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Glycation¹⁾ of Erythrocyte Superoxide Dismutase Reduces Its Activity

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Commercially available Cu, Zn superoxide dismutase (SOD) from bovine erythrocytes was purified. Purified SOD was incubated with 1 M glucose at 37 °C for 14 d under sterile conditions. Nonezymatic addition of glucose to SOD molecules increased linearly until 7 d, and then increased only slightly. The enzyme activity decreased to 88% after 7 d and 60% after 14 d. The glycated amino acid residue is not the N-terminal α -amino group but the ϵ -amino group of lysine. It seems that lysine at the active center, which assists the interaction of O₂⁻ and the SOD molecule, is affected during 14 d.

Keywords—glycation; nonenzymatic glycosylation; superoxide dismutase; lysine residue; diabetes

Interest in radical scavenger-enzymes, especially superoxide dismutase, has increased markedly in recent years in relation to aging and pathogenesis.²⁾ Cu, Zn superoxide dismutase (SOD) in erythrocytes, cooperating with catalase and glutathione peroxidase, protects the plasma membrane from oxygen toxicity due to active oxygen species such as O₂⁻, H₂O₂ and $\cdot$ OH. Increase in SOD activity in erythrocytes is observed in sickle cell anemia³⁾ and muscular dystrophy⁴⁾ and decrease is observed in diabetic children⁵⁾ and diabetic rats.^{6,7)}

On the other hand, it has been reported that, in diabetic patients⁸⁻¹¹⁾ or in experimental diabetes,^{12,13)} the amount of glucose adducts of various proteins increases as well as hemoglobin A_{1c}.¹⁴⁾ Glycation of proteins causes changes in their functions; a decrease in ligand binding capacity of human serum albumin,¹⁵⁾ an increase in oxygen affinity of hemoglobin,¹⁴⁾ and a reduction of the susceptibility of plasmin.¹⁶⁾ In enzyme proteins, glycation resulted in a decrease in the activities of pancreatic ribonuclease¹⁷⁾ and kidney β -N-acetyl-glucosaminidase.¹⁸⁾ Glycation has been extensively studied in relation to the pathogenesis of diabetic complications.¹⁹⁾ In this paper, in order to study the possible cause of the lowering of erythrocyte SOD activity in diabetes, we investigated the *in vitro* glycation of SOD.

Materials and Methods

Materials—SOD (92F-9340, 2800 U/mg) from bovine blood, cytochrome c (Type III) from horse heart, polylysine hydrobromide (55000 dalton) were obtained from Sigma Chemical Co. The content of reduced form of cytochrome c was estimated by the addition of sodium dithionite.²⁰⁾ Xanthine and xanthine oxidase (20 U/ml) from cow's milk were purchased from Wako Pure Chem. Ind., Ltd., and Boehringer Mannheim, respectively. Matrex gel (PBA-10) was obtained from Amicon Corp.

Purification and Assay of Superoxide Dismutase—SOD (50 mg) was applied to a diethyl aminoethyl (DEAE)-cellulose column (Whatman DE-52, 1.2 $\times$ 32 cm) equilibrated with 2.5 mM potassium phosphate buffer (pH 7.8) and was eluted with a linear gradient of buffer (200 ml) from 2.5 to 200 mM.²¹⁾ Three separate peaks observed at 280 nm, with area ratios of 0.33 (peak I), 0.31 (peak II) and 0.36 (peak III), corresponded to the buffer concentrations of 21.8, 27.8 and 36.7 mM, respectively. Peak III was dialyzed against distilled water followed by lyophilization and was used

as purified SOD. The purified SOD showed a broad single band on disc polyacrylamide gel electrophoresis.²²⁾ The SOD activity after glycation was assayed by the cytochrome c method using 50 mM sodium carbonate buffer (pH 10.2) containing 1×10^{-4} M ethylenediaminetetraacetic acid (EDTA).²³⁾

Copper Content—About 10 μ g of enzyme protein was added to a standard solution of copper and the copper content was analyzed by flameless atomic absorption spectroscopy (Shimadzu AA-646 atomic absorption spectrophotometer).

Glycation *in Vitro*—Purified SOD was dissolved to 0.5% (w/v) in 67 mM sodium phosphate buffer (pH 7.4) containing 1 M glucose. This solution was filtered through a Millipore filter (Millex-GS, 0.22 μ m) and poured into dry-heat sterilized tubes followed by incubation for 14 d at 37 °C. After adequate dialysis, aliquots were frozen at -20 °C until assayed. The same procedure without glucose was carried out as a control. The amount of glucose added to SOD was estimated by the thiobarbituric acid method²⁴⁾ by using ampules for acid hydrolysis. Protein concentration was assayed by the Lowry method.

