing the disulfide bond was prepared by first assembling t-boc-*S*-trityl-Cys-Gly-Met-Ser-(bzl)-Thr(bzl)-resin, then removing the trityl group with 20% trifluoroacetic acid (TFA)/5% triethylsilane in methylene chloride followed by neutralization and reacting the free sulfhydryl with excess t-boc cystine amide which had been oxidized with 0.9 equiv of *m*-chloroperbenzoic acid in DMF. The t-boc group was removed and the peptide acetylated. The peptide was cleaved from the resin with HF and purified by RP-HPLC. The molecular weights of the purified peptides were confirmed by mass spectrometry.

Oxidation of rhRlx in the Presence of H_2O_2. Oxidation of rhRlx was carried out, except when specified otherwise, at room temperature (22–24°C) and in 10 m*M* sodium acetate, pH 5.0. The concentration of rhRlx was 0.1 mg/mL (17×10^{-6} *M*) as determined by UV spectroscopy (extinction coefficient, 2.33 mg^{-1} cm^{-1} mL at 279.5 nm). Hydrogen peroxide 30% aqueous solution (J. T. Baker) was diluted with reaction buffer to the desired concentration range. To start the reaction, hydrogen peroxide stock solution was pipetted into a microcentrifuge tube (Sarstedt) containing the protein solution. The total reaction volume was maintained at 1.2 mL. At each predetermined time point, 0.10 mL of the solution was withdrawn and mixed with 0.02 mL of a 1.0 mg/mL crystallized bovine catalase solution (Sigma) to stop the reaction. The samples were stored at 5°C for later analysis by RP-HPLC.

Hydrogen peroxide concentrations studied were 8.4×10^{-4}, 1.7×10^{-3}, 8.4×10^{-3}, 17×10^{-3}, and 34×10^{-3} *M*, corresponding to H_2O_2:rhRlx molar ratios of 50, 100, 500, 1000, and 2000, respectively. The reaction kinetics were studied at pH values of 3.0 (0.01 *M* sodium lactate), 5.0 (0.01 *M* sodium acetate), 7.0 (0.01 *M* Tris–HCl), and 8.0 (0.01 *M* Tris–HCl). Reaction kinetics were also determined at pH 5.0 between 0.005 and 0.1 *M* sodium acetate, with the solution ionic strength maintained constant at 0.07 *M* with sodium chloride. The ionic strength effect was studied at 0.01 *M* sodium acetate, pH 5.0, with ionic strength adjusted to between 0.007 and 0.21 *M* with sodium chloride.

Oxidation of AcSer-Trp-Met-Glu-GluNH$_2$ and Ac-CysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ in the Presence of H_2O_2. Oxidation of the C-terminal acetylated and N-terminal amidated peptides, AcSer-Trp-Met-Glu-GluNH$_2$ (1) and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ (2), was performed at room temperature in 0.01 *M* sodium acetate, pH 5.0. The peptide concentrations as determined by amino acid analysis were 0.06 and 0.07 mg/mL for 1 and 2, respectively. To initiate the reaction a stock solution of H_2O_2 was transferred into a 2.0-mL microcentrifuge tube containing the peptide solution. The hydrogen peroxide concentration was 34×10^{-3} *M*. The reaction volume was kept at 1.0 mL. At selected time points, 0.1 mL of the reaction mixture was withdrawn and mixed with 0.02 mL of a 1.0 mg/mL catalase solution to hydrolyze excess H_2O_2. Samples were stored at 5°C until analyzed.

Oxidation of Methionine in the Presence of H_2O_2. The oxidation of methionine was performed at room temperature in 0.01 *M* sodium acetate, pH 5.0. The reaction was carried out at three concentrations of H_2O_2: 50×10^{-3}, 8.4×10^{-3}, and $3.4\ 10^{-3}$ *M*. The methionine concentration was 1.7×10^{-4} *M*. The reaction volume was kept at 2.0 mL. At selected time points, 0.2 mL of the reaction mixture was withdrawn and mixed with 0.02 mL of a 1.0 mg/mL catalase solution to hydrolyze excess H_2O_2. Samples were stored at 5°C until analyzed.

Reversed-Phase High-Performance Liquid Chromatography. Recombinant human relaxin and its oxidized degradation products were analyzed by RP-HPLC. The chromatography was performed on a Hewlett–Packard 1090L HPLC system using a Vydac C4 column (Alltech) equilibrated at 40°C. Mobile phase A was 0.1% aqueous TFA (Pierce); mobile phase B consisted of 0.1% TFA in 1:9 (v/v) water:acetonitrile (J. T. Baker, HPLC grade). The mobile-phase flow rate was 1.0 mL/min and peak detection was at 214 nm. Analysis started with 19% B and increased to 41% B in 12 min. The gradient was then ramped up to 100% B to

elute any adsorbed catalase from the column prior to the next injection.

