[Chem. Pharm. Bull. 34(3)1075—1079(1986)]

Disulfide Cleavage and Insulin Denaturation by Active Oxygen in the Copper(II)/Ascorbic Acid System

HIDESHI INOUE and MASAAKI HIROBE*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan

(Received August 20, 1985)

Cupric ion catalytically enhances the autoxidation of L-ascorbic acid. The cleavage of disulfide occurred in the copper(II)/ascorbic acid system and was inhibited by catalase and by scavengers of hydroxyl radical, so that the hydroxyl radical was considered to be formed and to cleave the disulfide. Insulin was denatured by this system, but in this case the inhibitors were less effective. It seems likely that cupric ion was bound to insulin and that locally generated hydroxyl radical caused the damage. Cytokinins, which are plant growth regulators and retard leaf senescence, effectively inhibited the above reactions in the copper(II)/ascorbic acid system.

Keywords——disulfide; superoxide; hydroxyl radical; copper; ascorbic acid; insulin; cytokinin

Superoxide and related active oxygen species, hydrogen peroxide, hydroxyl radical and singlet oxygen, play important roles in biological systems; they are known to participate in enzymatic reactions, lipid peroxidation, inflammation, carcinogenesis, diabetes, *etc.*[1] We have reported that the disulfide interchange reaction is initiated by superoxide in an aprotic solvent.[2] The first step of the reaction is the cleavage of disulfide by superoxide in an S_N2-like process.[3] In an aqueous solution, superoxide is not sufficiently strong as a nucleophile or as a reductant to cleave disulfide. However, disulfide cleavage occurs in the xanthine/xanthine oxidase system and is inhibited by superoxide dismutase (SOD), catalase, scavengers of hydroxyl radical and diethylenetriamine pentaacetic acid (DETAPAC); hydroxyl radical was suggested to be the ultimate reactant, formed by iron-catalyzed Haber–Weiss reaction.[3,4]

It is known that the autoxidation of ascorbic acid is catalytically enhanced by cupric ion, and in this process hydroxyl radical is thought to arise.[5,6] Disulfides play important roles in living systems, *e.g.*, in protein structure. The present paper therefore deals with disulfide cleavage and protein denaturation by the copper(II)/ascorbic acid system, and with the effects of natural chelators.

Experimental

Materials——Catalase (beef liver), SOD (bovine erythrocyte), bovine serum albumin (BSA) and insulin were obtained from Sigma Chemical Co., Ltd. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and 6-benzylaminopurine were obtained from Nakarai Chemicals Ltd. Copper(II) sulfate (99.6%) was obtained from Kanto Chemical Co., Inc. L-Ascorbic acid, EDTA and DMSO were obtained from Wako Pure Chemical Industries, Ltd. Sephadex G-25 was obtained from Pharmacia Fine Chemicals. Mannitol and thiourea were recrystallized from ethanol. Visible spectra were recorded on a Hitachi 557 double-beam spectrometer.

Cleavage of DTNB by Copper(II)/Ascorbic Acid System——DTNB (39.6 mg) and $NaHCO_3$ (15 mg) were dissolved in 4 ml of distilled water, then 5 ml of potassium phosphate buffer (0.1 M, pH 7.0) was added. The solution was adjusted to 10 ml with distilled water and was stocked below 0 °C until just before use. L-Ascorbic acid was dissolved in distilled water under an atmosphere of argon at 0 °C. The reaction mixture contained 0.1 mM DTNB, 0.02 mM $CuSO_4$ and 2 mM L-ascorbic acid in 50 mM potassium phosphate buffer (pH 7.8). The reaction was started by the addition of L-ascorbic acid solution, and the generation of 2-nitro-5-thiobenzoate anion (TNB^-) was detected at

412 nm ($\varepsilon = 13600\ M^{-1}\,cm^{-1}$). The inhibitors were added before the addition of L-ascorbic acid. The chelators were incubated with $CuSO_4$ for 5 min before the addition of L-ascorbic acid.