Poly-lysine (0.1% w/v) dissolved in 67 mM phosphate buffer (pH 7.4) containing 150 mM glucose was incubated at 37 °C for 5 d, followed by dialysis and lyophilization. The resulting powder was referred to glucose adduct of poly-lysine.

Amino Acid Analysis—The SOD solution incubated for 7 d with 1 M glucose was dialyzed and loaded on a phenylboronate affinity column (Matrex gel, PBA-10).¹⁵⁾ Glycated SOD eluted by 25 mM sodium phosphate buffer (pH 8.5) containing 100 mM sorbitol was dialyzed and lyophilized. The lyophilized protein (8 mg) was dissolved in 5 ml of 35 mM sodium phosphate buffer (pH 7.8) and reduced for 4 h at room temperature by the addition of 9 mg of sodium borohydride. The reaction was terminated by the addition of 1 M HCl, followed by dialysis and lyophilization. The reduced protein (2 mg) was hydrolyzed in 2 ml of distilled 6 M HCl containing phenol (0.1%) at 110 °C for 24 h. Reduction and acid hydrolysis of commercial SOD, purified SOD and poly-lysine for amino acid analysis were carried out by the same procedure. Amino acid analysis was carried out with a Hitachi 835 high-performance amino acid analyzer.

Preparation of Apoenzyme—Apoprotein fraction which had lost 95% of the theoretical copper content of SOD was obtained by overnight dialysis of purified SOD solution against 80 mM acetic acid/20 mM sodium acetate (pH 3.8) buffer.²²⁾ The sample was adequately dialyzed against distilled water and was used for measurement of circular dichroism (CD) spectra.

CD Spectra—Molar CD $\Delta\epsilon$ is defined as $\Delta\epsilon = [\theta]/3300$ where $[\theta]$ is the molar ellipticity. Spectra were measured in the wavelength range from 200 to 350 nm using a JASCO 500C spectropolarimeter. Protein concentrations ranged from 7 to 0.5 mg/ml. Cuvettes of 1 and 0.1 cm light path length were used to obtain the maximum sensitivity at any wavelength.

Results

Peak III, which was eluted from the ion exchange column at the buffer concentration of 36.7 mM (see Materials and Methods), showed an absorption spectrum having fine structure from 250 to 270 nm, which is characteristic of SOD of bovine erythrocytes,²¹⁾ and only this fraction showed the absorption of copper at 680 nm. In peak III, the excitation maximum of fluorescence spectrum was at 275 nm (305 nm emission), and the emission maximum was at 305 nm (275 nm excitation). Only peak III showed SOD activity by the cytochrome c method. The result of amino acid analysis of this purified SOD is given in Table I with the theoretical values.²⁵⁾ Though purified SOD showed lower contents of valine, isoleucine and histidine residues than the theoretical values, the contents of other amino acid residues agreed fairly well with the theoretical values.

The CD spectrum of purified SOD is shown in Fig. 1. The ellipticity band of purified SOD coincided in position with that given for the holoenzyme in the literature²⁶⁾ but the molar CD $\Delta\epsilon$ of purified SOD at 260 nm was about 55% of that of the holoenzyme. A striking difference of CD spectrum in the ultraviolet (UV) region between apo- and holoenzyme has been reported,²⁶⁾ that is, the positive ellipticity at 255–265 nm is destroyed in the apoenzyme. As the presence of apoenzyme without copper was suggested, the copper content of purified SOD was assayed. It was estimated to be $61.9 \pm 9.1\%$ ($n=9$) of the theoretical value. These results indicate the presence of 38.1% of apoenzyme in the purified SOD.