The peptide preparations were analyzed by RP-HPLC. Chromatography was done at ambient temperature on a Nucleosil C-18 (4.6 × 150-mm) column (Alltech). The mobile-phase flow rate was 0.8 mL/min and peak detection was at 214 nm. Mobile phases A and B were the same as those used in the analysis of rhRlx. The linear gradient used to separate 1 and its Met-sulfoxide derivative was from 15 to 28% B in 13 min, 28 to 100% B in 2 min, and 100 to 0% B in 2 min. Peptide 1 eluted at approximately 16.5 min and oxidized 1 eluted at 10 min. The linear gradient used in the analysis of 2 was from 4 to 17% B in 13 min, 17 to 100% B in 2 min, and 100 to 0% B in 2 min. The retention time of intact 2 was 16 min and that of the oxidized by-product was 10.5 min.

Amino Acid Analysis. Methionine and methionine sulfoxide were separated and quantitated using the Beckman 6300 automated amino acid analyzer (Beckman, CA). Briefly the amino acids were diluted to 40 nm/mL and injected into a 12-cm sodium high-performance column (Beckman). Elution buffers were NaD, NaE, and NaF (Beckman). The eluting amino acids were derivatized with ninhydrin (Nin-X; Beckman), and peak detection was carried out at 440 and 570 nm. Under these conditions, methionine sulfoxide and methionine eluted at 7.5 and 31 min, respectively.

Tryptic Mapping. Tryptic digestion of rhRlx and oxidized rhRlx and analysis of the resultant peptides by RP-HPLC were performed following closely the procedure described by Canova-Davis *et al.* (13). The protein at a concentration of 0.1 mg/mL was digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated bovine pancreatic trypsin (Worthington Biochemical) for 4 hr at 30°C in 10 m*M* Tris–acetate, pH 7.3, 0.2 m*M* $CaCl_2$. The enzyme:substrate ratio was 1:100 (w/w). The peptide fragments were separated by RP-HPLC on a Nucleosil C18 column (Alltech, CA) at room temperature. The mobile-phase flow rate was 1.0 mL/min and the linear elution gradient of acetonitrile containing 0.08% TFA was from 3 to 30% in 54 min. Peptide fragments of interest were collected from the column effluent and characterized by mass spectrometric analysis and N-terminal sequencing by Edman degradation.

Mass Spectrometric Analysis. A Sciex API III triple quadrupole instrument fitted with an ion spray (nebulized-assisted electrospray) ion source was employed to collect mass spectral data. Samples were dissolved in a 50:50 (v/v) water:methanol solution containing 0.1% formic acid. The instrument was calibrated with a mixture of polypropylene glycols. Molecular mass was calculated via the supplied HyperMass software.

Cyclic Adenosine Monophosphate (cAMP) Production Assay. The activity of rhRlx was evaluated by determining the ability of the protein to upregulate the production of cAMP by normal human uterine endometrial cells *in vitro* (21). The cells were grown in a 96-well microtiter plate. Recombinant human relaxin samples were diluted with assay buffer to a concentration range between 1.0 and 4.0 ng/mL. The cells were incubated with 0.25 mL of sample for 30 min at 37°C. After incubation, the rhRlx solutions were discarded and the cells were lysed with 0.1 *N* HCl. Intracellular cAMP released was determined by radioimmunoassay and expressed as picomoles per milliliter. A rhRlx standard curve was generated and used to correlate cAMP production and rhRlx concentration. The bioactivity of rhRlx samples was then expressed as mass equivalents of rhRlx standard.

Isolation and Characterization of Degradation Products. The protein peaks separated by RP-HPLC were collected, vacuum-dried (Speedvac, Savant), and reconstituted into 0.01 *M* acetate, pH 5.0, for analysis by peptide mapping, mass spectrometry (13,14), and cAMP bioassay (21).

RESULTS AND DISCUSSION

Degradation Product Isolation and Characterization

A representative chromatogram of an oxidized rhRlx sample analyzed by RP-HPLC is shown in Fig. 2. The main peak (peak 4) coeluted with intact rhRlx reference at 10.5 min. Three additional peaks corresponding to three hydrophilic reaction by-products were separated on the same chromatogram at 5.2 min (peak 1), 8.2 min (peak 2), and 8.8 min (peak 3).

