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Subunit Association and Side-Chain Reactivities of Bovine Erythrocyte Superoxide Dismutase in Denaturing Solvents†

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ABSTRACT: The copper- and zinc-containing superoxide dismutase of bovine erythrocytes retains its native molecular weight of 32 000 in 8.0 M urea for at least 72 h at 25 °C, as evidenced by sedimentation equilibrium analysis. Subsequent to prolonged exposure to urea, the dimeric enzyme could be dissociated by sodium dodecyl sulfate in the absence of reductants, indicating the absence of unnatural disulfide cross-links. The sulfhydryl group of cysteine-6 was unreactive toward 5,5′-dithiobis(2-nitrobenzoic acid) or bromoacetic acid in both neutral buffer and 8.0 M urea. The histidine residues of the enzyme were resistant to carboxymethylation in neutral buffer and 8.0 M urea. However, when the enzyme was exposed to bromoacetic acid in the presence of 6.0 M guanidinium chloride and 1 mM (ethylenedinitrilo)tetraacetic acid (EDTA), both sulfhydryl and histidine alkylation were observed. Guanidinium chloride (6.0 M) increased the reactivity of the sulfhydryl group of cysteine-6 and allowed the oxidative formation of disulfide-bridged dimers. This was prevented by 1 mM EDTA. It follows that 8.0 M urea neither dissociates the native enzyme into subunits nor produces a conformation detectably different than that possessed under native conditions.

The copper- and zinc-containing superoxide dismutase from bovine erythrocytes (BESOD)[1] is a homodimer of molecular weight 32 000 (Evans et al., 1974; Steinman et al., 1974; Abernethy et al., 1974). X-ray diffraction analysis has allowed elucidation of the structure and identification of the ligands at the active site of this enzyme (Richardson et al., 1975a,b). The copper is held within the active site through the imidazole rings of histidine-44, -46, -61, and -118, arranged in a distorted square-planar configuration. The copper participates in the catalytic process through a Cu(II)/Cu(I) redox cycle (Klug et al., 1972; Klug-Roth et al., 1973; Rotilio et al., 1972). The zinc, which is 6 Å away from the copper, is liganded by histidine-61, -67, and -78 and by aspartate-81 in a tetrahedral arrangement. The zinc appears to serve a secondary structural role (Forman & Fridovich, 1973; Lippard et al., 1977).

The enzyme is unusually stable and is unchanged by an isolation procedure which utilizes organic solvents (McCord & Fridovich, 1969). Its catalytic activity is unaffected by 8.0 M urea or by 4% sodium dodecyl sulfate ($NaDodSO_4$) and

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[1] Abbreviations used: EDTA, (ethylenedinitrilo)tetraacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BESOD, bovine erythrocyte superoxide dismutase; $NaDodSO_4$, sodium dodecyl sulfate.

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is only gradually denatured by 6.0 M guanidinium chloride plus EDTA (Forman & Fridovich, 1973; Abernethy et al., 1974). The suggestion that the enzyme exhibited half-of-the-sites reactivity (Fielden et al., 1974), which was subsequently withdrawn (Cockle & Bray, 1977), generated great interest in subunit interactions. This led to several studies on the effects of urea on the activity and subunit structure of several copper- and zinc-containing superoxide dismutases. Marmocchi et al. (1978) reported that 8.0 M urea promoted a dissociation of BESOD into active subunits, while Bannister et al. (1978) reported similar results with the comparable enzyme from swordfish liver. The method employed in these studies was gel exclusion chromatography. Due to the previously mentioned studies concerning the resistance of BESOD to denaturing stress, the published reports on dissociation were intriguing and the phenomenon was reexamined by using a different experimental technique. The present work describes the effect of 8.0 M urea and 6.0 M guanidinium chloride on the subunit association and side-chain reactivity of BESOD. The results reported below indicate that BESOD retains its native dimeric structure in 8.0 M urea.

