Essentiality of the active-site arginine residue for the normal catalytic activity of Cu,Zn superoxide dismutase

Charles L. BORDERS, Jr.,*†, Jan E. SAUNDERS,* Dianne M. BLECH* and Irwin FRIDOVICH†

*Department of Chemistry, College of Wooster, Wooster, OH 44691, U.S.A., and

†Department of Biochemistry, Duke University Medical Center, Durham, NC 27710, U.S.A.

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Chemical modification of bovine and yeast Cu,Zn superoxide dismutases with phenylglyoxal diminishes the catalytic activities by $\geq 98\%$, and treatment of these enzymes with butanedione plus borate leads to $\geq 96\%$ inactivation. The activity loss is accompanied by the modification of less than two arginine residues per subunit with no concomitant loss of Cu or Zn. The phenylglyoxal-modified enzymes require at least a 20-fold greater concentration of cyanide for 50% inhibition than do the corresponding native enzymes. Polyacrylamide-gel electrophoresis and activity staining of the phenylglyoxal-inactivated enzymes demonstrate that the residual activity is largely associated with modified forms that bear lower net positive charge than the native superoxide dismutases.

The structure, function and evolution of the superoxide dismutases (SODs) has been examined in satisfying detail (Steinman, 1981). X-ray crystallographic studies have provided a view of the active-site region of the bovine enzyme at 0.2 nm (2 Å) resolution (Tainer *et al.*, 1982, 1983; Getzoff *et al.*, 1983). An arginine residue is situated in the active-site cleft of this enzyme within 0.5 nm (5 Å) of the Cu(II). Since this particular arginine residue is conserved in the sequences of all of the Cu,Zn-SODs examined to date (Bannister & Parker, 1985) and since chemical modification of this arginine residue causes loss of activity (Malinowski & Fridovich, 1979; Borders & Johansen, 1980*a,b*; Bermingham-McDonogh *et al.*, 1982), it seems clear that this residue is important for the catalytic action of Cu,Zn-SOD.

However, there has been some disagreement concerning just how important this active-site arginine residue is. Thus Malinowski & Fridovich (1979), working with the bovine enzyme, noted inactivation by phenylglyoxal or butanedione in borate up to a limit of 80–90%, concomitant with

modification of Arg-141. Furthermore, when the butanedione/borate-inactivated enzyme was dialysed, there was a restoration of full activity even when the dialysis was in the presence of EDTA, establishing that reversible modification of Arg-141 was not accompanied by labilization of the Cu(II). Cu,Zn-SODs from wheat germ, chicken liver and several mammalian sources were all comparably sensitive to inactivation by arginine-modifying reagents (Malinowski & Fridovich, 1979), as were the enzymes from teleost fishes and from *Photobacterium leiognathi* (Martin & Fridovich, 1981). Mota de Freitas & Valentine (1984) also noted 10–20% residual activity in the phenylglyoxal-modified bovine Cu,Zn-SOD and further reported that the modified enzyme was less susceptible to inhibition by phosphate than was the native enzyme. This indicated that the residual activity was due to the modified enzyme and not simply due to residual unmodified Cu,Zn-SOD. Borders & Johansen (1980*a,b*), working with the yeast enzyme, also noted that modification of the active-site arginine residue (Arg-143) by phenylglyoxal caused inactivation, but they noted a retention of $< 2\%$ of the original activity and concluded that this arginine residue was essential for normal catalytic activity.

There is a substantial quantitative difference between 10–20% residual activity and 2% residual activity. The former suggests a facilitative role for

Abbreviations used: SOD, superoxide dismutase; Cu,Zn-SOD, copper- and zinc-containing superoxide dismutase.

† On research leave from the College of Wooster during 1984–1985 when most of this work was carried out.

the active-site arginine residue, whereas the latter suggests an obligatory role for this residue. Moreover, there is also the question of a qualitative difference. Is the activity seen after modification of the arginine residue due to the modified enzyme or to a residuum of native enzyme that escaped modification? We now report data that resolve these questions.