The protein under each of the four peaks was collected from the column effluent and characterized for identity and bioactivity. The molecular weights of the isolated protein species obtained by mass spectrometric analysis are shown in Table I. Recombinant human relaxin reference and the protein collected under peak 4 exhibited a mass of 5962.51 ± 0.32, which agrees with the expected value of 5963 for intact rhRlx. Peak 2 and peak 3, which were pooled into one fraction, and peak 1 exhibited masses of 5978.76 ± 1.12 and 5994.39 ± 0.25, respectively. These results are consistent with the degradation products being the monooxidized (rhRlx + 16) and dioxidized (rhRlx + 32) forms of rhRlx. The corresponding sulfones were not produced under these conditions.

In a separate experiment peaks 1, 2, 3, and 4 were digested with trypsin and analyzed by RP-HPLC on a Vydac C-18 column as described previously (13). The peptides resulting from tryptic digestion of rhRlx have been characterized (15). Most relevant to this study are peptide fragments T6 and T5–T9. The peptide T6, which contains Met B(4), elutes at 46 min and the disulfide-linked peptide T5–T9 elutes at 47 min (Fig. 3d). Figure 3b is a tryptic map of monooxidized rhRlx under RP-HPLC peak 2. The position

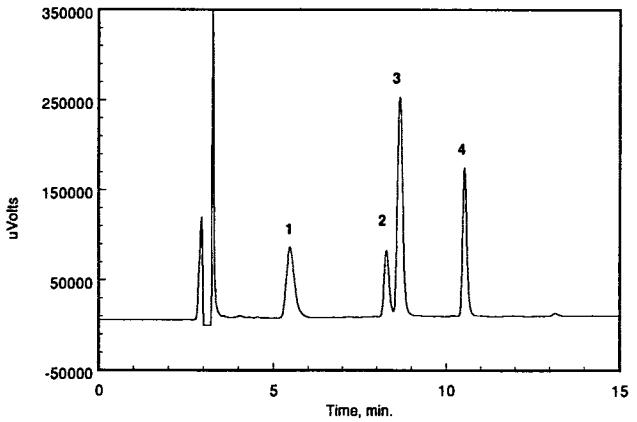


Fig. 2. RP-HPLC chromatogram of an oxidized rhRlx sample. Peak 1, Met B(4), Met B(25) disulfoxide rhRlx; peak 2, Met B(4) sulfoxide rhRlx; peak 3, Met B(25) sulfoxide rhRlx; peak 4, native rhRlx.

Table I. Mass Spectrometric Analysis of Protein Peaks Collected from RP-HPLC Following Oxidation by H_2O_2

RP-HPLC peak	Average mass
Reference	5962.51 ± 0.32
1	5994.59 ± 0.25
2 and 3	5978.76 ± 1.12
4	5962.51 ± 0.32

of peptide fragment T5–T9 remained unchanged, whereas the retention time of the T6 fragment shifted from approximately 46 to 36 min (T6′). Mass spectrometric and N-terminal analyses of the isolated T6′ peptide (Table II) indicate that this shift was due to the conversion of the methionine residue to methionine–sulfoxide. Thus, RP-HPLC peak 2 can be positively identified as Met B(4) sulfoxide rhRlx. Similarly, the tryptic map of monooxidized rhRlx obtained under RP-HPLC peak 3 (Fig. 3c) exhibited an altered T5–T9 pep-