Figure 2 summarizes the result obtained after incubation of purified SOD with 1M glucose for 14 d. The amount of glucose added to SOD molecules during incubation, which

TABLE I. Amino Acid Composition of Superoxide Dismutase

Amino acid residue	Theoretical value ^{a)}	Commercial SOD	Purified SOD	Glycated SOD ^{c)}
Asp, Asn ^{b)}	22, 12	26.45	33.10	32.52
Thr	24	16.02	23.00	22.35
Ser	16	11.36	15.59	15.55
Glu, Gln ^{b)}	16, 6	19.82	22.65	23.09
Gly	50	31.55	48.06	46.32
Ala	18	14.88	18.36	19.26
Cys	6	— ^{b)}	—	—
Val	30	19.67	23.32	21.71
Met	2	1.96	1.64	1.79
Ile	18	10.21	13.19	12.69
Leu	16	16.00	16.00	16.00
Tyr	2	0.54	1.75	2.07
Phe	8	7.96	7.90	10.90
Lys	20	15.44	18.75	15.26
His	16	9.97	13.50	13.20
Arg	8	7.07	7.74	7.84
Pro	12	9.46	10.86	10.49

a) The data of Steinman *et al.*²⁵⁾ b) Present but not quantitated. c) This was obtained from the adsorbed fraction on the phenylboronate affinity column.

Fig. 1. Circular Dichroism Spectra of (1) Apoenzyme, (2) Purified SOD and (3) Holoenzyme

Solutions of apoenzyme (7.03 mg/ml) and purified SOD (1.87 mg/ml) were measured in a cuvette of 1 cm light path length. The spectrum of the holoenzyme is taken from the literature.²⁶⁾

Fig. 2. Addition of Glucose to Purified SOD and Its Activity

Addition of glucose to SOD incubated with (—○—) and without (—●—) 1 M glucose was estimated by measuring the absorbance at 443 nm after reaction with thiobarbituric acid (0.2—1.0 mg protein was used). SOD activity during incubation with (—△—) and without (—▲—) 1 M glucose was estimated by the cytochrome c method at pH 10.2.

was estimated by measuring absorbance at 443 nm in the thiobarbituric acid method, increased linearly until 7 d, and after that increased only slightly. The enzyme activity decreased to about 88% after 7 d and 60% after 14 d. On the other hand, incubation without glucose caused no alteration in the enzyme activity.

To separate glycated SOD from nonglycated SOD, purified SOD incubated for 7 d with 1 M glucose was applied to a phenylboronate affinity column. The amino acid analysis of

Fig. 3. Elution Patterns of (1) Purified SOD, (2) Glycated SOD and (3) Glucose Adduct of Poly-lysine from the Amino Acid Analysis Column

HL and HL' are hexitol-lysine and its acid rearrangement product, respectively, and U is an unknown product. An arrow indicates a new peak in glycated SOD.

Fig. 4. Circular Dichroism Spectra of Purified SOD Incubated with and without 1 M Glucose

Solutions of SOD (0.5 mg/ml) were measured in a cuvette with 0.1 cm light path length. —□—, control (not incubated, without glucose); —△—, incubated with glucose for 7 d; —○—, incubated with glucose for 14 d; —●—, incubated without glucose for 14 d.

glycated SOD is also listed in Table I. Comparison of purified SOD with glycated SOD clarified the glycated amino acid residues. The numbers of glycine, valine and lysine decreased (1.7, 1.6, and 3.5 respectively), but on the other hand, the number of phenylalanine increased (3.0). The other amino acid residues remained unchanged.

The elution profiles of the amino acid analysis column are shown in Fig. 3. In glycated SOD a new peak appeared between phenylalanine and lysine. Elbe *et al.*¹⁷⁾ reported that the new peak can be used for quantitating of glycated lysine residues, that is, the content of fructose lysine. To confirm the correspondence of this new peak to the acid rearrangement product of ϵ -(1-deoxyhexitolyl)-lysine observed by Elbe *et al.*, the acid hydrolysate after reduction of the glucose adduct of poly-lysine was applied to the amino acid analysis column. One peak (HL) appeared at the same position as that of phenylalanine and the other (HL') was eluted between phenylalanine and lysine. The area of HL' was 36% of the sum of HL and HL', agreeing with Elbe's result. This indicates that the yield of HL' is reproducible. So, the number of fructose lysine in glycated SOD estimated to be 3.6, by using the area of the new peak between phenylalanine and lysine, and the color factor of alanine. This value agreed well with the decrease in the number of lysine residues, 3.5 (Table I). The increased number (3.0) of phenylalanine in glycated SOD (Table I) clearly reflects the elution of HL at the same position.

Figure 4 shows the CD spectra from 200 to 240 nm of incubated SOD with or without 1 M glucose and control (no incubated) SOD. The control SOD has negative ellipticity with the maximum at 210 nm, indicating typical β -sheet structure.²⁷⁾ The molar ellipticity at 210 nm of the SOD incubated with glucose for 7 d gave almost the same value as that of the control SOD. On the other hand, in the case of the SOD incubated with glucose for 14 d, a slight

decrease of negative ellipticity at 210 nm was observed, though SOD incubated without glucose showed no significant change.

