Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 596-603

INTERCHANGE REACTION OF DISULFIDES AND DENATURATION OF OXYTOCIN BY COPPER(II)/ASCORBIC ACID/O_2 SYSTEM

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Received April 24, 1987

SUMMARY: The interchange reaction of disulfides was caused by the copper(II)/ascorbic acid/O_2 system. The incubation of two symmetric disulfides, L-cystinyl-bis-L-phenylalanine (PP) and L-cystinyl-bis-L-tyrosine (TT), with L-ascorbic acid and $CuSO_4$ in potassium phosphate buffer (pH 7.2, 50 mM) resulted in the formation of an asymmetric disulfide, L-cystinyl-L-phenylalanine-L-tyrosine (PT), and the final ratio of PP:PT:TT was 1:2:1. As the reaction was inhibited by catalase and DMSO only at the initial time, hydroxyl radical generated by the copper(II)/ascorbic acid/O_2 system seemed to be responsible for the initiation of the reaction. Oxytocin and insulin were denatured by this system, and catalase and DMSO similarly inhibited these denaturations. As the composition of amino acids was unchanged after the reaction, hydroxyl radical was thought to cause the cleavage and/or interchange reaction of disulfides to denature the peptides.

Active oxygen species such as superoxide, hydrogen peroxide, singlet oxygen and hydroxyl radical cause damage to various biomolecules and participate in many biological phenomena.[1] Disulfides play important roles in living systems, e.g., in maintaining protein structure. We have reported that superoxide cleaves disulfides and initiates their interchange reaction in an aprotic solvent,[2,3] and that in an aqueous solution superoxide is not sufficiently strong as a nucleophile or as a reductant to cleave disulfides but hydroxyl radical is formed through the iron-catalyzed Haber-Weiss reaction[4] and cleaves disulfide.[3] We

Abreviations: SOD, superoxide dismutase; PP, L-cystinyl-bis-L-phenylalanine; TT, L-cystinyl-bis-L-tyrosine; PT, L-cystinyl-L-phenylalanine-L-tyrosine;

0006-291X/87 $1.50

also reported that the copper(II)/ascorbic acid/O_2 system generates hydroxyl radical,[5] which cleaves disulfide and denatures insulin.[6,7] The cleavage or interchange reaction of disulfides by active oxygen results in protein denaturation. This is believed to be an important mechanism of oxygen toxicity. The present paper deals with disulfide interchange reaction and oxytocin denaturation caused by the copper(II)/ascorbic acid/O_2 system.[7]

MATERIALS AND METHODS

Materials: L-Cystinyl-bis-L-phenylalanine (PP) and L-cystinyl-bis-L-tyrosine (TT) were obtained from Bachem. Catalase (beef liver), SOD (bovine erythrocyte), oxytocin (Grade X) and insulin were obtained from Sigma Chemical Co. Ltd. Copper(II) sulfate (99.6 %) was obtained from Kanto Chemical Co., Inc. L-Ascorbic acid and DMSO were obtained from Wako Pure Chemical Industries, Ltd.

Interchange reaction of disulfides: PP (0.5 mM) and TT (0.5 mM) were incubated with $CuSO_4$ and L-ascorbic acid in potassium phosphate buffer (pH 7.2, 30 mM) at 25°C. The reaction was initiated by the addition of L-ascorbic acid. The concentrations of $CuSO_4$ and L-ascorbic acid used are given in the table and figure footnotes. The disulfides were detected by HPLC using a Shim-pac WAX-1 column (4 mm x 50 mm) (Shimadzu). The column was eluted with potassium phosphate buffer (pH 7.2, 30 mM) at 1.0 ml/min. Absorbance (254 nm) was constantly monitored. The determination of the yields of PT was carried out by assuming the absorption coefficient to be equal to the average of those of PP and TT.