Cleavage of BSA–TNB by Copper(II)/Ascorbic Acid System——BSA (272 mg), DTNB (39.6 mg) and $NaHCO_3$ (15 mg) were dissolved in 2 ml of potassium phosphate buffer (50 mM, pH 7.0) under argon. The solution was incubated for 2 h at room temperature, then passed through a Sephadex G-25 column. The concentration of protein was determined by Bensadoun and Weinstein's method.[7] To determine the content of TNB, BSA–TNB was mixed with a large excess of 2-mercaptoethanol and the released TNB^- was detected at 412 nm. The cleavage of BSA–TNB by the copper(II)/ascorbic acid system was followed by the same method as used in the case of DTNB cleavage. The reaction mixtures contained 1.2 μM BSA–TNB, 20 μM $CuSO_4$ and 2 mM L-ascorbic acid in 50 mM potassium phosphate buffer (pH 7.8) at 25 °C.

Denaturation of Insulin by Copper(II)/Ascorbic Acid System——The reaction mixture containing 0.2 mM insulin, 2 mM L-ascorbic acid and 0.2 mM $CuSO_4$ in 50 mM potassium phosphate buffer (pH 7.8) was incubated at 37 °C for 60 min. Insulin was determined by high performance liquid chromatography (HPLC) using a Partisil-5 ODS column (4.5 × 150 mm) (Waters Associates). The column was eluted with a 50 min linear gradient of 0—60% acetonitrile/sodium phosphate buffer (0.1 M, pH 2.1) at 1.5 ml/min. Absorbance (225 nm) was constantly monitored. The retention time of insulin was 29 min.

Results and Discussion

The autoxidation of ascorbic acid is catalyzed by cupric ion.[5] During this process DTNB was cleaved, and the rate of TNB^- formation was proportional to the concentration of $CuSO_4$ (Fig. 1). This reaction was inhibited by catalase, scavengers of hydroxyl radical and EDTA. SOD and heat-denatured catalase did not inhibit the reaction (Table I). These results suggested that the disulfide was cleaved by hydroxyl radical formed during the process of ascorbic acid autoxidation catalyzed by cupric ion (Chart 1).

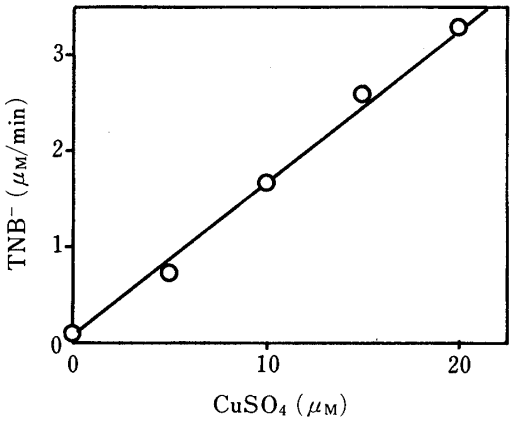

Fig. 1. Cleavage of DTNB by Ascorbic Acid in the Presence of Copper(II)

$$AH_2 + O_2 \xrightarrow{Cu^{2+}} A + H_2O_2$$
$$AH_2 + Cu^{2+} \longrightarrow AH\cdot + Cu^{+} + H^{+}$$
$$H_2O_2 + Cu^{+} \longrightarrow OH\cdot + OH^{-} + Cu^{2+}$$

(AH_2 = ascorbic acid, A = dehydroascorbic acid)

Chart 1

Table I. Effects of Inhibitors on DTNB Cleavage by the Copper(II)/Ascorbic Acid System

Addition		TNB^- (μM/min)	Inhibition (%)
No addition		3.14	0
SOD	1.0 U/ml	3.13	0
Catalase	61.3 U/ml	1.74	45
Catalase (Heat-denatured)	61.3 U/ml	3.14	0
DMSO	10 mM	0.24	92
Mannitol	10 mM	1.84	41
Thiourea	5 mM	0.38	88
EDTA	40 μM	0.04	99