Experimental

Materials

Bovine Cu,Zn-SOD was prepared by the method of McCord & Fridovich (1969) or was obtained from Diagnostic Data, Mountain View, CA, U.S.A.; the *Saccharomyces cerevisiae* enzyme was a product of Pharmacia, Uppsala, Sweden. Butane-2,3-dione and phenylglyoxal monohydrate were from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and organic buffer salts were from the Sigma Chemical Co., St. Louis, MO, U.S.A. Coomassie Brilliant Blue G-250 was purchased from Eastman Kodak Co., Rochester, NY, U.S.A.

Determination of enzyme activity

SOD activity was measured by the pyrogallol autoxidation assay (Marklund & Marklund, 1974) as previously described (Borders & Johansen, 1980a; Blech & Borders, 1983).

Inactivation of enzyme by butanedione

Cu,Zn-SOD (0.31 mM) was incubated with 25 mM-butanedione in 50 mM-sodium borate buffer, pH 8.3, at 25°C. At intervals samples were removed and diluted into ice-cold 50 mM-borate buffer, pH 8.3, for assay. The reaction of butanedione with arginine residues in borate buffer is readily reversible (Riordan, 1973; Borders & Riordan, 1975). It was therefore necessary to assay these samples immediately after dilution to observe maximal inactivation. Borate could not be incorporated into the Tris/cacodylate/diethylenetriaminepenta-acetate buffer in which pyrogallol autoxidation was measured because it inhibited pyrogallol autoxidation, probably by forming an adduct with vicinal phenolic hydroxy groups of pyrogallol. For this reason, secondary dilution of the inactivated enzyme into the assay mixture was followed by progressive re-activation of the SOD during the 2–3 min of each assay. Data reported below reflect initial rates in order to minimize the error introduced by this re-activation.

Inactivation of enzyme by phenylglyoxal

Cu,Zn-SOD (31 μ M) was incubated with 20 mM-phenylglyoxal in 50 mM-Bicine/100 mM-NaHCO₃ buffer, pH 8.3, at 25°C. At intervals samples were removed for assay of residual activity. No detect-

able reversal of the inactivation caused by phenylglyoxal was seen. In one experiment, bovine SOD was analysed for amino acid and for metal content after incubation with 25 mM-phenylglyoxal at 25°C for 6 h in the Bicine/bicarbonate buffer. The enzyme was separated from the reagents by gel-exclusion chromatography on a 0.9 cm $\times$ 23 cm column of Sephadex G-25 equilibrated and eluted with 25 mM-NaCl/5 mM-sodium phosphate buffer, pH 7.0, that had been freed of trace metals as described by Thiers (1957). Amino acid analyses were performed on a Durrum D-500 amino acid analyser after hydrolysis in 6.0 M-HCl/1% (v/v) thioglycolic acid for 20 h at 114°C. Metal content was measured with a Perkin-Elmer 2280 atomic-absorption spectrometer.

Cyanide-sensitivity of enzyme and electrophoresis

Cu,Zn-SOD (78 μ M) was incubated with 20 mM-phenylglyoxal in the Bicine/bicarbonate buffer at pH 8.3 at 25°C and then freed of reagents by gel-exclusion chromatography on Sephadex G-25 in 25 mM-sodium phosphate, pH 6.9. Native and phenylglyoxal-inactivated SODs so prepared were compared with respect to cyanide-sensitivity and electrophoretic mobility. Electrophoresis was performed on 7.5% polyacrylamide gel as described by Davis (1964), with the modification that the stacking gel was omitted and the enzyme was applied to the running gel in 20% (v/v) glycerol. Gels were stained for protein with 0.05% Coomassie Blue in 12% (w/v) trichloroacetic acid or for SOD activity (Beauchamp & Fridovich, 1971). Sensitivity towards cyanide was measured as described by Beauchamp & Fridovich (1973) except that the pyrogallol autoxidation assay was used to determine SOD activity. Controls for the slight effect of cyanide, as such, on the rate of pyrogallol autoxidation were performed and used to correct for this effect.