Materials and Methods

BESOD was isolated by slight modifications (Abernethy et al., 1974; Malinowski & Fridovich, 1979) of the original procedure (McCord & Fridovich, 1969). Xanthine oxidase was purified as described by Waud et al. (1975). The following proteins were obtained from the indicated sources: horse heart cytochrome *c* (type III), bovine erythrocyte carbonic anhydrase, ovalbumin, and bovine serum albumin (Sigma); lysozyme and pepsin (Worthington); and bovine liver catalase (Boehringer Mannheim). Ultrapure guanidinium chloride and sodium dodecyl sulfate were from Schwartz/Mann, and urea was from Baker and Adamson. Urea solutions (10.0 M) were prepared and stored at 23 °C and were deionized over a mixed-bed ion-exchange column of Amberlite MB-3 prior to use. Bromoacetic acid (Aldrich) was recrystallized from petroleum ether and stored over a desiccant in the dark at 4 °C. Sephadex G-25 (fine) was from Pharmacia, 5,5′-dithiobis(2-nitrobenzoic acid) from Pierce, and succinic anhydride from Eastman.

Activity Assays. Enzyme samples were assayed according to McCord & Fridovich (1969). Samples were dialyzed 18–24 h at 4 °C against 50 mM potassium phosphate, pH 7.8, prior to assay. Protein concentrations of native samples were measured by the method of Murphy & Kies (1960) and those of denatured and carboxymethylated samples by the method of Lowry et al. (1951) with native BESOD as the standard.

Sedimentation Equilibrium Analysis. Native BESOD (~2.6 mg) was dissolved in 3.0 mL of 8.0 M urea and 50 mM potassium phosphate, pH 7.8, and was dialyzed against the same solution for 18 h at 23 °C. The sample was then brought to sedimentation equilibrium (Yphantis, 1964). The solvent density was determined according to Tanford et al. (1974). The partial specific volume, $\bar{v}$, was calculated to be 0.727 cm^3/g (Cohn & Edsall, 1943). This value was assumed to be unchanged in 8.0 M urea since urea does not denature or inactivate this enzyme. Furthermore, denaturation by urea (4.0–8.0 M) decreased $\bar{v}$ by only 0.01–0.02 cm^3/g, as shown with carbomonoxyhemoglobin (Steinhardt, 1938), ovalbumin (Charlwood, 1957), and bovine serum albumin (Katz & Ferris, 1966).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. Enzyme samples, after dialysis against water overnight at 4 °C and lyophilization, were redissolved to 1.0 mg/mL in one of the following solutions: 0.01 M sodium phosphate and 1% $NaDodSO_4$ in the presence or absence of 6.0 M urea, 0.05 M EDTA, or 1% (v/v) 2-mercaptoethanol, pH 7.1. The samples were incubated for 5 h at 37 °C or for 1 h at 60 °C, followed by 5 h at 37 °C. After incubation, 25 μL of the samples was mixed with 5 μL of 0.05% (w/v) bromphenol blue in 0.1 M sodium phosphate, 0.02% NaN_3, pH 7.1, and 40% glycerol and applied to 10% polyacrylamide gels made up and used as described by Weber & Osborn (1969). The gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Colab), 9% acetic acid, and 45% methanol and then destained by diffusion in 14% acetic acid and 8% methanol. The protein molecular weight standards were bovine serum albumin (68 000), catalase (60 000), ovalbumin (43 000), pepsin (35 000), carbonic anhydrase (29 000), lysozyme (14 300), and cytochrome *c* (12 300).

Effects of Denaturants on Enzyme Activity and Subunit Association. Native BESOD (0.2–0.4 mg/mL) in potassium phosphate (pH 7.0 or 7.8) in the presence or absence of 8.0 M urea, 0.5–1.0 mM EDTA, or 6.0 M guanidinium chloride was incubated at 23 °C. At intervals, aliquots (0.2–1.0 mL) were removed and either dialyzed against 50 mM potassium phosphate, pH 7.8, or water at 4 °C or desalted on a column of Sephadex G-25 (1.0 × 66 cm) equilibrated in 1.0 M ammonium bicarbonate followed by lyophilization to remove the volatile buffer salts. The samples were then assayed for protein concentration and residual enzymatic activity and were analyzed by $NaDodSO_4$ gel electrophoresis.