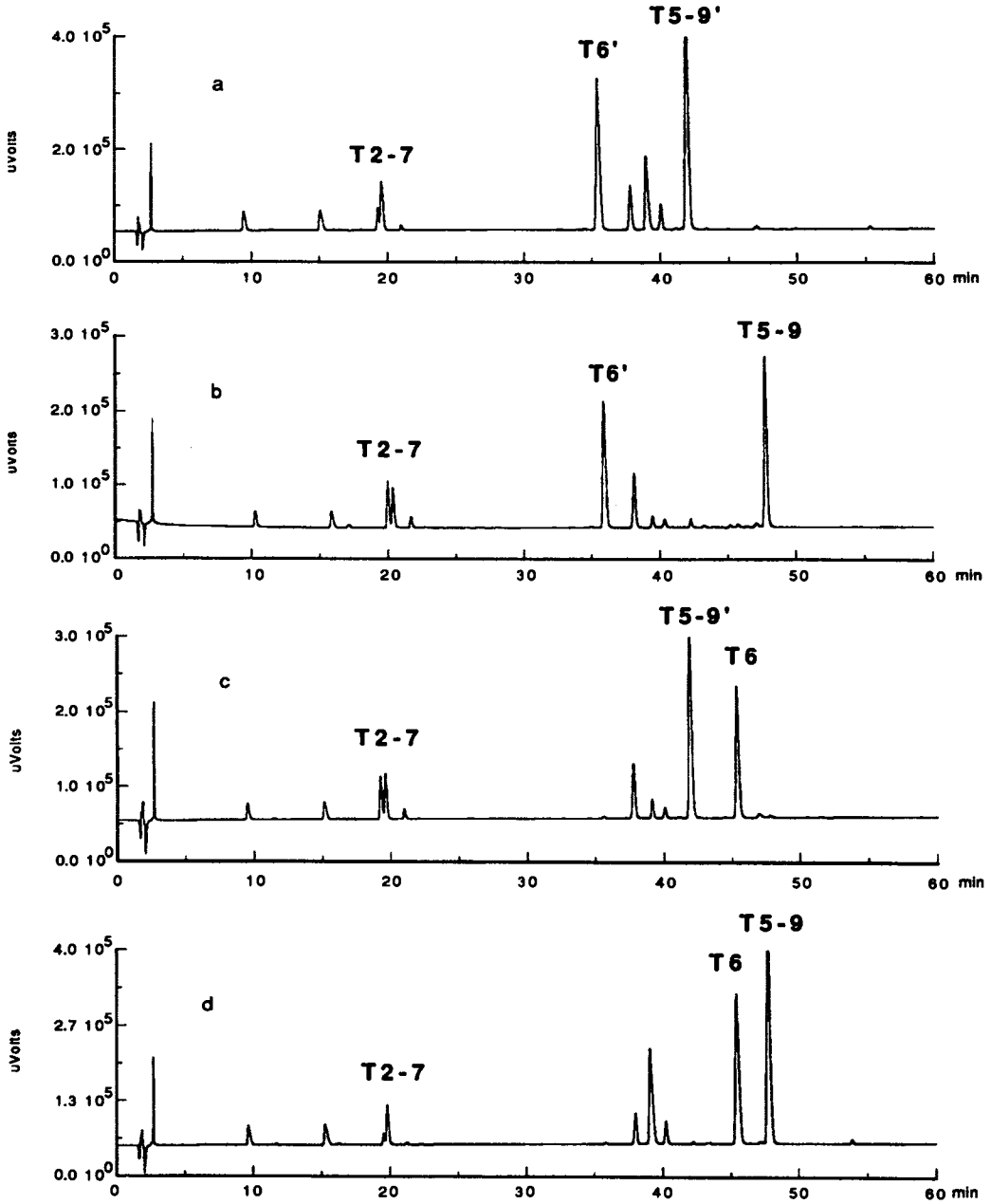


Fig. 3. Peptide map of various forms of rhRlx isolated by RP-HPLC. (a) Peak 1, showing the disappearance of T6 and T5–T9, and the appearance of T6′ and T5–T9′; T6′ contains Met B(4) sulfoxide and T5–T9′ contains Met B(25) sulfoxide. (b) Peak 2, showing the disappearance of T6 and the appearance of T6′; T5–T9 remains unaltered. (c) Peak 3, showing the disappearance of T5–T9′; T6 remains unaltered. (d) Peak 4, showing T6 and T5–T9 peptide fragments containing Met B(4) and Met B(25), respectively.

Table II. Primary Structure of Peptide Fragments T6, T6′, T5–T9, and T5–T9′

Peptide fragment	Retention time (min)	Mass	Primary structure[a]
T6	46	1136.6[b]	DSWMEEVIK
T6′	36	1152.4	DSWMEEVIK
T5–T9	47	1533.7[b]	AQIAICGMSTWS
T5–T9′	42	1549.0	AQIAICGMSTWS

[a] The N-terminal analysis involved a reduction step; Met-sulfoxide was reduced back to methionine and T5 was not detected.
[b] From Ref. 15.

tide eluting at approximately 42 min (T5–T9′), which contained the sulfoxide form of Met B(25) (Table II). Consequently, peak 3 was assigned to Met B(25) sulfoxide rhRlx. The tryptic map of dioxidized rhRlx collected under RP-HPLC peak 1 exhibited altered T6 and T5–T9 peptide fragments, confirming that both methionine residues on the B chain were oxidized to their corresponding sulfoxide forms (Fig. 3a).

