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Peroxidation of Lysozyme Treated with Cu(II) and Hydrogen Peroxide

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Abstract

When lysozyme was treated with Cu(II) and H_2O_2 at pH 7.4, the protein underwent polymerization as well as changes in its fluorescent characteristics. Upon prolonged incubation, most of the protein aggregates were degraded into smaller peptides. Amino acid analysis indicated that the basic amino acid residues were most susceptible to the oxidation. Tryptophan residues were converted to N-formylkynurenine and kynurenine, and lysine residues were deaminated to form α-aminoadipic acid δ-semialdehyde. During Cu(II)–H_2O_2 treatment, the formation of carbonyl groups was accompanied by the loss of free amino groups in the protein. Succinylation of free amino groups protected lysine residues from oxidation by Cu(II)–H_2O_2, but failed to prevent polymerization. The studies with the modified lysozyme suggest that Cu(II)–H_2O_2 can oxidize various amino acid residues in addition to lysine to generate different types of carbonyl compounds and these carbonyl compounds may be responsible for the formation of crosslinks in the polymerization process.

Introduction

In recent years it has become evident that a large number of biological destructive processes are the consequence of reactions with various reactive oxygen species. Some of these reactions involve catalysis by metal ions such as iron and copper. Earlier studies from various laboratories have shown that the combination of Cu(II) and H_2O_2 can serve as a potent oxidant for biomolecules such as catechols [1], nucleotides [2, 3], proteins [4, 5] and phospholipids [6]. Some of these oxidative processes may be related to tissue damage in certain disease states.

Feeney *et al.* [7] observed that lysozyme activity was destroyed irreversibly when incubated with Cu(II) in an alkaline medium. Chung *et al.* [8] found that at neutral pH, $CuSO_4$ alone did not have any effect on the enzymatic activity of lysozyme unless H_2O_2 (1 mM or higher) was added. With increasing concentration of the oxidant, structural modification of the enzyme was also detected. This included polymerization, followed by degradation of the peptide chain, and changes in the fluorescent characteristics [8]. In this paper, we examine the nature and scope of these alterations.

Experimental

Lysozyme (chicken egg white) was obtained from Worthington Biochemical Corp.; catalase from Boehringer Mannheim Corp.; 30% H_2O_2 from Baker Chemical Co.; protease (Streptomyces griseus, Type XIV), kynurenine and succinic anhydride from Sigma Chemical Co.; N-formylkynurenine from Chemalog Corp.; 2,4-dinitrophenylhydrazine (DNPH) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) from Eastman Organic Chemicals; Sephadex G-50 and Sephacryl S-300 from Pharmacia Fine Chemicals.

Treatment of Lysozyme with Cu(II) and H_2O_2

Lysozyme (1 mg) was incubated with $CuSO_4$ (0.075 mmol) and H_2O_2 (50 mmol) in 1 ml of 10 mM sodium phosphate buffer, pH 7.4 at 37 °C for varying periods of time. After a few min, turbidity developed which progressed with time. The reaction was terminated by the addition of 1 μmol of EDTA and 5 μg of catalase. The precipitate could be redissolved by warming at 67 °C for 2–3 min with gentle agitation after pH was adjusted to 9–10 with 1 M NaOH. After cooling to room temperature, the pH was adjusted to 7 with 1 M HCl.

In order to obtain the aggregated polymerized fraction, lysozyme was allowed to react with $CuSO_4$ for 30 min, at which point the reaction was terminated and the mixture was centrifuged at 1000 × g for 10 min at 4 °C. The sediment was washed three times with deionized water.

