Inactivation of Cu,Zn-Superoxide Dismutase by Intermediates of Maillard Reaction and Glycolytic Pathway and Some Sugars

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Human Cu,Zn-superoxide dismutase (SOD) was incubated with various intermediates of the Maillard reaction and glycolytic pathway (arabinose, glyoxal, glycolaldehyde, glyceraldehyde, glyceraldehyde 3-phosphate, and dihydroxyacetone) and some reducing sugars (sorbose, xylose, and ribose). The change of the activity and the molecular weight were measured and compared with that of SOD incubated with glucose or fructose. Sorbose, xylose, and ribose decreased the activity with a rate comparable to fructose. Site-specific and random fragmentation were observed upon the incubation with them. Arabinose showed a similar inactivation rate as glucose. The intermediates other than arabinose had a high inactivation rate. Especially, glyceraldehyde, glyceraldehyde, and glyoxal most strongly lowered the activity in a concentration-dependent manner and a significant inactivation was recognized even at 1 mM level. SDS-PAGE band patterns indicated that the inactivation by those carbonyl compounds occurred by both crosslinking and site-specific fragmentation of SOD.

Key words: superoxide dismutase; glycation; Maillard reaction; inactivation; glycolytic pathway

Superoxide dismutases (SODs) are ubiquitous metalloproteins that catalyze the dismutation of superoxide anion into hydrogen peroxide and molecular oxygen. It is generally accepted that SODs play a major protective role in living cells and have been widely used as pharmacological tools in the study of pathophysiological mechanisms.

Taniguchi's group indicated that human Cu,Zn-SOD undergoes glycation (Maillard reaction) at specific lysine residues and that the enzyme is inactivated by glycation in vitro as well as in vivo. The level of glycated Cu,Zn-SOD is increased in the erythrocytes of patients with diabetes mellitus, as well as patients with Werner's syndrome, an age-accelerated disease.

Although an elevated level of glucose has been thought to play a primary role via the Maillard reaction in increased glycation and crosslinking in diabetic tissues, the non-enzymatic glycation is also known to result from the action of various metabolites and the intermediates of the Maillard reaction other than glucose. Especially, glyoxal and glycolaldehyde have been recently reported to contribute to N\textsuperscript{\textalpha}-carboxymethyllysine (CML) formation, one of the advanced glycosylation end products (AGE), and protein crosslinking under physiological conditions. Baynes' group suggested that glyoxal and arabinose are intermediates in the oxidative glycosylation and crosslinking of protein by glucose. Moreover, multiple intermediates in the glycolytic and polyol pathways were capable of nonenzymatically modifying proteins and, among them, dihydroxyacetone phosphate, glyceraldehyde, and glyceraldehyde 3-phosphate were highly reactive agents that in the micromolar range of concentrations formed more AGEs much faster than 20 mM glucose. Although it was reported that fructose resulted in rapid and marked fragmentation of Cu,Zn-SOD among sugars other than glucose, no information on the interaction of Cu,Zn-SOD with other sugars and the intermediates of Maillard reaction and glycolytic pathway has been available.

The purpose of this study is to clarify whether those intermediates are potent enough to cause a significant inactivation of Cu,Zn-SOD compared with glucose and fructose. We incubated Cu,Zn-SOD with various concentrations of intermediates of the Maillard reaction and glycolytic pathway, d-arabinose, glyoxal, glycolaldehyde, glyceraldehyde, glyceraldehyde 3-phosphate, and dihydroxyacetone, and observed the change of the activity and the molecular weight. For comparison, the interactions with some sugars (l-sorbose, d-xylose, and d-ribose) were also examined.

Materials and Methods

Recombinant human Cu,Zn-SOD was a kind gift from Ube Industries, Japan. The amino acid sequence of the recombinant enzyme is the same as that of the human erythrocyte enzyme except that the N terminus Alpha is not acetylated. D-Glucose, d-fructose, and d-arabinose were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). L-Sorbose, d-xylose, and d-ribose were obtained from Wako Pure Chemical industries (Osaka, Japan). Dihydroxyacetone, glycolaldehyde (as dimer), d,l-glyceraldehyde and d,l-glyceraldehyde 3-phosphate were from Sigma Chemical Co. (St. Louis, U.S.A.). Glyoxal (40% aqueous solution) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). The concentration of glycolaldehyde was checked by Girard-T assay. All other chemicals were of the highest grade available and were used without further purification. All solutions were prepared with water purified by a Milli-Q system (Millipore, Tokyo, Japan).