Results

Inactivation of Cu,Zn-SOD by butanedione

Treatment of bovine SOD with 25 mM-butanedione in 50 mM-borate buffer, pH 8.3, caused a rapid inactivation, such that after 25 min only 3% of the control activity remained (Fig. 1, ● symbols). There was no further loss of activity during 24 h of modification. Although this 97% inactivation is extensive, it may not represent the maximum inactivation attainable, owing to the ready reversibility of the butanedione/borate reaction with arginine residues (Riordan, 1973). The inactivation involves a dynamic equilibrium between the arginine residue, butanedione and borate. If the concentration of any one of these reactants is decreased (i.e. by dilution), the

Fig. 1. Inactivation of bovine Cu,Zn-SOD by butanedione

The enzyme (0.31 μ M) was incubated at 25°C with 25 mM-butanedione in 50 mM-borate buffer, pH 8.3. At the indicated times, samples were diluted into 50 mM-borate buffer, pH 8.3, and assayed immediately for SOD activity (●). The activities in every instance were determined from the initial rate of pyrogallol autoxidation in the presence of SOD, as described in the text. The samples labelled 1, 2, 3 and 4 were diluted 201-, 61-, 28.5- and 13-fold respectively. After the immediate assay these diluted samples were kept at 25°C overnight and then assayed again (○) for SOD activity. Data point 5 (○) is described in the text. It should be noted that, beginning with the 40 min sample, no dilution was made before the activity determination, but instead a small portion was added directly to the assay. The native SOD retained full activity throughout the time course of this experiment.

equilibrium is perturbed and re-adjusts itself. When SOD was diluted to different extents into 50 mM-borate buffer during the course of modification and assayed immediately and again after incubation overnight, the activity changed with time (Fig. 1, ○ symbols). When a sample was diluted 201-fold into 50 mM-borate after 3 min and after 440 min of modification, the initial relative activities of 53% and 3% respectively were changed to 37% and 35% after incubation at 25°C overnight (Fig. 1, data points 1 and 5).

A 200-fold dilution of the modified bovine SOD into 50 mM-Tris buffer, pH 8.3, and subsequent incubation at 25°C for 24 h gave complete re-activation, confirming that the 97% inactivation is due to modification of the arginine residue and not to the loss of the Cu(II) ion under these conditions. Treatment of yeast SOD with butanedione under the conditions of Fig. 1 gave 96% inactivation after 30 min of modification, and the activity remained at this level for 24 h. Dilution of the modified yeast enzyme into Tris buffer and incubation overnight again led to complete re-activation.

Fig. 2. Inactivation of bovine Cu,Zn-SOD by phenylglyoxal

The enzyme (31 μ M) was incubated at 25°C with 10 mM- (■) or 20 mM-phenylglyoxal (●) in 50 mM-Bicine/100 mM-NaHCO₃ buffer, pH 8.3. Samples were withdrawn and diluted at the indicated times, and assayed immediately for SOD activity. The native enzyme retained full activity during the time course of this experiment.

Inactivation of bovine Cu,Zn-SOD by phenylglyoxal

When bovine SOD was treated with phenylglyoxal in 50 mM-Bicine/100 mM-NaHCO₃ buffer, pH 8.3, it suffered progressive inactivation, which not only followed pseudo-first-order kinetics for nearly four half-lives, but continued after the point of deviation from linearity (Fig. 2). Treatment of the bovine enzyme with 25 mM-phenylglyoxal for 6 h under these conditions caused a loss of 96% of the catalytic activity and a decrease of 1.89 arginine residues per subunit, but had no effect on the native complement of 1.0 Cu atom and 1.0 Zn atom per subunit (results not shown).