Molecular-sieve Chromatography

The products of Cu(II)–H_2O_2 treatment were analyzed on a Sephadex G-50 column (19 × 480 mm)

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at room temperature. The elution buffer consisted of 10 mM sodium phosphate, 0.1 M NaCl and 0.1 mM EDTA, pH 7.5. The flow-rate was 48 ml/h and was regulated by a proportioning pump (Technicon Instrument Corp.) on the effluent side. The effluent was monitored continuously for UV absorption at 230 nm in a Gilford Spectrophotometer Model 2400 and for fluorescence emission at wavelengths greater than 420 nm (Filter 2A) with excitation maximum at 360 nm (Filter 7-60) in an Aminco Fluro-Colorimeter 4-744. The molecular weight was estimated with Sephacryl S-300 (23 × 480 mm). The elution buffer consisted of 20 mM Tris, 0.1 mM EDTA and 0.5 M NaCl, pH 8.2

Polymerization Kinetics

Lysozyme (3 mg) was treated with Cu(II) and H_2O_2 in 3 ml of 10 mmol sodium phosphate as described above. The rate of the polymerization process was followed by turbidity measurements at 550 nm in a Cary 210 spectrophotometer. To confirm the validity of the turbidity measurements, the weight of the protein aggregates was determined. The reaction mixture was filtered through a pre-weighed 0.45 μ membrane filter immediately after measurement of the turbidity. The protein aggregate on the filter was dried under an infrared lamp for 30 min and then weighed.

Amino Acid Analysis

Protein (2 mg) in 4 ml of 6 M HCl was sealed under vacuum and heated at 110 ℃ for 24 h. After the hydrolysate was dried in a vacuum desiccator over a silica gel desiccant and NaOH pellets, the sample was redissolved in 1.2 ml of 0.2 M sodium citrate, pH 2.25, containing 2% thioglycol. Hydrolysis of protein for tryptophan and kynurenine determination was carried out according to the method of Simpson *et al.* [9].

Amino acid analysis was carried out on a Beckman Model 120C Amino Acid Analyzer. Glycine and alanine were used as internal standards since they were found to be stable under the conditions of Cu(II) and H_2O_2 treatment. The values for other amino acid residues were calculated based on these standards and expressed in terms of the molecular weight of native lysozyme.

Determination of α-Aminoadipic Acid

Dissolved aggregated protein was oxidized with performic acid [10] prior to amino acid analysis. The solution was evaporated on a rotary evaporator and the dried sample was hydrolyzed with 6 M HCl and then treated as described above. The hydrolysate was run on the long column of the amino acid analyzer. Elution was performed with 20 ml 0.16 M sodium citrate, pH 2.68, followed by 0.16 M sodium citrate, pH 2.91. Under these conditions, α-aminoadipic acid appears as a peak after glycine.

Acid Hydrolysis of N-Formylkynurenine

This experiment was carried out according to the method of Yamasaki *et al.* [11].

Determination of Carbonyl Groups and Free Amino Groups

Carbonyl groups were measured by the method of Bowes and Moss [12] with slight modifications. Lysozyme treated with Cu(II) and H_2O_2 was digested with 2% protease at 37 ℃ for 24 h. The hydrolyzed protein solution (0.2 ml) was treated for 30 min at room temperature with 0.1 ml of a saturated solution of 2,4-dinitrophenylhydrazine in 1 M HCl. Absorbance at 460 nm was determined after addition of 3 ml 0.3 M KOH. The amounts of carbonyl groups were expressed as absorbance at 460 nm, since molecular extinction coefficients of various carbonyl compounds vary considerably. Free amino groups were determined in the presence of SDS according to the method of Habeeb [13] using 2,4,6-trinitrobenzene sulfonic acid (TNBS) as a reagent.