The potency of the different rhRlx species isolated under peaks 1, 2, 3, and 4 relative to intact rhRlx was evaluated based on their ability to upregulate the production of cAMP by human endometrial cells in culture. The results are shown in Fig. 4. Within the error of the assay, there was no significant difference in bioactivity between intact rhRlx and its oxidized derivatives. This observation is not totally unexpected since Met B(4) and Met B(25) are located at the two termini of the B chain which are relatively remote from the putative receptor binding sites, Arg B(13) and Arg B(17) (22,23).

Kinetics of rhRlx Oxidation by H_2O_2

The kinetics of rhRlx oxidation by H_2O_2 were followed by plotting the areas under peak 1, peak 2, peak 3, and peak 4 (Fig. 2) as a function of time. Figure 5 is a representative plot of the time course of the reaction carried out in 10 m*M* acetate buffer, pH 5.0, at room temperature. The relaxin

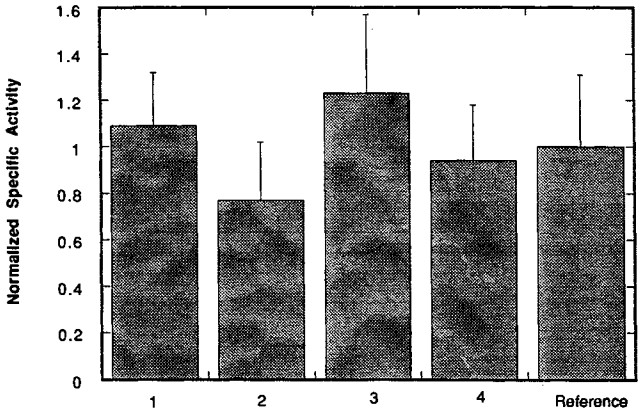


Fig. 4. Bioactivity of various oxidized forms of rhRlx as measured by the upregulation of cAMP production in human uterine endometrial cells. (1) Met B(4), Met B(25) disulfoxide rhRlx; (2) Met B(4) sulfoxide rhRlx; (3) Met B(25) sulfoxide rhRlx; (4) native rhRlx.

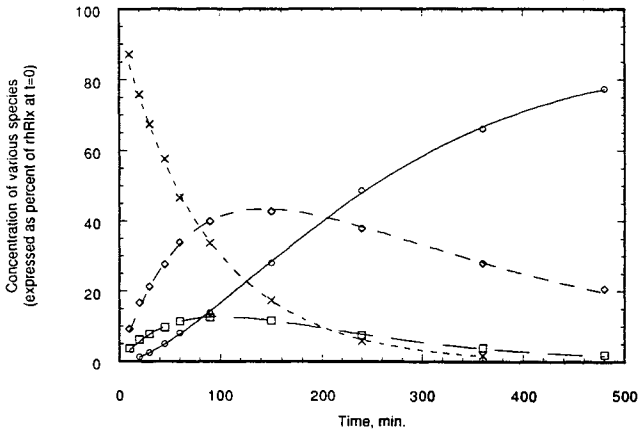
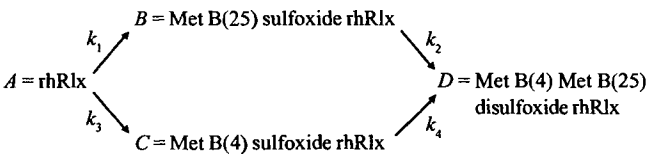


Fig. 5. Oxidation of recombinant human relaxin at 22–24°C: relaxin, 17×10^{-6} *M*; hydrogen peroxide, 17×10^{-3} *M*. Plot of the concentration of different species versus time. Points are experimental data; solid lines are generated from the kinetic model. (○) Met B(4), Met B(25) disulfoxide rhRlx; (◇) Met B(25) sulfoxide rhRlx; (□) Met B(4) sulfoxide rhRlx; (×) native rhRlx.

concentration was 0.1 mg/mL (17×10^{-6} *M*) and the H_2O_2 concentration was 17×10^{-3} *M*. Peaks 2 and 3, corresponding to Met B(4) sulfoxide rhRlx and Met B(25) sulfoxide rhRlx, were first detected with the concomitant disappearance of intact rhRlx. Met B(25) sulfoxide rhRlx was produced at a significantly faster rate than Met B(4) sulfoxide rhRlx. In the later phase of the reaction, there was an accumulation of the dioxidized species, while Met B(4) sulfoxide rhRlx and Met B(25) sulfoxide rhRlx decreased slowly. When the reaction was allowed to proceed to completion, Met B(4), Met B(25) disulfoxide rhRlx was the final degradation product.