Assay of Cu,Zn-SOD. Cu,Zn-SOD activity was assayed using the nitrite method. The absorbance at 550 nm was recorded with a Pharmacia Biotech Ultraspec 3000 spectrophotometer. The activity of each sample was indicated as an average of triplicate measurements.

Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) and measurement of polymer structures. SDS-PAGE was done as described after the sample was reduced with 5% mercaptoethanol. Fifteen percent slab gels (90 x 55 mm) were used. The gels were stained with 0.025% Coomassie Brilliant Blue R-250. The ratio of polymer was calculated from the staining intensity as judged by densitometric scanning with an imaging densitometer Model GS-700 (Bio-Rad, California, U.S.A.).

Incubation of Cu,Zn-SOD with various carbonyl compounds. The stan-
Standard reaction mixture contained 50 mM potassium/sodium phosphate buffer, pH 7.4, 150 mM NaCl, 0.025% NaN₃, 100 mM carboxyl compounds, and 2 mg/ml Cu,Zn-SOD. After incubation at 37°C for the indicated duration, the reaction was stopped by freezing the mixture. Each sample was stored at -25°C until use.

Results
In this investigation, recombinant human Cu,Zn-SOD was chosen because the specific residue at which the SOD undergoes glycation was elucidated⁴ and the results obtained in the experiment can be expected to reflect physiological effects of glycation in the human body best among SODs available from various sources judged from the fact that the SOD has the same amino acid sequence as SOD occurring in human tissues, except for the N-terminus.¹²

Incubation of Cu,Zn-SOD with d-glucose (100 mM) for 9 days lowered the activity to about 70%, while no decrease in the activity was observed when Cu,Zn-SOD was alone incubated in the buffer (Fig. 1). Incubation with d-fructose (100 mM) rapidly inactivated Cu,Zn-SOD compared with d-glucose and the inactivation rate was about 10 times that of d-glucose judged from the slope of those inactivation curves. As pointed out by Oohara et al.,¹² site-specific (the formation of 15 kDa-band) and random fragmentation of Cu,Zn-SOD was recognized in both reaction mixtures depending on the decrease in the activity (Fig. 2).

Figure 1 also shows the effects of other reducing sugars on the activity of Cu,Zn-SOD. L-Sorbose, which is a ketose similar to d-fructose, caused to decrease in the activity with a rate comparable to d-fructose. Aldoses such as d-xylose and d-ribose also inactivated Cu,Zn-SOD. The rate was much bigger than that of d-glucose and comparable to d-fructose. In either incubation with these three sugars, a distinct 15 kDa band appeared (Fig. 2), followed by that the fragment was largely replaced by nonspecific degradative products. This result indicates that the site-specific and random fragmentations of Cu,Zn-SOD, which were observed upon incubation with d-glucose and d-fructose, occurred in the incubation with these sugars as well. d-Arabinose had a similar inactivation rate to d-glucose (Fig. 1). With the decrease of the activity, the site-specific and random fragmentation was recognized in a similar manner to that with d-glucose (Fig. 2).

Next we examined the effects of dihydroxyacetone, glyceraldehyde, glycolaldehyde, and glyoxal on the activity of Cu,Zn-SOD. In all of those carboxyl compounds, the activity of Cu,Zn-SOD decreased with incubation time. The inactivation rate of dihydroxyacetone was almost equivalent to that of d-ribose (Fig. 1). The other three compounds showed a much bigger inactivation rate than that of dihydroxyacetone. Figure 3 indicates the dependence of the concentration of those carboxyl compounds on the inactivation of Cu,Zn-SOD. As can be seen, a concentration-dependent manner was recognized in all of them. From a comparison with 10 mM glyceraldehyde, glyceraldehyde 3-phosphate possibly seemed to have a similar inactivation rate to that of glyceraldehyde (Fig. 3A). Although, among them, glyoxal could inactivate Cu,Zn-SOD most rapidly, the residual activity after a long incubation was a minimum in the incubation with glycolaldehyde. Glycolaldehyde significantly inactivated Cu,Zn-SOD even at a lower concentration than 1 mM (Fig. 3B).