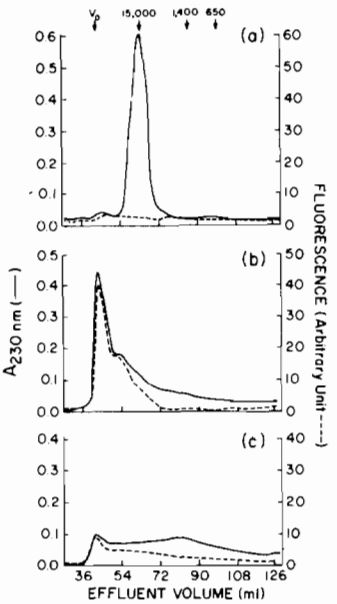

Fig. 1. Lysozyme treated with Cu(II)–H_2O_2. Lysozyme (1 mg) was incubated with 0.075 mM $CuSO_4$, 50 mM H_2O_2 and 10 mM sodium phosphate, pH 7.4, in a total volume of 1.0 ml at 37 ℃ for different periods of time. (a) 0 min, (b) 30 min (c) 240 min. The reaction mixture was redissolved as described in Methods. The sample was chromatographed on a Sephadex G-50 column (19 × 480 mm) at room temperature. The elution buffer contained 10 mM sodium phosphate, 0.2 M NaCl and 0.1 mM EDTA, pH 7.5. The flow rate was 48 ml/h. The peptide elution was monitored continuously at 230 nm and the fluorescence with maximum excitation at 360 nm was measured as described in Methods.

Succinylation

Lysozyme was treated with succinic anhydride according to the method of Glazer *et al.* [14].

Results and Discussion

Treatment of Lysozyme with Cu(II) and H_2O_2

When lysozyme was treated with $CuSO_4$ and H_2O_2 for short periods of time, high molecular weight aggregates were formed which, upon further incubation, were degraded to smaller peptides (Fig. 1). The high molecular weight material formed during a 30 min incubation period exhibited a strong fluorescence and appeared in the void volume of a Sephadex G50 column (Fig. 1b). After 240 min of incubation, most of the aggregated protein dissolved and the bulk of the protein peaks had shifted to much smaller peptides (Fig. 1c).

The aggregated protein formed after 30 min of incubation was collected by centrifugation, washed and redissolved by adjusting the pH to 9–10 and warming to 65 °C. Molecular weight estimation on a Sephacryl S300 column revealed a broak peak with average molecular weight of 73 K daltons (data not shown). The aggregate was not disrupted by treatment with 2% mercaptoethanol, 10 M urea, 20% SDS or 1 M hydroxylamine.

Aggregation Kinetics

The aggregation and dissolution phenomenon was measured in 2 ways. Turbidity measurements at 550 nm were found to closely correspond to gravimetric measurements of the filtered precipitate (Fig. 2). The most extensive aggregation occurred at about 30 min followed by fragmentation of the polymers.

Amino Acid Analysis

Table I shows a comparison of the amino acid composition of the native, control lysozyme (column 2) with that of the aggregate (column 3). In general, the basic amino acids were most labile. Over 80% of the histidine and tryptophan residues were destroyed as well as 55% of the lysine and 46% of the arginine residues. The other amino acids were relatively unaffected.

TABLE I. Amino Acid Analysis of Control, Aggregated and Suc-lysozyme.

	Theoretical	Lysozyme Cu(II)–H_2O_2		Suc-lysozyme Cu(II)–H_2O_2	
		Control	Treated	Control	Treated
Aspartic Acid	21	20.6	19.3	21.6	22.2
Threonine	7	6.7	7.5	7.2	6.7
Serine	10	9.8	9.8	10.6	7.7
Glutamic Acid	5	5.2	5.8	6.0	6.0
Proline	2	2.0	2.1	2.0	1.3
Glycine	12	12.3	12.7	12.5	13.1
Alanine	12	11.7	11.2	12.2	12.6
Half-cystine	8	8.1	5.2	6.7	4.3
Valine	6	5.1	5.5	6.2	4.9
Methionine	2	2.1	2.4	1.7	1.1
Isoleucine	6	6.1	6.1	5.0	4.9
Leucine	8	8.8	7.6	6.7	6.6
Tyrosine	3	3.4	2.2	2.9	1.5
Phenylalanine	3	3.5	3.1	3.1	2.4
Lysine	6	6.0	2.7	5.8	5.6
Histidine	1	0.9	0.2	1.5	0.0
Arginine	11	10.1	5.9	11.0	8.8
Tryptophan	6	5.9	1.2	5.8	0.0
Cysteic acid		0.0	0.6	N.D.[b]	N.D.[b]
Kynurenine		0.0	2.1	0.0	4.1
α-Aminoadipic acid[a]		0.0	1.5	N.D.[b]	N.D.[b]
Total	129	128.3	114.7	128.5	113.8
Ammonia		13.5	11.5	11.4	12.6

The preparation of samples was described in Methods. The number of composite amino acid residues were expressed in terms of the molecular weight of native lysozyme. [a]After performic acid oxidation. [b]N.D. = Not determined.