The following kinetic model was used to analyze the data:



The differential equations describing the system are

$$\frac{dA}{dt} = -k_T [A]$$

$$\frac{dB}{dt} = k_1 [A] - k_2 [B]$$

$$\frac{dC}{dt} = k_3 [A] - k_4 [C]$$

$$\frac{dD}{dt} = k_2 [B] + k_4 [C]$$

The analytical solutions to these equations can be expressed as follows:

$$A = A_0\, e^{-k_T t}$$

$$B = \left(\frac{k_1 A_0}{k_T - k_2}\right)(e^{-k_2 t} - e^{-k_T t})$$

$$C = \left(\frac{k_3 A_0}{k_T - k_4}\right)(e^{-k_4 t} - e^{-k_T t})$$

$$D = A_0 - A_0\left[e^{-k_T t} + \frac{k_1}{k_T - k_2}(e^{-k_2 t} - e^{-k_T t}) + \frac{k_3}{k_T - k_4}(e^{-k_4 t} - e^{-k_T t})\right]$$

In the presence of a large excess of H_2O_2, k_T, k_1, k_2, k_3, and k_4 are the pseudo-first-order rate constants of the various reactions identified in the model. They were estimated by nonlinear regression analysis fitting experimental data points to the above equations using the KaleidaGraph graphic software package for the Macintosh computer (Abelbeck Software). The correlation coefficients R were greater than 0.99 and the coefficients of variation associated with the estimation were less than 10% in all cases.

The rate constants k_T, k_1, k_2, k_3, and k_4 were directly proportional to the H_2O_2 concentration. The corresponding bimolecular rate constants for the individual reactions (k_T', k_1', k_2', k_3', k_4') shown in Table III were derived from the slope of the straight lines which result from the plot of the pseudo-first-order rate constants versus H_2O_2 concentration. The rate of oxidation of intact rhRlx to Met B(25) sulfoxide rhRlx (k_1') and the rate of oxidation of Met B(4) sulfoxide rhRlx to Met B(4), Met B(25) disulfoxide rhRlx (k_4') are not almost identical. Similarly the rate of oxidation of intact rhRlx to Met B(4) sulfoxide (k_3') is similar to the rate of oxidation of Met B(25) rhRlx to Met B(4), Met B(25) disulfoxide rhRlx (k_4'). Thus, the two oxidation steps proceeded independently of each other and the rate of oxidation at Met B(25) was approximately 2.5 times faster than that at Met B(4).

Influence of pH, Buffer, Ionic Strength, and Temperature on the Rate of Oxidation

The kinetics of rhRlx oxidation by H_2O_2 was studied at pH's 3.0 (0.01 M lactate), 5.0 (0.01 M acetate), 7.0 (0.01 M Tris–HCl), and 8.0 (0.01 M Tris–HCl) at room temperature; the solution ionic strength was maintained at 0.01 M by the addition of NaCl. The rate of disappearance of native rhRlx, defined as $k'_T = k'_1 + k'_3$, were 1.1×10^{-2}, 1.1×10^{-2}, 1.0×10^{-2}, and 0.95×10^{-2} $sec^{-1}\, M^{-1}$ at pH's 3.0, 5.0, 7.0, and 8.0, respectively. Thus, over the pH range studied, which covers 5 orders of magnitude in H_3O^+ activity, the rate differs by less than 20%. The small decrease in k'_T with pH may be due to the slow degradation of H_2O_2 at high pH's.

The effect of ionic strength and buffer concentration on the rate of oxidation of rhRlx was studied at room temperature in acetate buffer, pH 5.0. The first experiment was carried out in 0.01 M buffer and the solution ionic strength was adjusted to cover the range between 0.007 and 0.21 M with NaCl. In the second experiment, the solution ionic strength was maintained at 0.07 M with NaCl and the acetate concentration was varied between 0.005 and 0.100 M. The second-order rate constant for the disappearance of native rhRlx (k'_T) varied between 1.0×10^{-2} and 1.3×10^{-2} $sec^{-1}\, M^{-1}$. There was no apparent trend in the variations and the differences among the values were not statistically different. Thus, the reaction appeared to be insensitive to changes in ionic strength and acetate concentration in the ranges studied.

The thermodynamic parameters of the reaction are derived from the Eyring plot of the kinetic data obtained in 0.01 M acetate, pH 5.0, at 5, 15, 25, and 35°C. The enthalpy (ΔH) of activation of the reaction was obtained directly from the slope of the straight lines. The entropy of activation (ΔS) of the reaction was calculated from the intercept of the lines with the y axis using the relationship

$$\text{Intercept} = \ln\left(\frac{k}{h}\right) + \frac{\Delta S}{R}$$

where k is the Boltzman constant, h is the Planck constant, and R is the gas constant. Values obtained for ΔH and ΔS for the individual reactions are listed in Table IV. The thermodynamic parameters associated with the oxidation of Met B(25) sulfoxide to the dioxidized form appear to be slightly different from those of the other three reactions. However, this difference is probably due to a slight under estimation of k_2 at low temperatures, which tends to decrease the slope of the line and decrease the magnitude of ΔS. Overall the reaction ΔH was estimated to be around 10 to 15 kcal mol^{-1} with highly negative ΔS (≈ -25 to -30 cal $K^{-1}\, mol^{-1}$).