Sulfhydryl Group Titrations. Thiol titrations with DTNB were done as described in the text below. An ϵ_m of 13 600 M^{-1} cm^{-1} for the 5-mercapto-2-nitrobenzoate anion was used (Habeeb, 1972). At the protein concentrations employed, the absorbance of BESOD at 412 nm would be less than 0.004. This small contribution to the total absorbance of the samples treated with DTNB was ignored and resulted in an overestimate of sulfhydryl content of only 0.02 sulfhydryl group/dimer.

Carboxymethylation of Bovine Erythrocyte Superoxide Dismutase. BESOD (0.2 mg/mL) was incubated in 0.1 M bromoacetic acid and 0.2 M potassium phosphate (pH 7.0), 0.1 M bromoacetic acid, 8 M urea, and 0.2 M potassium phosphate (pH 7.0), 0.1 M bromoacetic acid, 8 M urea, 0.2 M potassium phosphate, and 5×10^{-4} M EDTA (pH 7.0), and 0.1 M bromoacetic acid, 6 M guanidinium chloride, 1 mM EDTA, and 0.2 M potassium phosphate (pH 7.0). The pH was adjusted to 7.0 with NaOH. Incubation was at 23 to 24 °C in the dark. At intervals, 1.0-mL aliquots were removed from each sample and dialyzed against 0.05 M ammonium bicarbonate at 4 °C in the dark. After exhaustive dialysis, the samples were thrice lyophilized (twice from H_2O) and subjected to amino acid analysis. A second aliquot of the enzyme, incubated in 0.1 M bromoacetic acid, 8.0 M urea, 0.2 M potassium phosphate, and 5×10^{-4} M EDTA, pH 7.0, was removed, dialyzed against 50 mM potassium phosphate, pH 7.8, at 4 °C, and then assayed for protein concentration and enzymatic activity as described above.

Amino Acid Analysis. Desalted samples of protein were hydrolyzed in 2.0 mL of 6 N HCl and 0.1% (w/v) phenol in vacuo for 24 h at 110 °C. The hydrolysates were analyzed on a Beckman 119 automatic amino acid analyzer equipped with a 1-mV range card, employing the standard two-column methodology (Moore & Stein, 1963; Hubbard, 1965). The identities of the various carboxymethyl derivatives of histidine were determined by their elution behavior as previously described (Gurd, 1972). The absorptivity constants for the carboxymethyl derivatives of histidine were taken as those for the nearest eluting amino acid (Harris & Hill, 1969). The

Table I: Effect of Denaturants on Activity and Sulfhydryl Reactivity of Bovine Erythrocyte Superoxide Dismutase

sample conditions[a]	sp act. (units/mg)	S-(carboxymethyl)-cysteine[b] (residues/subunit)	thiol/subunit
control	4000		
KP_i, 24 h	4438	0	
KP_i, 48 h	4256	0	
KP_i, 72 h	4000	0	
8.0 M urea, 6 h	4660	0	
8.0 M urea, 12 h	3893	0	
8.0 M urea, 24 h	4539	0	
8.0 M urea, 48 h	4701	0	
8.0 M urea, 72 h	4717	0	
8.0 M urea, 57 μM EDTA, 24 h[c]	3781		0.05[d]
8.0 M urea, 57 μM EDTA, 48 h	4010		0.05
8.0 M urea, 57 μM EDTA, 72 h	3603		0.03
8.0 M urea, 0.5 mM EDTA, 24 h[e]	3617	0.17	
8.0 M urea, 0.5 mM EDTA, 48 h	3186	0.15	
8.0 M urea, 0.5 mM EDTA, 72 h	3061	0.29	
6.0 M GdmCl, 1 mM EDTA, 24 h	461	0.65	
6.0 M GdmCl, 1 mM EDTA, 48 h	463	0.68	
6.0 M GdmCl, 1 mM EDTA, 72 h	306	0.88	