TABLE II. Effects of Inhibitors on BSA–TNB Cleavage by the Copper(II)/Ascorbic Acid System

Addition		TNB^- (μM/min)	Inhibition (%)
No addition		43.7	0
SOD	1.7 U/ml	44.0	0
Catalase	30.7 U/ml	13.3	70
Catalase (Heat-denatured)	30.7 U/ml	44.0	0
DMSO	10 mM	17.8	59
Mannitol	9 mM	36.8	16
Thiourea	5 mM	5.1	88
EDTA	23 μM	0.6	99

TABLE III. Denaturation of Insulin by the Copper(II)/Ascorbic Acid System

L-Ascorbic acid (mM)	$CuSO_4$ (mM)	Time (min)	Peak height of insulin
0	0	0	100
0	0.1	60	100
2.0	0	60	94
2.0	0.02	60	74
2.0	0.1	60	26
2.0	0.2	60	4
10.0	1.0	60	3

TABLE IV. Effects of Inhibitors on the Denaturation of Insulin by the Copper(II)/Ascorbic Acid System

Inhibitors		Denaturation (%)	Inhibition (%)
None		96	0
SOD	8.3 U/ml (5 μg/ml)	91	5
Catalase	128.4 U/ml (3.7 μg/ml)	82	15
Denat. catalase	(5.3 μg/ml)	92	4
DMSO	500 mM	74	23

BSA–TNB, in which a sulfhydryl group of BSA was modified with Ellman's reagent (DTNB), was also cleaved by the copper(II)/ascorbic acid system and released TNB^-. This reaction was inhibited by catalase, scavengers of hydroxyl radical and EDTA, and was not inhibited by SOD or denatured catalase (Table II). Again, it appears that hydroxyl radical was involved in cleaving the disulfide in this protein.

Insulin has three disulfide bonds which are essential for the natural conformation, and it was denatured by the copper(II)/ascorbic acid system in a $CuSO_4$ concentration-dependent manner. The intact insulin (determined by HPLC) was no longer detectable when the concentration of $CuSO_4$ was equal to that of insulin (Table III). This reaction was also inhibited by catalase and scavengers of hydroxyl radical, but higher concentrations of inhibitors were required than in the case of DTNB cleavage (Table IV). These results

Table V. Concentrations of Chelators for 50% Inhibition of DTNB Cleavage by the Copper(II)/Ascorbic Acid System

Chelators	μM	Chelators	μM
Guanine	6.8	L-Glutamine	>80
Kinetin	8.4	L-Phenylalanine	
Adenine	8.7	L-Aspartic acid	
6-Benzylaminopurine	8.8	L-Lysine	
DETAPAC	12.0	Adenosine	—
EDTA	12.2	ADP	—
L-Histidine	16.0	Cytosine	—
L-Tryptophan	71.5	Salicylic acid	—

Table VI. Effects of 6-Benzylaminopurine on the Denaturation of Insulin by the Copper(II)/Ascorbic Acid System

$CuSO_4$ (mM)	6-Benzylaminopurine (mM)	Denaturation (%)	Inhibition (%)
0.2	0	96	0
0.2	0.2	37	60
0.1	0	74	0
0.1	0.1	30	60
0.1	0.2	0	100

suggested that the cupric ion was bound to the insulin, and that locally generated hydroxyl radical reacted with the protein. It appears likely that the hydroxyl radical causes the denaturation of protein by the cleavage of disulfide,[8] but further investigations are required to identify the reaction sites.