Bannard *et al.* (24) have summarized the various characteristics of the oxidation reaction of organic sulfides by hydrogen peroxide and alkyl peroxides. The reactions are first order with respect to sulfide and peroxy compounds. It is acid catalyzed and the reaction rate varies with the nature and composition of the solvent. Catalysts and inhibitors of free radical reactions including oxygen do not exhibit enhancement or inhibitory effect on the reaction kinetics. They proposed a one-step mechanism where sulfide interacts with a peroxide–solvent complex in which a series of concerted electronic displacements leads to hydrogen exchange and oxygen transfer. This mechanism assumes that the peroxidic oxygen is electrophilic and readily released in the presence of a nucleophilic group. This assumption has been verified

Table III. Bimolecular Rate Constants for the Oxidation of Methionine Residues in 10 mM Acetate, pH 5.0, T = 22–24°C

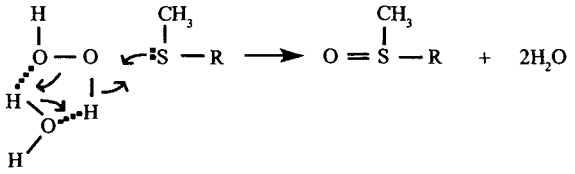
Reaction	Bimolecular rate constant $k'_i \times 10^{-2}$ ($sec^{-1}\, M^{-1}$)
rhRlx → Met B(25) sulfoxide rhRlx (k'_1)	0.85 ± 0.04
rhRlx → Met B(4) sulfoxide rhRlx (k'_3)	0.34 ± 0.02
Met B(25) sulfoxide rhRlx → Met B(4), Met B(25) disulfoxide rhRlx (k'_2)	0.38 ± 0.07
Met B(4) sulfoxide rhRlx → Met B(4), Met B(25) disulfoxide rhRlx (k'_4)	0.83 ± 0.10
AcSer-Trp-Met-Glu-GluNH$_2$	1.07 ± 0.03
AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$	1.00 ± 0.02
Methionine	0.93 ± 0.16

Table IV. Thermodynamic Parameters for the Various Oxidation Reactions in rhRlx at pH 5.0

Reaction	ΔS (cal mol^{-1} K^{-1})	ΔH (kcal mol^{-1})
rhRlx → Met B(25) sulfoxide rhRlx	−31	10
Met B(25) sulfoxide rhRlx → Met B(4), Met B(25) disulfoxide rhRlx	−20	14
rhRlx → Met B(4) sulfoxide rhRlx	−28	12
Met B(4) sulfoxide rhRlx → Met B(4), Met B(25) disulfoxide rhRlx	−28	11
Disappearance of native rhRlx	−29	11

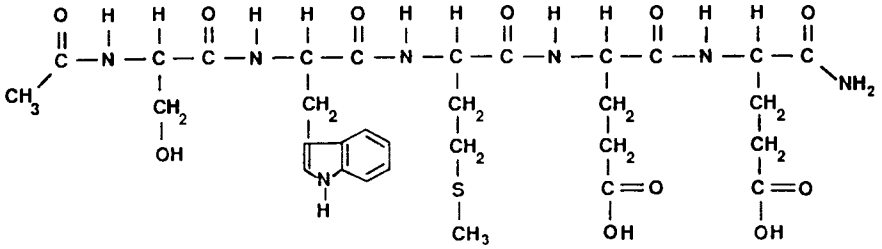
by the fact that electron-attracting groups on peroxide increased the rate of reaction by increasing the electrophilic character of oxygen and electron-releasing groups attached to the vicinity of sulfur increased its electron density, with a corresponding increase in oxidation rate (25). Furthermore, this mechanism is consistent with the highly negative entropy of activation of the reaction, which is in the range of 30 to 40 entropy units (24,30).