Denaturation of oxytocin: A solution of 0.93 mM oxytocin was incubated with $CuSO_4$ and L-ascorbic acid in potassium phosphate buffer (pH 7.2, 50 mM) at 37°C. Oxytocin was determined by HPLC on a Partisil-5 ODS column (4.5 mm x 150 mm) (Waters Associates). The column was eluted with a 30 min linear gradient of 12-48% CH_3CN/sodium phosphate buffer (pH 2.1, 0.1 M) or with a 22% CH_3CN/sodium phosphate buffer (pH 2.1, 0.1 M) at 1.5 ml/min. Absorbance (225 nm) was constantly monitored.

Amino acid analysis[8]: The reaction mixture or HPLC-purified sample, which was diluted to contain 10 μg of peptide in 40 μl, was put into a Pyrex tube and dried *in vacuo*. Then the precipitated peptide was hydrolyzed in 20 μl of constant-boiling HCl containing 4% thioglycolic acid for 20 hours at 110°C. The hydrolyzate was concentrated in vacuo, the residue was dissolved in 0.2 ml of 0.02% HCl, and this solution was subjected to amino acid analysis. Amino acids were separated on a 4 x 50 mm column packed with fine particles of strongly acidic cation exchanger, reacted with o-phthalaldehyde at 50-60°C, and detected by fluorometry. The eluate from the column was oxidized with NaOCl before the reaction with o-phthalaldehyde in this system.

RESULTS AND DISCUSSION

It was previously reported that disulfides are cleaved by hydroxyl radical generated by the copper(II)/ascorbic acid system.[4] Interchange reaction of disulfides by hydroxyl radical was investigated by use of the copper(II)/ascorbic acid system. The two symmetric disulfides, PP and TT, were incubated with L-ascorbic acid and $CuSO_4$ in potassium phosphate buffer (pH 7.2, 50 mM) and analyzed by HPLC. A new peak appeared between the peaks of PP and TT with a concomitant decrease of the two disulfides (Fig. 1). The product was isolated by HPLC; its retention time and UV spectra were consistent with those of the product obtained by base-induced interchange reaction of PP and TT. When the isolated product was incubated with aqueous alkali, formation of equimolecular quantities of PP and TT was observed by HPLC. Therefore the product was considered to be an asymmetric disulfide, L-cystinyl-L-phenylalanine-L-tyrosine (PT). When equimolecular amounts of PP and TT were incubated with the copper(II)/ascorbic acid system, the final ratio of PP:PT:TT was 1:2:1 (Fig. 2). This reaction was inhibited by catalase and by

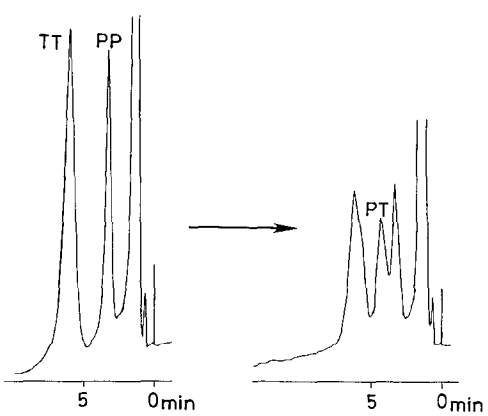

Fig. 1. HPLC charts of disulfide interchange reaction by the copper(II)/ascorbic acid system.
A solution of 0.5 mM PP and 0.5 mM TT was incubated with 0.5 mM $CuSO_4$ and 2 mM L-ascorbic acid in 30 mM potassium phosphate buffer (pH 7.2) at 25°C. The conditions of the HPLC analysis were as described in "MATERIALS AND METHODS".