[a] Reaction mixtures contained 0.2 M potassium phosphate (KP_i), pH 7.0, plus the additional components indicated (final pH 7.0). Bromoacetate (0.1 M) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (0.59 mM) were absent in samples employed for activity measurements. [b] Reaction mixture was identical with that in footnote *a* with the addition of 0.1 M bromoacetate (final pH 7.0). [c] Reaction mixture contained 8.0 M urea, 5.7×10^{-5} M EDTA, and 0.05 M potassium phosphate, pH 7.8. [d] Reaction mixture was identical with that in footnote *c* with the addition of 5.9×10^{-4} M DTNB. [e] Reaction mixture contained 0.1 M bromoacetate for both activity measurements and amino acid analysis.

absorptivity constant for *S*-(carboxymethyl)cysteine was determined from a standard amino acid mixture purchased from Pierce.

Results

Ultracentrifugation in 8.0 M Urea. BESOD was brought to sedimentation equilibrium in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, at 20 000 and 26 000 rpm. The equilibrium distributions, plotted according to Yphantis (1964), are shown in Figure 1. The enzyme was centrifugally homogeneous, and a molecular weight of 32 000 ± 1200 was calculated. Since this is the molecular weight of the native enzyme (McCord & Fridovich, 1969), we conclude that a dimeric structure is retained in 8.0 M urea. Retention of full activity in this solvent (Table I) further indicates that the native dimeric structure is unaffected by 8.0 M urea. The remote possibility of unnatural disulfide-bridged dimers, created by oxidative coupling or by disulfide interchange involving the thiol group on cysteine-6 which lies within the β barrel structure and which is ordinarily not exposed to solvent (Richardson et al., 1975a), was explored and eliminated by electrophoresis in the presence of $NaDodSO_4$.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$ per se does not dissociate the subunits of BESOD (Keele et al., 1971; Abernethy et al., 1974). However, heating with $NaDodSO_4$ in 6.0 M urea plus EDTA or treating with 6.0 M guanidinium chloride and EDTA does dissociate the subunits of BESOD (Abernethy et al., 1974). BESOD is thus not disulfide bridged, and if the dimeric species seen in 8.0 M urea is likewise, it too should be dissociated by these nonreducing treatments with $NaDodSO_4$.

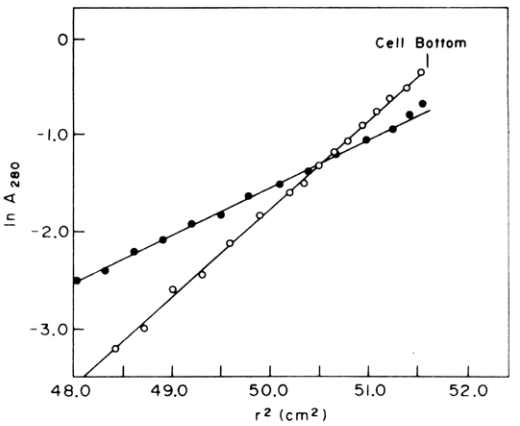

FIGURE 1: Sedimentation equilibrium analysis of BESOD in 8.0 M urea and 50 mM potassium phosphate, pH 7.8. Ultracentrifugation was performed at 20 000 (closed circles) and 26 000 (open circles) rpm at 25 °C. Recovery of total protein absorbance was 100%.