The data obtained in this series of experiments agree with published literature, except that within the pH range studied, the reaction is fairly independent of pH. Toennies and Callan (26) reported that the oxidation of methionine residues in several proteins is independent of pH over the range from 1.0 to 5.0. Thus, if there is an acid-catalyzed component of the reactions, it must be fairly weak, and in aqueous solution of moderate acidity, it is masked by a water-catalyzed reaction which is consistent with the following proposed mechanism:

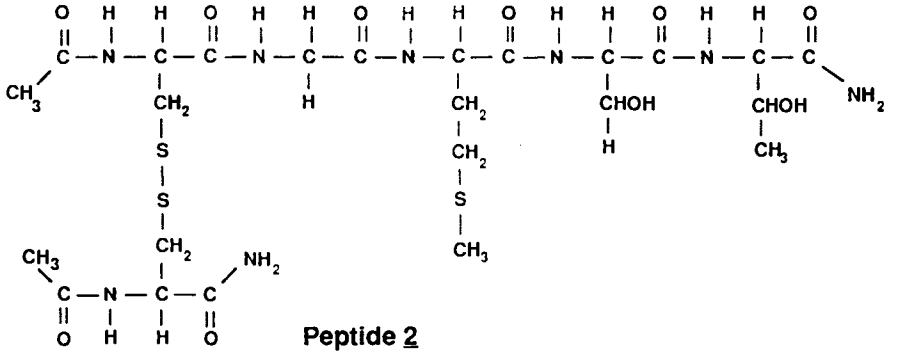


Oxidation of AcSer-Try-Met-Glu-GluNH$_2$ and AcCysNH$_2$-S-S-AcCys-Gly-Met-Ser-ThrNH$_2$ Peptides

Methionine residues buried inside of the hydrophobic core of globular proteins are normally not susceptible to oxidation by H_2O_2 (27–29). The structure of rhRlx obtained from X-ray crystallographic studies indicated that the methyl thioether side chains of Met B(4) and Met B(25) are juxtaposed on opposite sides of the Trp B(3) indole side chain (23). Although Met B(25) is located at the end of the B(11)-



Peptide 1



Peptide 2

Fig. 6. Primary structure of peptide 1 and peptide 2.

to-B(25) α-helix loop, both residues are exposed to the solvent and easily oxidized as observed experimentally. The difference between the rate of oxidation at Met B(4) and that at Met B(25) was small but noticeable. This difference may be due to neighboring group effects, in which case the primary sequence around the two methionine residues would play a dominant role. Peptide 1 (Fig. 6) represents residues B(2) to B(6), the pentapeptide fragment containing Met B(4). Peptide 2 (Fig. 6) is a replicate of the pentapeptide fragment B(23) to B(27) linked to Cys A(24) which contains Met B(25). Oxidation of these two peptides in 10 m*M* acetate, pH 5.0, at room temperature followed pseudo-first-order kinetics and yielded the corresponding Met-sulfoxide peptides as determined by RP-HPLC and mass spectrometry. The second-order rate constants for the oxidation of 1 and 2 were 1.1×10^{-2} and 1.0×10^{-2} sec^{-1} M^{-1}, respectively. These values were identical to each other and very similar to the oxidation rate constant obtained for Met B(25) under the same conditions (0.8×10^{-2} sec^{-1} M^{-1}). Thus, the difference in the oxidation rate between Met B(4) and Met B(25) on rhRlx was apparently not affected by the amino acids flanking the two methionine residues of interest. More specifically, the two glutamic acids adjacent to Met B(4) did not facilitate the oxidation of this residue via possible intramolecular acid catalysis. This was supported by the insensitivity of the reaction to the pH of the solution and the fact that in the solid phase, Glu B(5) and Glu B(6) formed salt bridges with Arg A(18) and Lys A(17), respectively (23). The disulfide bridge formed by Cys A(24) and Cys B(23) apparently did not present any steric barrier to the oxidation of Met B(25).

Oxidation of Methionine

The intrinsic oxidation rate of methionine was monitored by amino acid analysis. The bimolecular rate constant of the reaction was obtained from a plot of the pseudo-first-order rate constant for the disappearance of methionine versus H_2O_2 concentration. Data shown in Table III indicate that oxidation of Met B(25) proceeded at a rate similar to that of free methionine. This result, coupled with the data obtained for the model peptides, suggests that Met B(25) is fully exposed and accessible to H_2O_2, whereas the lower reactivity at Met B(4) is probably due to steric hindrance.

ACKNOWLEDGMENTS

The authors wish to thank Reed Harris for his assistance with the amino acid and N-terminal analysis, Victor Ling and Andrew Guzzetta for their assistance with the mass spectrometric analysis, the bioassay services group for the cAMP assay, and Drs. Thomas Patapoff, Michael Powell, and Tom Bewley for their helpful comments.

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