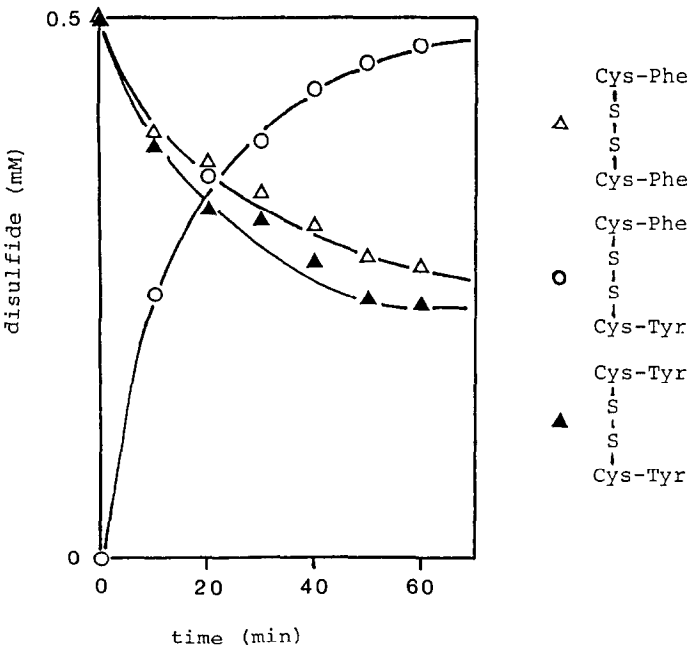

Fig. 2. The time course of the interchange reaction of disulfides by the copper(II)/ascorbic acid system. A solution of 0,5 mM PP and 0.5 mM TT was incubated with 0.5 mM $CuSO_4$ and 2 mM L-ascorbic acid in 30 mM potassium phosphate buffer (pH 7.2) at 25°C.

DMSO only at the initial time (Table I). Hydroxyl radical formed by the copper(II)/ascorbic acid system was considered to cleave the disulfides, and the resulting thiolate anions cause the interchange reaction (Scheme I). As catalase and DMSO can inhibit the initiation step but not the following chain reaction, slight cleavage of symmetric disulfides was supposed to initiate the

Table I. Effects of inhibitors on disulfide interchange reaction by the copper(II)/ascorbic acid system

Inhibitor	Decrease of PP (mM)	Decrease of TT (mM)	Yield of PT (mM)
none	0.132 (0 %)	0.114 (0 %)	0.158 (0 %)
Catalase 100 U/ml	0.060 (-55 %)	0.045 (-61 %)	0.113 (-29 %)
SOD 500 mM	0.055 (-58 %)	0.054 (-53 %)	0.105 (-34 %)

PP (0.5 mM), TT (0.5 mM), $CuSO_4$ (0.02 mM), L-ascorbic acid (0.6 mM), and sodium phosphate buffer (pH 7.2, 20 mM) were used. The reaction was carried out at 25°C for 12 min.

$$2Cu^{2+} + AH_2 \longrightarrow 2Cu^{+} + A + 2H^{+}$$

$$2Cu^{+} + O_2 + 2H^{+} \longrightarrow 2Cu^{2+} + H_2O_2$$

$$H_2O_2 + Cu^{+} \longrightarrow \cdot OH + OH^{-} + Cu^{2+}$$

$$RSSR + \cdot OH \longrightarrow RSOH + RS\cdot$$

$$2RS\cdot + AH_2 \longrightarrow 2RSH + A$$

$$RSOH + AH_2 \longrightarrow RSH + H_2O + A$$

$$RSH \rightleftharpoons RS^{-} + H^{+}$$

$$R'SSR' + RS^{-} \rightleftharpoons RSSR' + R'S^{-}$$

(AH_2: ascorbic acid)

(A: dehydroascorbic acid)

Scheme I

chain reaction and to result in the formation of a large amount of asymmetric disulfide.

Hydroxyl radical formed by the copper(II)/ascorbic acid can cleave disulfide bonds in proteins.[6] Thus, the denaturation of oxytocin by the copper(II)/ascorbic acid system was investigated. Oxytocin is a peptide which is composed of nine amino acid residues and one disulfide bond. Oxytocin was incubated with L-ascorbic acid and $CuSO_4$ in potassium phosphate buffer (pH 7.2). The disappearance of oxytocin and the concomitant appearance of a product peak were observed by HPLC, and a precipitate was formed. As this reaction was inhibited by catalase and by DMSO at the initial time (Table II), the participation of hydroxyl radical was suggested. For the purpose of identifying the reaction site of hydroxyl radical, the composition of amino acids was analyzed. The composition of amino acids was unchanged after the reaction (Table III) and cysteic acid was not detected. Therefore hydroxyl radical was considered to denature oxytocin by causing cleavage and/or interchange of the disulfide bond without modification of the amino acid residues.