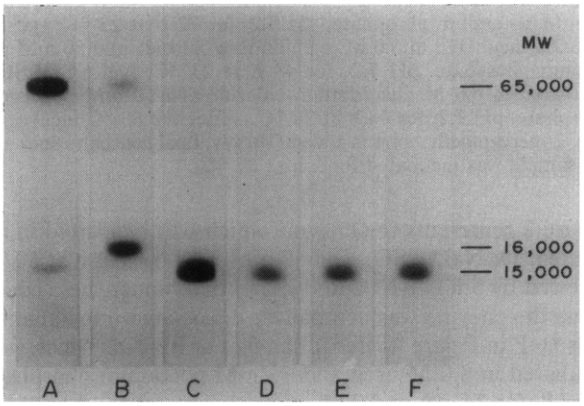

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of native and 8.0 M urea incubated BESOD. Prior to incubation in $NaDodSO_4$ and electrophoresis, the protein samples were dialyzed against H_2O and lyophilized. (Gel A) Native BESOD incubated in 1% $NaDodSO_4$ and 0.01 M sodium phosphate, pH 7.1, for 5 h at 37 °C; (gel B) native BESOD incubated in 1% $NaDodSO_4$, 1% 2-mercaptoethanol, and 0.01 M sodium phosphate, pH 7.1, for 5 h at 37 °C. The following samples were each incubated in 6.0 M urea, 1% $NaDodSO_4$, 0.05 M EDTA, and 0.01 M sodium phosphate, pH 7.1, for 1 h at 60 °C and for 5 h at 37 °C prior to electrophoresis. (Gel C) Native BESOD; (gel D) BESOD incubated in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, for 24 h; (gel E) BESOD incubated in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, for 48 h; (gel F) BESOD incubated in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, for 72 h.

BESOD was incubated in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, at 23 to 24 °C. Samples were taken at 24-h intervals for $NaDodSO_4$ gel electrophoresis. Gels A and B in Figure 2 show the native enzyme incubated in 1% $NaDodSO_4$ ± 1% 2-mercaptoethanol, respectively. This corroborates previous reports that the dimeric structure is very resistant to dissociation into subunits. As shown, the nondissociated enzyme exhibited an apparent molecular weight of 65 000. This behavior has been noted previously (Weser et al., 1971; Hartz & Deutsch, 1972; Abernethy et al., 1974) and probably represents anomalous $NaDodSO_4$ binding due to incomplete denaturation of the polypeptide chain. Gel C in

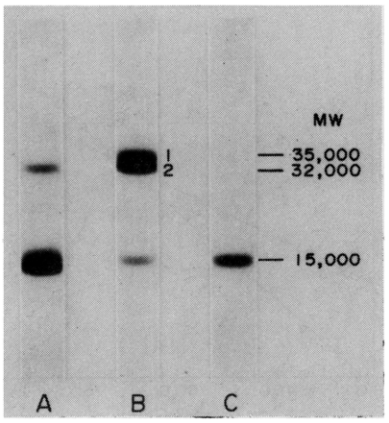

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of 6.0 M guanidinium chloride incubated BESOD. The following samples, after desalting and lyophilization, were incubated in 6.0 M urea, 1% $NaDodSO_4$, 0.05 M EDTA, and 0.01 M sodium phosphate, pH 7.1, for 1 h at 60 °C and for 5 h at 37 °C prior to electrophoresis: (gel A) BESOD incubated in 6.0 M guanidinium chloride, 1 mM EDTA, and 50 mM potassium phosphate, pH 7.2, for 72 h at 23 °C; (gel B) BESOD incubated in 6.0 M guanidinium chloride and 50 mM potassium phosphate, pH 7.2, for 48 h at 23 °C; (gel C) BESOD incubated in 6.0 M guanidinium chloride and 50 mM potassium phosphate, pH 7.2, for 48 h at 23 °C. After the 60 °C incubation step, 2-mercaptoethanol was added (1% v/v, final concentration) and the sample was incubated for 5 h at 37 °C.

Figure 2 represents the enzyme which was incubated in 6.0 M urea, 1% $NaDodSO_4$, and 50 mM EDTA for 1 h at 60 °C followed by 5 h at 37 °C and shows that, under these conditions, the enzyme was completely dissociated into subunits. Gels D–F in Figure 2 refer to samples of BESOD which were incubated in 8.0 M urea and 50 mM potassium phosphate, pH 7.8, for 24, 48, and 72 h, respectively, followed by treatment in 6.0 M urea, 1% $NaDodSO_4$, 50 mM EDTA, and 10 mM sodium phosphate, pH 7.1, for 1 h at 60 °C and for 5 h at 37 °C. As shown, all of the applied protein was completely dissociated into subunits of 15 000 molecular weight. It follows that treatment of BESOD with 8.0 M urea does not generate disulfide-bridged dimers. We conclude that the 32 000 molecular weight species seen in 8.0 M urea (Figure 1) was the native, noncovalently linked dimeric enzyme.