Discussion

The purity of SOD was confirmed by observations of a characteristic absorption spectrum, tyrosine fluorescence and amino acid analysis. Bovine erythrocytes SOD contains two tyrosine residues, but no tryptophan residue.²⁵⁾ When even a trace of tryptophan coexists with tyrosine, a characteristic fluorescence of tyrosine at 305 nm is hidden by a strong fluorescence at 340 nm of tryptophan. Though the contents of the amino acid residues partially disagreed with the theoretical values (Table I), we considered that there was almost no contamination of other proteins, especially carbonic anhydrase B, which is a usual contaminant and contains 8 tryptophan residues. The SOD molecule consists of two identical subunits and each unit contains one copper atom at the catalytic site. As this purified SOD contained 38.1% apoprotein (Fig. 1), copper and zinc were added to the purified SOD. However, the enzyme activity was not increased. Thus, we used this purified SOD for our *in vitro* glycation experiments.

The glycation of a protein proceeds by bimolecular condensation of glucose with amino groups such as N-terminal and ϵ -amino of lysine residues in the protein. This reaction is apparent first order with respect to the concentration of glucose,²⁸⁾ since the Schiff-base formation is fast and the subsequent Amadori rearrangement is slow. The extent of glycation either *in vivo* or *in vitro* is dependent upon the number of potentially reactive amino groups on the protein, the concentration of glucose in the medium and the life time of the protein. It seems that SOD in erythrocytes can not escape continuous modification by glucose during the lifespan of red cells (120 d). In this experiment, a high concentration of glucose (1 M, which is 40 times higher than that of about 25 mM in diabetic blood plasma) was chosen to shorten the incubation period to about one-tenth of the lifespan.

SOD has net negative charge at physiological pH, since the pI value is 4.95.²⁹⁾ The interaction of O₂⁻ with this anionic enzyme was shown to be assisted by the positive charge on lysine residues which provides a long-range electrostatic guidance for O₂⁻.³⁰⁾ Lysine 134 has an important role in directing O₂⁻ to the highly positive catalytic binding site at the bottom of the β -barrel.³¹⁾ To clarify the glycosylated amino acid residues, amino acid analysis was carried out after separation of glycosylated SOD by phenylboronate affinity column. In glycosylated SOD, 3.5 lysine residues were lost and 3.6 fructose lysine were formed (confirmed by a new peak on the amino acid analysis column). The fact that there was no decrease in alanine residues in glycosylated SOD indicates the absence of glycation at the α -amino group of N-terminal alanine. The main glycosylated amino acid residue is lysine. The reason for the decrease in glycine (1.7) and valine (1.6) is not clear.

The fact that incubation for 14 d caused a decrease to 60% in SOD activity, though only a little more glucose was added to SOD incubated for 7 d, suggests that it resulted from secondary changes induced by glycation of some lysine residues. In general, the CD signals at 200–240 nm originate from conformations sensitive to secondary structures of proteins.³²⁾ The decrease of negative ellipticity at 210 nm after 14 d incubation with glucose (Fig. 4) suggests a decrease in β -structure. Wood *et al.*²⁶⁾ showed that the addition of 8 M urea to the holoenzyme caused only a small decrease in negative ellipticity at 210 nm, though in the case of the apoenzyme it caused a larger decrease, showing a CD spectrum similar to that of random coil structure. This means that copper and zinc contributed to stabilizing the final structure and even the β -barrel through the complex network of hydrogen bonds. This network may be perturbed by the glycation of lysine residues. We found nondisulfide cross-linking of human serum albumin in *in vitro* glycation.³³⁾ The secondary structural change

caused by glycation of lysine residues during 14d incubation may involve intra- or intermolecular cross-linking. We propose the post-translational modification of SOD by glucose as one possible cause of the lowering in erythrocytes SOD activity observed in diabetes.⁵⁻⁷⁾

According to the topological model of SOD proposed by Richardson *et al.*,³⁴⁾ five lysine residues, 3, 9, 23, 89, and 151 are oriented to the outside of the barrel. It seems that these residues may be protonated, since they are in contact with the bulk solution. As it is considered that a protonated lysine residue is difficult to be glycated, the other five lysine residues, 67, 68, 73, 120, and 134, located in the two large loops, may be more easily glycated. As lysines 120 and 134 are only 12 Å and 13 Å from the copper at the active center,³⁰⁾ the easily glycated lysine residues may be those at positions 67, 68, and 73.

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