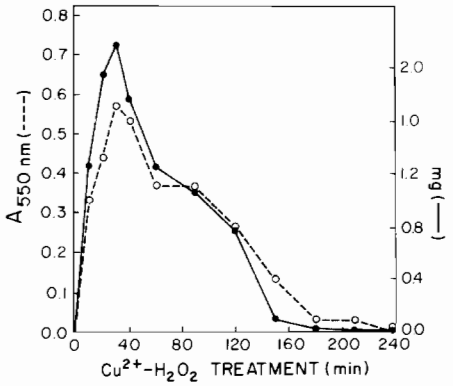

Fig. 2. Aggregation kinetics. The reaction mixtures were the same as described in Fig. 1. Samples were incubated for different periods of time. Immediately after the reaction was stopped, each sample was read at 550 nm, and the mixture was filtered through a 0.42 μ membrane filter which was then dried under an infrared lamp for 30 min, cooled to room temperature, and weighed.

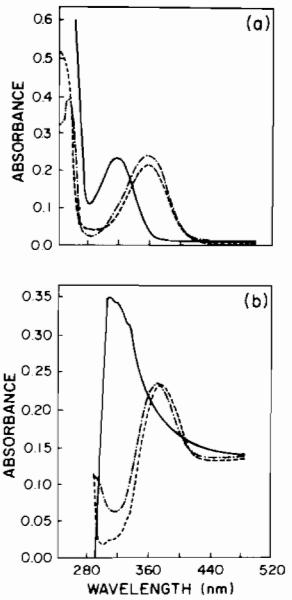

Fig. 3. Absorption spectra following acid hydrolysis of N-formylkynurenine. Mild acid hydrolysis (50 mM HCl) was carried out at −10 °C for 24 h as described in Methods. The absorption spectra were measured in a Cary 210 Spectrophotometer. (a) Spectra of authentic N-FK and kynurenine. – – – N-FK 30.3 μM in 16.7 mM HCl. –·–·– kynurenine 30.3 μM in 16.7 mM HCl. – – – HCl hydrolyzed N-FK 30.3 μM in 16 mM HCl. (b) Difference Spectra. Protein concentrations are 5 mg in 3 ml of 16.7 mM HCl. —— aggregated *vs.* native lysozyme. –·–·– HCl hydrolyzed aggregated *vs.* aggregated lysozyme. – – – HCl hydrolyzed N-FK *vs.* N-FK (concentrations: same as a.).

Following performic acid oxidation, α-aminoadipic acid was detected. This indicates the presence of α-aminoadipic acid δ-semialdehyde, a deamination product of lysine, in the polymerized protein.

Amino acid analysis revealed the presence of kynurenine residues in the insoluble fraction, N-formylkynurenine is often found as an oxidation product of tryptophan. Hydrolysis under mild conditions, in 50 mM HCl, converts all N-formylkynurenine to kynurenine (Fig. 3a). This transformation can be detected by changes in the ultraviolet absorption spectrum at 320 and 360 nm [11, 14–16] (Fig. 3). Spectroscopic scanning of the redissolved polymerized protein *versus* control lysozyme showed a broad peak between 305 and 330 nm. Hydrolysis with 50 mM HCl shifted the peak to 370 nm, close to that seen when N-formylkynurenine was hydrolyzed to kynurenine under similar conditions.

By use of the molar extinction coefficient 3.3×10^3 M^{-1} cm^{-1} for N-formylkynurenine and 3.9×10^3 M^{-1} cm^{-1} for kynurenine, it was possible to estimate their relative abundance. The polymerized protein contained 2.1 residues of N-formylkynurenine and 0.6 residues of kynurenine. This is reasonably close to the 2.1 residues of kynurenine found by amino acid analysis which should be the sum of both forms.