Disulfide-bridged dimers of BESOD can be produced by aerobic incubation of the enzyme in 6.0 M guanidinium chloride and 50 mM potassium phosphate, pH 7.2, for 48 h at 23 °C. EDTA present at 1.0 mM in this denaturing solvent largely prevented this disulfide bridging, presumably because this oxidative bridging is catalyzed by metals such as copper(II). These results are shown in Figure 3. The sample incubated in the presence of EDTA could still be largely converted to the monomeric subunit during heating in the presence of both urea and $NaDodSO_4$ with only a small amount of a 32 000 molecular weight species present (Figure 3, gel A). In contrast, the sample incubated in the absence of EDTA was largely a 32 000 molecular weight species resistant to dissociation by heating in urea and $NaDodSO_4$, with only a minor amount of the 15 000 molecular weight subunit present. The latter result suggests that the bovine dismutase, when denatured by guanidinium chloride in the absence of a metal chelator, is capable of undergoing a dimerization reaction which renders the protein resistant to subsequent dissociation during heating in urea and $NaDodSO_4$, conditions known to dissociate the native enzyme. Such an association between the subunits might be covalent in nature, possibly due to an intermolecular disulfide bond between the subunits. For a test of this proposal, the enzyme sample, after being heated in urea and $NaDodSO_4$, which caused complete inactivation, was incubated in 1% 2-mercaptoethanol for 5 h at 37 °C as described under Materials and Methods. This treatment altered the electrophoretic mobility of the protein to that expected for the monomeric subunit (Figure 3, gel C).

The presence of the two closely spaced protein bands (1 and 2 of gel B, Figure 3) is not completely understood, although one band might represent an oxidatively generated disulfide bond between two cysteine residues at the 6 position on adjacent subunits while the other band might represent a disulfide generated by a thiol–disulfide exchange reaction between cysteine-6 and cystine-55(144). Interestingly, continued thiol–disulfide exchange reactions to produce high molecular weight aggregates of BESOD did not occur, within the time scale of these experiments, in the presence of 6.0 M guanidinium chloride.

Sulfhydryl Reactivity of BESOD in 8.0 M Urea. Since disulfide-bridged dimers were not formed in 8.0 M urea but were produced in guanidinium chloride, we conclude that the single thiol of cysteine-6 is unreactive in the native conformation present in the former solvent. This was explored with DTNB. BESOD failed to react with 0.4 mM DTNB during 96 h at 23 to 24 °C in 8.0 M urea and 50 mM potassium phosphate, pH 6.8. However, when the enzyme was unfolded by heating to 60 °C for 2 h in 8.0 M urea, 10 mM EDTA, and 50 mM potassium phosphate, pH 6.8, it exhibited 0.9 reactive thiol/dimer. This is approximately half the expected stoichiometry and may represent incomplete denaturation of the enzyme. The apparent lack of thiol reactivity could have been due to the presence of copper(II) in the incubation mixture. This metal will react both with free cysteine to prevent its reaction with DTNB and with the product of the reaction, 5-mercapto-2-nitrobenzoate anion, to decrease its visible absorbance between 380 and 450 nm (data not shown). This experiment was therefore repeated in the presence of 5.7×10^{-5} M EDTA. The results, shown in Table I, reveal that no significant loss of enzymatic activity occurred during a 72-h incubation at 23 °C. Furthermore, there was only a small extent of sulfhydryl exposure, which did not increase with time. These results lend support to the interpretation that the sulfhydryl group of cysteine-6 was unreactive to DTNB even in the presence of 8.0 M urea.