Cleavage of DTNB by the copper(II)/ascorbic acid system was effectively inhibited by chelating agents, especially adenine, guanine[9] and cytokinins (kinetin and 6-benzylaminopurine) (Table V). Adenosine, ADP, cytosine and the amino acids were less effective inhibitors. The denaturation of insulin by the copper(II)/ascorbic acid system was effectively inhibited by 6-benzylaminopurine (Table VI). Since a decrease of oxygen consumption was observed by using an oxygen electrode, 6-benzylaminopurine seemed to inhibit the catalytic participation of cupric ion in the oxidation of ascorbic acid.[10]

Superoxide is highly reactive with disulfide in an aprotic solvent, and causes rapid cleavage or interchange reaction.[2,3] Thus, superoxide generated in a hydrophobic site may be similarly reactive. In an aqueous solution, superoxide does not react with disulfides, but hydroxyl radical, which is considered to be derived from superoxide in living systems,[4] cleaves them.[3] Thus, the generation of superoxide can lead to disulfide cleavage in both hydrophilic and hydrophobic sites.

Hydroxyl radical is also generated by the cupric ion-catalyzed autoxidation of ascorbic acid.[6,11] The cleavage of DTNB by the copper(II)/ascorbic acid system was effectively inhibited by chelating agents, especially adenine, guanine and cytokinins (kinetin and 6-benzylaminopurine) (Table V), and the denaturation of insulin by the copper(II)/ascorbic acid system was effectively inhibited by 6-benzylaminopurine (Table VI). Ascorbic acid is a ubiquitous substance present in rather large amounts in both animal and plant tissues. Since chloroplast stroma is abundant in ascorbic acid, it is particularly interesting that the cytokinins, which retard leaf senescence in plants, inhibited the generation of hydroxyl radical and the denaturation of protein during the autoxidation of ascorbic acid catalyzed by cupric ion.

References and Notes

1) A. M. Michelson, J. M. McCord and I. Fridovich (ed.), "Superoxide and Superoxide Dismutase," Academic Press, New York, 1977; L. W. Oberley (ed.), "Superoxide Dismutase," Vol. I and II, CRC Press, Florida, 1982; E. W. Kellogg III and I. Fridovich, *J. Biol. Chem.*, **250**, 8812 (1975); J. M. McCord, *Science*, **185**, 529 (1974); Y. Oyanagi, *Biochem. Pharmacol.*, **25**, 1465 (1976); A. M. Michelson and M. F. Buckingham, *Biochem. Biophys. Res. Commun.*, **58**, 1079 (1974); F. Hirata and O. Hayaishi, *J. Biol. Chem.*, **250**, 5960 (1975); G. Cohen, "Free Radicals in Molecular Biology, Aging, and Disease," ed. by D. Armstrong, R. S. Sohal, R. G. Cutler and T. F. Slater, Raven Press, New York, 1984, pp. 307—316.
2) T. Nagano, K. Arakane and M. Hirobe, *Tetrahedron Lett.*, **21**, 5021 (1980).
3) H. Inoue, T. Nagano and M. Hirobe, *Tetrahedron Lett.*, **25**, 317 (1984).
4) B. Halliwell, *FEBS Lett.*, **92**, 321 (1978).
5) V. S. Butt and M. Hallaway, *Arch. Biochem. Biophys.*, **92**, 24 (1961).
6) J. M. C. Gutteridge and S. Wilkins, *FEBS Lett.*, **137**, 327 (1982).
7) A. Bensadoun and D. Weinstein, *Anal. Biochem.*, **70**, 241 (1976).
8) S. Ishimitsu, S. Fujimoto and A. Ohara, *Chem. Pharm. Bull.*, **26**, 3289 (1978).
9) E. Frieden and J. Alles, *J. Biol. Chem.*, **230**, 797 (1958).
10) The rate of oxygen consumption was reduced to one-half by 8 μM 6-benzylaminopurine. Conditions: 2 mM L-ascorbic acid; 0.02 mM $CuSO_4$; 50 mM potassium phosphate buffer; 25 °C.
11) D. A. Rowley and B. Halliwell, *Arch. Biochem. Biophys.*, **225**, 279 (1983).