Effect of inactivation of Cu,Zn-SOD by phenylglyoxal on sensitivity to cyanide

If the activity seen after extensive treatment with phenylglyoxal represents residual native enzyme, rather than the activity of a phenylglyoxal-modified enzyme, then its sensitivity to CN⁻ should be identical with that of the native enzyme. Bovine and yeast SODs were inactivated to different degrees by exposure to phenylglyoxal, and the sensitivity of residual SOD activity

Fig. 3. Polyacrylamide-gel electrophoresis of native and phenylglyoxal-inactivated bovine (a) and yeast (b) Cu,Zn-SODs

The odd-numbered gels were stained for protein with Coomassie Blue, and the others were stained for SOD activity by the method of Beauchamp & Fridovich (1971). The inactivated SODs were prepared by treating the appropriate enzyme (78 μM) with 20 mM-phenylglyoxal in 50 mM-Bicine/100 mM- NaHCO_3 buffer, pH 8.3, at 25°C for an appropriate time, stopping the reaction by gel filtration as described in the text, and assaying for specific SOD activity. The native enzymes were subjected to the same conditions in the absence of phenylglyoxal. Only running gels were used in each instance, and proteins were layered on the gels in 20% glycerol. (a) For the bovine SOD samples, the following were applied (percentage activity, μg of protein, total pyrogallol units of activity for the gels subjected to activity stains): 1 (100, 29), 2 (100, 1.4, 2.0), 3 (23, 39), 4 (23, 7.5, 2.5), 5 (1.7, 41) and 6 (1.7,

towards CN^- was explored. As shown in Table 1, modification of these enzymes to approx. 45% and approx. 15% residual activity caused only a minor increase in the amount of CN^- required for 50% inhibition. However, modified SODs with <2% residual activity had a markedly decreased sensitivity towards CN^- . In fact, titration of the residual <2% activity with cyanide revealed a biphasic response for both the bovine and yeast SODs, in which 10–20% of the activity was as responsive to CN^- as was the native enzyme, whereas the remainder was very much less sensitive. The values given in Table 1 are the concentrations of cyanide needed to inhibit by 50% the total residual activity. For this reason, these results underestimate the difference in sensitivity between the native and the phenylglyoxal-modified enzymes.

Electrophoretic behaviour of phenylglyoxal-inactivated SODs

Highly purified bovine and yeast Cu,Zn-SODs give two major bands and one minor band on polyacrylamide-gel electrophoresis under non-denaturing conditions. All bands have a high SOD activity, as shown in Figs. 3(a) and 3(b), gels 1 and 2. When bovine SOD was 77% inactivated by phenylglyoxal, its gel pattern showed the occurrence of three new bands, two major and one minor, which migrated to the anode more rapidly than the corresponding bands of the native enzyme (Fig. 3a, gels 3 and 4). A likely cause of this phenomenon is that upon phenylglyoxal treatment a pool of modified enzymes is generated in which the net positive charge per SOD dimer decreases by 1, 2 or 3 units. It is significant that all six bands showed clear SOD activity (Fig. 3a, gel 4). Inactivation to 1.7% of the native activity caused a total loss of the two major bands in native SOD and an increased population of more rapidly migrating forms (Fig. 3a, gel 5). It is pertinent that all bands still had SOD activity (Fig. 3a, gel 6), although it should be noted that a 23-fold greater amount of protein was applied to gel 6 than to gel 2 to compensate for the lower specific activity of the phenylglyoxal-modified enzyme. It is also seen that the slowest protein band, which is most likely to have any SOD with an unmodified active site, had the greatest specific activity, when one compares the activity bands to the protein-stained bands. Yeast SOD showed patterns similar to the bovine enzyme, but here it is much more obvious that the slowest-migrating bands had a disproportionately

33, 0.8). (b) For the yeast SOD samples (same units): 1 (100, 40), 2 (100, 1.9, 2.3), 3 (12, 43), 4 (12, 12.7, 1.7), 5 (0.8, 44) and 6 (0.8, 44, 0.4).