Carboxymethylation of BESOD. The stability of BESOD was further explored by noting the effects of various solvents on its activity and on its reactivity with bromoacetic acid. The results, shown in Table I, reveal that the enzyme during incubation in 0.2 M potassium phosphate, pH 7.0, in the presence of absence of 8.0 M urea for 72 h at 23 °C lost no enzymatic activity. In contrast, incubation in 6.0 M guanidinium chloride, 1 mM EDTA, and 0.20 M potassium phosphate, pH 7.0, produced a substantial loss of enzymatic activity. There was no evidence of *S*-(carboxymethyl)cysteine present in the samples incubated in potassium phosphate buffer in the presence or absence of 8.0 M urea. In contrast, the sample incubated in guanidinium chloride and EDTA showed near stoichiometric amounts of *S*-(carboxymethyl)cysteine. Incubation of the enzyme with bromoacetic acid in the presence of 8.0 M urea and 0.5 mM EDTA showed a progressive loss of activity over a 72-h period and a detectable amount of *S*-(carboxymethyl)cysteine. It is clear that the sulfhydryl group of cysteine-6 is reactive toward alkylating reagents in BESOD denatured in 6 M guanidinium chloride plus 1 mM EDTA but is not reactive in neutral phosphate buffer in the presence or absence of 8.0 M urea, under which conditions the enzyme is fully active. The sulfhydryl group is thus not exposed to

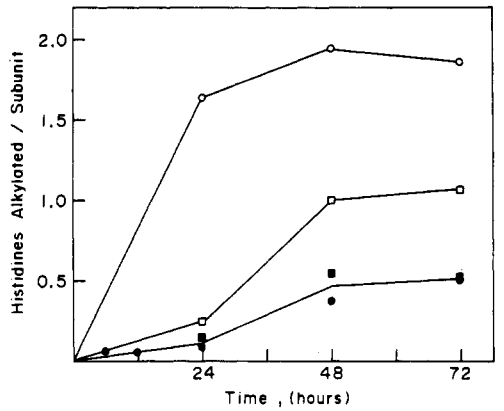

FIGURE 4: Effect of denaturants on the rate of histidine alkylation in BESOD. Native BESOD was incubated in 0.1 M bromoacetic acid and 0.2 M potassium phosphate, pH 7.0, with the following additions: (●) none; (■) 8.0 M urea; (□) 8.0 M urea; 5×10^{-4} M EDTA; (○) 6.0 M guanidinium chloride and 1 mM EDTA. At the indicated times, samples were analyzed for histidine alkylation as described under Materials and Methods.

reaction during incubation of the enzyme in 8.0 M urea.

Carboxymethylation of histidine residues was explored, and the results are summarized in Figure 4. The histidine residues of native BESOD in neutral phosphate buffer are quite unreactive toward bromoacetic acid, and 8.0 M urea does not perceptibly change this situation. In contrast, 8.0 M urea plus 0.5 mM EDTA did modestly increase the reactivity of the histidine residues (presumably reflecting the modification or only those protein molecules which are denatured) while 6.0 M guanidinium chloride plus 1 mM EDTA increased it markedly.[2] The incomplete histidine modification with the fully denatured BESOD is not unexpected since the reaction conditions employed are not optimal for this modification (Bradshaw et al., 1969). Modification of histidine residues of certain proteins, in the presence of 6.0 M guanidinium chloride, does not always proceed to completion (Harris & Hill, 1969).

Discussion

The copper–zinc superoxide dismutase from bovine erythrocytes is an unusually sturdy enzyme, retaining activity in 10.0 M urea or 4% $NaDodSO_4$ (Forman & Fridovich, 1973). Dissociation of this homodimeric enzyme in $NaDodSO_4$ requires additional denaturing stress, such as heat, urea, mercaptoethanol, or EDTA. The copper–zinc superoxide dismutases from diverse sources are remarkably similar and probably all share this great stability. Indeed, the human (Hartz & Deutsch, 1972) and the wheat germ (Beauchamp & Fridovich, 1973) enzymes have also been seen to be stable to $NaDodSO_4$ in the absence of additional denaturing stress.