Fluorescence Studies

The native fluorescence of an unmodified protein has characteristic spectra contributed mainly by its tryptophan and tyrosine residues. Chio and Tappel [17] observed production of fluorescence in proteins following their reaction with malonaldehyde. The resulting conjugated Schiff bases had maximal fluorescence at 465 nm when excited at 400 nm. Fujimori [18] suggested that the fluorescence observed in calf lens α-crystallin was due to conjugated Schiff base formation resulting from reaction of N-formylkynurenine residues and ε-amino groups of lysine.

The fluorescence contributed by conjugated Schiff bases can be abolished by reduction with $NaBH_4$. When the fluorescent lysozyme polymer formed as a result of Cu(II)–H_2O_2 treatment was reacted with $NaBH_4$, 42% of the fluorescence was destroyed (Table II). The fluorescence due to N-formylkynurenine and kynurenine was also abolished by this treatment. Relative fluorescence is significantly influenced by the microenvironment within the protein structure [19], thus it is difficult to estimate quantitatively the participation of each of these factors to the overall fluorescence. The loss of only 42% of the fluorescence by this treatment suggests that more than half of the fluorescence is due to structures other than Schiff bases and kynurenine derivatives.

Succinylation of Lysozyme

The possible involvement of the ε-amino group of lysine in the polymerization process was tested by preliminary treatment of lysozyme with succinic

TABLE II. $NaBH_4$ Reduction.[a]

Sample	Control Fluor.	Reduced Fluor.	% of control
Aggregated lysozyme	4.4	2.6	58
N-FK	3.4	0.2	6
Kynurenine	4.6	0.0	0

[a]Aggregated lysozyme (1 mg) in 2.0 ml of 10 mM sodium phosphate, pH 9.5, was incubated with 1 mg $NaBH_4$ in 0.01 M NaOH (14 μl of 72 mg/ml). After 30 min of incubation at 37 °C, the volume was adjusted to 3 ml with water. Fluorescence in arbitrary units was measured in an Aminco Fluoro-Colorimeter at wavelength over 420 nm with maximum excitation at 360 nm. For N-FK and kynurenine, the sample concentration was adjusted to 0.266 mM. The final concentration of $NaBH_4$ was 19 mM.

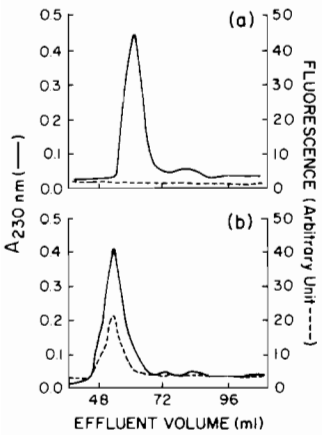

Fig. 4. Chromatography of succinylated lysozyme. The protein was treated with Cu(II)–H_2O_2 for (a) 0 min and (b) 30 min and then chromatographed on Sephadex G-50 as described in Fig. 1.

anhydride. When the succinylated lysozyme (suc-lys) was treated with Cu(II)–H_2O_2, polymerization still occurred (Fig. 4). However, the product was very different. The average molecular weight of the polymers was smaller (45 K *vs.* 73 K daltons), and they did not precipitate out of the solution as did those formed from native lysozyme. In addition, their fluorescence was about 30% less. The increased solubility may have resulted from the smaller size of the peptides as well as the increased negative charges contributed by the added succinyl groups.

Amino acid analysis showed that the succinylation process protected the lysine residues (Table I). However, in addition to tryptophan, arginine and histidine, a number of other amino acids which were previously resistant to destruction were now altered. These included serine, proline, valine, methionine and phenylalanine. Succinylation may have changed the protein conformation, exposing these amino acids to the oxidative process. It is also possible that the new copper-ligand characteristics of the derivatized protein influenced the type of destructive process.