SDS-PAGE of Cu,Zn-SOD incubated with 100 mM glyceraldehyde, glycolaldehyde and glyoxal for 2 days showed a pattern differing from that incubated with reducing sugars (lane 1 in Figs. 4A, B, and C). The three discrete bands with molecular masses of about 40 kDa appeared along with the indiscernible band of a larger molecular mass than them. A band of a slightly larger molecular mass than the original one (20 kDa) was also observed. The bands of a larger molecular mass than 40 kDa corresponded to the polymer structures such as dimer, trimer, tetramer, and so on. In the native form, Cu,Zn-SOD is known to be present as

![Figure 1](image1.jpg)  
**Fig. 1.** Inactivation of Cu,Zn-SOD during Incubation with Some Reducing Sugars and Carboxyl Compound.

![Figure 2](image2.jpg)  
**Fig. 2.** Fragmentation of Cu,Zn-SOD after Incubation with 100 mM d-glucose (1), d-fructose (2), d-xylose (3), d-ribose (4), L-sorbose (5), and d-arabinose (6) and in the Absence of Sugars (7).

![Figure 3](image3.jpg)  
**Fig. 3.** Dependence of the Concentration of Glyceraldehyde (A), Glycerolaldehyde (B), and Glyoxal (C) on the Activity of Cu,Zn-SOD.
a dimer composed of two identical subunits. In SDS-PAGE, the dimer of native Cu,Zn-SOD dissociates into the monomer and thus only the monomer subunit appears. The appearance of the bands corresponding to polymer structures larger than dimer means that these carbonyl compounds could crosslink between molecules or subunits of Cu,Zn-SOD. With a decrease in the concentration of those compounds, the ratio of the polymer formation decreased and the band with molecular mass of 15 kDa appeared at a lower concentration than 2 mM. The appearance of the polymers in Cu,Zn-SOD incubated with glycolaldehyde was assessed by densitometric scanning of staining intensity on SDS-PAGE (Fig. 5). The figure represents percent contents and is based on a measure of weight. Therefore, assuming that the staining intensity is equal per peptide mass, at equimolar amounts the staining of a large fragment would be more intense. As clearly in the figure, the decrease of the relative activity was related to the increase of the polymer content.

**Discussion**

Kato’s group investigated the reaction of lysozyme with \( \alpha \)-dicarbonyl compounds such as diacetyl, glyoxal and methylglyoxal and the reaction of lysozyme, ovalbumin, ribonuclease A, and serum albumin with some reducing sugars. By the changes of amino acids composition and the SDS–PAGE band patterns, it was indicated that the \( \alpha \)-dicarbonyl compounds and reducing sugars tested could polymerize some proteins. It was noted, in particular, that 3-deoxyglucosone, which is an intermediate of the Maillard reaction, was found to be important as a cross-linker in the polymerization reaction of some proteins with the reducing sugars. On the other hand, Cu,Zn-SOD is known to undergo not the polymerization reaction but the specific fragmentation upon reaction with the reducing sugars. This fragmentation possibly results from the formation of superoxide anion derived from the Amadori product generated in the course of the Maillard reaction followed by the formation of hydrogen peroxide produced from it by the catalytic reaction of Cu,Zn-SOD. Furthermore, the presence of Cu ion in the molecule of Cu,Zn-SOD easily produces hydroxyl radical from hydrogen peroxide. Based on the catalytic reaction and the presence of the metal ion, therefore, Cu,Zn-SOD can be considered to be characteristically sensitive against the Maillard reaction. Of interest was how Cu,Zn-SOD having such a property behaves in the reaction with the reactive carbonyl compounds of a low molecular weight.

We found that glycolaldehyde, glyoxal, glyceraldehyde, and glyceraldehyde 3-phosphate, intermediates of the Maillard reaction and glycolytic pathway, inactivate Cu,Zn-SOD much more strongly than glucose and fructose. Among them, glyoxal inactivated Cu,Zn-SOD almost completely and glycolaldehyde lowered the activity to the lowest level in a comparison at the same concentration of each compound. Arabinose formed as an intermediate in the antioxidative glycosylation also caused an inactivation of Cu,Zn-SOD. However, the extent was low and the rate was almost equivalent to that of glucose.