We reported that insulin was denatured by hydroxyl radical generated by the copper(II)/ascorbic acid system.[6] The amino

Table II. Effects of inhibitors on oxytocin denaturation by the copper(II)/ascorbic acid system

Inhibitor		Denaturation (%)	Inhibition (%)
none		66.5	0
SOD	16.7 U/ml	67.3	-1
catalase	110 U/ml	60.1	10
DMSO	500 mM	61.5	8

Oxytocin (0.93 mM), L-ascorbic acid (20 mM), $CuSO_4$ (2mM) and inhibitor were dissolved in sodium phosphate buffer (pH 7.2, 50 mM) and the solution was incubated at 25°C for 30 sec.

acid composition of insulin was analyzed after treatment with the copper(II)/ascorbic acid system (Table IV). The results show that histidine[9] and phenylalanine were lost at high concentrations of $CuSO_4$ and L-ascorbic acid. However, at low concentrations, the denaturation of insulin was caused without the loss of amino acid residues. These results suggest that hydroxyl radical reacted with disulfide bonds of cystine and denatured insulin.

The cleavage and interchange reaction are caused by superoxide in an aprotic solvent and by hydroxyl radical in an aqueous solution. As hydroxyl radical is derived from superoxide through the Haber-Weiss reaction,[3,4] these results suggest that the generation of superoxide can cause, cleavage and/or interchange reaction of disulfides in both hydrophilic and

Table III. Amino acid composition of oxytocin and denatured oxytocin.

Run	Asp	Glu	Pro	Gly	Ile	Leu	Tyr	Oxytocin
1	1.1	1.0	0.8	1.0	1.0	1.0	1.0	1.0
2	1.1	0.9	0.9	1.0	0.8	1.0	0.8	0.0
3	1.2	1.0	1.0	1.0	0.8	0.9	0.8	0.0

Run 1: oxytocin.
Run 2: a crude reaction mixture containing precipitate. Oxytocin (0.93 mM), L-ascorbic acid (20 mM) and $CuSO_4$ (2 mM) were dissolved in sodium phosphate buffer (pH 7.2, 50 mM) and the solution was incubated at 37°C for 60 min.
Run 3: a water-soluble product purified by HPLC.

Table IV. Amino acid composition of insulin and denatured insulin.

Ascorbic acid	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile
0 mM	3.2	0.9	2.1	6.9	1.2	4.0	3.0	4.7	0.8
0.2 mM	3.2	0.9	2.1	7.0	1.2	4.0	3.0	4.6	0.8
0.5 mM	3.5	1.0	2.3	7.3	1.3	4.0	3.2	4.8	0.9
1.0 mM	3.3	1.0	2.2	7.1	1.2	4.0	3.1	4.7	0.8

Ascorbic acid	Leu	Tyr	Phe	His	Lys	Arg	Insulin
0 mM	5.8	3.6	2.8	1.7	1.0	1.0	1.00
0.2 mM	5.6	3.7	2.7	1.6	1.0	1.0	0.50
0.5 mM	5.9	3.7	2.6	1.4	1.0	1.0	0.19
1.0 mM	5.8	3.7	2.4	1.1	0.9	1.0	0.05

Insulin (0.2 mM), $CuSO_4$ (0.2 mM) and L-ascorbic acid were dissolved in potassium phosphate buffer (pH 7.8, 50 mM) and the solution was incubated at 37°C for 60 min.

hydrophobic regions of cells. Since disulfides play an important role in maintaining protein structure, the cleavage and the interchange reaction of disulfides by active oxygen species are considered to be one of the most significant mechanisms of oxygen toxicity.

ACKNOWLEDGMENT: The authors are grateful to Prof. Terumi Nakajima of University of Tokyo for amino acid analysis.

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