Table 1. Sensitivities of native and phenylglyoxal-inactivated yeast and bovine Cu,Zn-SODs to inhibition by cyanide

SOD (78 μM) was allowed to react with 20mM-phenylglyoxal in 50mM-Bicine/100mM- NaHCO_3 buffer, pH 8.3, at 25°C for the samples modified to intermediate activities and at 37°C for the maximally inactivated samples, and the reaction was terminated for each modified sample by gel filtration (after 25 min and 75 min for the samples of intermediate activities and after 4 h for the maximally inactivated samples). The controls were subjected to the same procedures, but in the absence of phenylglyoxal. The specific activity of each sample was determined by the pyrogallol autoxidation assay (Marklund & Marklund, 1974), and the amount of NaCN required to cause 50% inhibition of the residual activity was determined by the method of Beauchamp & Fridovich (1973), with the pyrogallol assay.

Cu,Zn-SOD	Activity (%)	$K_{50\%}$ for NaCN (μM)
Bovine	100	27
	46	25
	13	32
	1.7	> 600
Yeast	100	6.5
	43	7.0
	17	7.4
	0.8	> 130

high percentage of the residual SOD activity (Fig. 3b, gels 2, 4 and 6).

Discussion

The data reported in the present paper reconcile reports from different laboratories on the activity of arginine-modified Cu,Zn-SOD. Thus modification with α -dicarbonyl compounds caused a loss of approx. 98% of the SOD activity, in agreement with the results reported by Borders & Johansen (1980a,b). The remaining activity is truly a reflection of the modified enzyme, rather than being due to residual native enzyme, in agreement with Malinowski & Fridovich (1979), Martin & Fridovich (1981) and Mota de Freitas & Valentine (1984).

The phenylglyoxal-modified enzyme was at least 20-fold more resistant to the inhibitory effect of CN^- than was the native enzyme. This may indicate that the active-site arginine residue facilitates the binding of CN^- to the active-site Cu(II) ion, much as it does the approach of O_2^- to that site. Arginine has often been identified as the positively charged residue providing for the binding of anions to functional sites of enzymes and other proteins (Riordan *et al.*, 1977; Riordan, 1979; Bjerrum *et al.*, 1983), and such a role, vis-à-vis O_2^- , has been proposed for the arginine residue

at the active site of Cu,Zn-SOD (Malinowski & Fridovich, 1979; Borders & Johansen, 1980a,b; Tainer *et al.*, 1983; Osman & Basch, 1984). Polyacrylamide-gel electrophoresis of phenylglyoxal-inactivated Cu,Zn-SOD indicates that the modification causes a loss of positive charge concomitant with a massive decrease in specific activity (Fig. 3), thus supporting the proposed role of the active-site arginine residue (see above). To our knowledge this is the first direct demonstration that chemical modification of an active-site arginine residue causes a loss of positive charge.

Given the size of phenylglyoxal and the dimensions of the solvent-access channel to the active site (Tainer *et al.*, 1983), one must also consider steric effects. This can only be definitely ruled out by elimination of the charge on the essential arginine residue without affixing bulky substituents, perhaps by site-directed mutagenesis (Zoller & Smith, 1983). There may be reason for discounting the importance of steric effects. Thus the inhibition by CN^- depends upon the association constant for the CN^- -Cu(II) interaction, which is the ratio of the on rate to the off rate. Purely steric hindrances might be expected to affect the on and off rates to the same degree and should thus not affect the association constant. Elimination of an electrostatic attraction could, in contrast, greatly decrease the association constant for the interaction of oppositely charged species. We conclude that the positive charge of the arginine residue at the active site of Cu,Zn-SOD provides greater than a 50-fold acceleration of the catalytic process.

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