Taniyagchi et al. indicated that Cu,Zn-SOD was inactivated by glycation at specific lysine residues, Lys and Lys, and that glycation of Cu,Zn-SOD initially brings about site-specific cleavage of the molecule between Pro and His followed by random fragmentation. As the glycation reaction is initiated by electrophilic attack of the carbonyl group of glucose against amino group of the protein molecule, reducing sugars with a higher proportion of carbonyl forms are assumed to have a higher reactivity with Cu,Zn-SOD and cause to inactivate it more quickly. It was unexpected, however, that the inactivation rate of arabinose was almost equivalent to that of glucose in spite of the observation that arabinose has a ten-fold or more proportion of carbonyl forms than glucose. This reason has been unclear. However, this result may suggest...
that the proportion of carbonyl group is much lower than the estimated one under these experimental conditions.

Although the concentration of glyceraldehyde and glyceraldehyde 3-phosphate in human tissues varies greatly among individuals and investigations, they appear to range in concentration from a few μmol/liter to less than 100 μmol/liter.20,21 Judged from the concentration dependence of those compounds on Cu,Zn-SOD inactivation, it is difficult to consider that they may mainly involve in the inactivation of Cu,Zn-SOD at the physiological level. Glyceraldehyde and glyoxal showed a much higher inactivation rate than glyceraldehyde. Glyceraldehyde significantly inactivated Cu,Zn-SOD even at lower concentrations than 1 mm. Judged from the inactivation rate and the residual activity, glyoxal and glycolaldehyde can be expected to affect the activity of Cu,Zn-SOD not only in vitro but also in vivo. Unfortunately, the concentration of glycolaldehyde and glyoxal in human tissues are not available and thus it is difficult to estimate the extent of their contributions to inactivation of Cu,Zn-SOD in human tissues. It is unlikely, in addition, to assume that both compounds are present as the free form in human tissues because of their high reactivity. In order to evaluate how extent those compounds are responsible for inactivation of Cu,Zn-SOD, therefore, we need to estimate their amounts formed for a given time in human tissues under physiological circumstances.

The SDS-PAGE band patterns clearly indicate that Cu,Zn-SOD incubated with glyceraldehyde, glycolaldehyde, and glyoxal was inactivated by a different mechanism from that incubated with reducing sugars. Cu,Zn-SOD incubated with those carbonyl compounds of lower concentrations than 2 mm yielded a large fragment (15 kDa), suggesting that the site-specific fragmentation occurred in the same manner as that incubated with reducing sugars. In contrast, at a higher concentration, more polymer formation was observed than the site-specific fragmentation and the ratio of the polymer formation was closely related to the inactivation (Fig. 5). The formation of Cu,Zn-SOD polymer was possibly caused by the ability of these compounds to polymerize amino compounds, i.e., to crosslink between amino groups.22–24 Therefore, it is possible that both the crosslinking and the site-specific fragmentation may be involved in the inactivation of Cu,Zn-SOD. The SDS-PAGE band patterns of Cu,Zn-SOD incubated with 100 mm glyceraldehyde, glycolaldehyde, and glyoxal showed characteristic three bands near 40 kDa and a band of a slightly larger molecular mass than 20 kDa. Understanding the formation mechanism of those bands might contribute to the analysis of the inactivation mechanism by those compounds. Therefore, the structural analysis, such as amino acid compositions and N-terminal sequence, of those bands is in progress.

In conclusion, Cu,Zn-SOD was inactivated by the intermediates of Maillard reaction and glycolytic pathway, glyoxal, glycolaldehyde, glyceraldehyde, glyceraldehyde 3-phosphate, and dihydroxyacetone, much more rapidly than by glucose. Especially, glycolaldehyde, glycolaldehyde, and glyoxal lowered the activity most and a significant inactivation was recognized even at 1 mm level of each compound. The inactivation with those compounds may be caused by both crosslinking and site-specific fragmentation of the Cu,Zn-SOD molecule.

References