Production of Carbonyl and Amino Groups during Cu(II)–H_2O_2 Treatment

During the oxidation process several amino acids were destroyed, some polypeptide bonds were cleaved, and there was formation of new carbonyl functional groups. The time course for the formation of carbonyl and amino groups during the incubation with either native lysozyme or suc-lys is shown in Figs. 5 and 6, respectively. In spite of the protection afforded the lysine groups by the succinylation procedure, suclys yielded more carbonyl groups than did native lysozyme at any time period. This suggests that they were formed from other amino acid residues [20, 21]. Neither N-formylkynurenine nor kynurenine could be the major source of these carbonyl groups since their contribution could only account for less than 10% of the observed value.

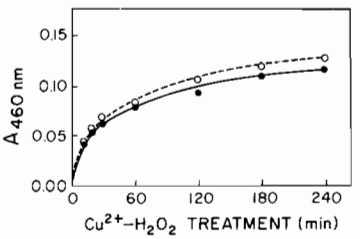

Fig. 5. Formation of carbonyl groups during Cu(II)–H_2O_2 treatment. Lysozyme was treated with Cu(II)–H_2O_2 as described in Fig. 1. The entire sample mixture was digested with 2% protease at 37 °C for 24 h. The solution equivalent to 0.2 mg of protein was treated with 0.1 ml of DNPH at room temperature for 30 min. Absorbance at 460 nm was measured after addition of 3 ml 0.5 M KOH. Closed circles represent native lysozyme and open circles represent succinylated lysozyme.

The TNBS reagent measures amino groups and not free ammonia. Thus the loss of amino groups seen during the first 30 min with native lysozyme probably resulted from the deamination of the ε-amino group of lysine (Fig. 6). The slight increase with time thereafter is probably due to the cleavage of peptide bonds. Since the ε-amino groups of lysine were protected in suc-lys, the steady increase in amino groups detected with time in the modified protein is probably a reflection of peptide bond cleavage. This could also be visualized by Sephacryl S300 chromatography (data not shown). The lack of detectable free amino groups at zero time indicates that the succinylation process was essentially complete.

The results reported here show that under appropriate conditions, H_2O_2 in the presence of Cu(II)

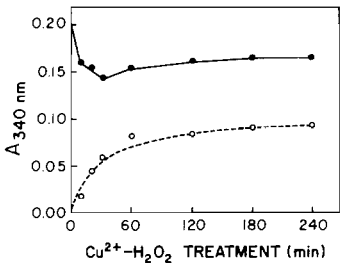

Fig. 6. Determination of free amino groups. Lysozyme was treated with Cu(II)–H_2O_2 as described in Fig. 1. An aliquot of the whole sample (0.2 ml) was diluted with water to make 1 ml. After addition of 1 ml 10% SDS, the mixture was incubated at 80 °C for 3 min. To the cooled sample, 1 ml 4% sodium carbonate, pH 9.0 and 1 ml 0.1% TNBS in water were added. The reaction mixture was incubated at 40 °C for 2 h. 0.5 ml HCl was then added and absorbance at 340 nm was measured. Closed circles represent native lysozyme and open circles represent succinylated lysozyme.

can produce remarkable alterations in the structure of lysozyme. Neither of these reactants alone can produce this effect so their combination has been suggested as a means for production of more potent oxidants such as OH radicals and $^1O_2$ [22–24]. By use of selective scavengers of these two species, their participation was excluded in the oxidation of lysozyme [8], NADH [2] and phospholipids [6]. Several investigators have postulated various Cu(II)–H_2O_2 complexes as the oxidizing agents for catechol [1], NADH [2], nucleotides [3], protein [4] and phospholipids [6]. Levitzki *et al.* [20] have suggested a mechanism involving Cu(III) in the cleavage of peptide bonds with concurrent generation of carbonyl groups. Alternatively, it is also possible that the selective binding of Cu(II) by various ligands on the target molecule may not allow scavenger molecules in solution to quench the site-specific production of OH radicals or other oxygen species [25, 26].

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