Recent reports that the bovine (Marmocchi et al., 1978) and the swordfish (Bannister et al., 1978) enzymes are dissociated into active monomers by 8.0 M urea appeared to question the great stability of these enzymes and were of special interest because they indicated that subunit interactions were not important for the catalytic activity of the native dimers. Both of these reports were based upon gel exclusion chromatography in the presence of 8.0 M urea. In contrast, we note that ultracentrifugation of the bovine enzyme in 8.0 M urea yields an equilibrium distribution characteristic of a single species with a molecular weight of 32 000 ± 1200. It was further shown that this dimeric species, in urea, was not disulfide bridged since it could be dissociated into 16 000-dalton subunits by $NaDodSO_4$ in the absence of reductants. Urea, at 8.0 M, did not detectably increase the reactivity of thiol or of imidazole residues. We must conclude that the dimeric species seen by sedimentation equilibrium in 8.0 M urea was the native form rather than an unnatural disulfide-bridged dimer. This conclusion fits comfortably with the complete retention of enzymatic activity during several days of incubation at 23 to 24 °C in 8.0 M urea.

We can readily explain the discrepant results obtained by others using gel exclusion chromatography in 8.0 M urea. Thus, the gel columns were calibrated with several proteins, such as insulin, cytochrome *c*, carbonic anhydrase, ovalbumin, and serum albumin (Marmocchi et al., 1978), and elution volume was related to the molecular weights of these proteins. In fact, elution volume, in this method, relates to radius of gyration. Most proteins are denatured in 8.0 M urea, and this denaturation increases their radius of gyration. BESOD, in contrast, retains its native globular structure in this solvent. A comparison of elution volumes between the globular BESOD and the denatured molecular weight standards then leads directly to a gross underestimation of the molecular weight of BESOD.

Marmocchi et al. (1978) showed that BESOD could form molecular hybrids with the wheat germ copper–zinc enzyme. This was offered as unequivocal evidence that the enzyme had dissociated into subunits in the presence of 8 M urea. Indeed, in a number of enzymes, the formation of hybrids through the association of nonidentical subunits occurs through the dissociation of the multimeric protein into subunits followed by the reassociation of the polypeptide chains in a random manner (Klotz et al., 1975). However, extensive dissociation of subunits is not necessary for subunit hybridization to occur. Freezing and thawing will produce hybrids of lactate dehydrogenase (Chilson et al., 1965). Functional hybrids will form between various superoxide dismutases upon mild heating (Tegelström, 1975; Edwards et al., 1978). The existence of an equilibrium between the native dimer and the separate monomers is sufficient to allow hybrid formation, even when the equilibrium greatly favors the dimer. The only requirement is that the hybrid have comparable stability to the native dimers and greater stability than the separated monomers. The extent of BESOD dissociation in the presence of 8 M urea need not be greater than a small fraction of 1% in order to lead to formation of measurable amounts of hybrid. This degree of dissociation would be too small to be measured by equilibrium sedimentation or by gel exclusion chromatography.

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[2] In this sample, there were 0.8 residue of 1-(carboxymethyl)histidine per subunit and 1.0 residue of 3-(carboxymethyl)histidine per subunit.

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Guanylate Cyclase of Isolated Bovine Retinal Rod Axonemes†

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ABSTRACT: The guanylate cyclase activity of axoneme–basal apparatus complexes isolated from bovine retinal rods has been investigated. The Mg^{2+} and Mn^{2+} complexes of GTP^{4-} serve as substrates. Binding of an additional mole of Mg^{2+} or Mn^{2+} per mole of enzyme is required. Among cations which are ineffective are Ca^{2+}, Ni^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Co^{2+}. The kinetics are consistent with a mechanism in which binding of Mg^{2+} or Mn^{2+} to the enzyme must precede binding of MgGTP or MnGTP. The apparent dissociation constants of the Mg–enzyme complex and the Mn–enzyme complex are 9.5×10^{-4} and 1.1×10^{-4} M, respectively. The apparent dissociation constants for binding of MgGTP and MnGTP to the complex of the enzyme with the same metal are 7.9×10^{-4} and 1.4×10^{-4} M, respectively. The cyclase activity is maximal and independent of pH between pH 7 and 9. KCl and NaCl are stimulatory, especially at suboptimal concentrations of Mg^{2+} or Mn^{2+}. Ca^{2+} and high concentrations of Mg^{2+} and Mn^{2+} are inhibitory. Ca^{2+} inhibition appears to require the binding of 2 mol of Ca^{2+} per mol of enzyme. The dissociation constant of the Ca_2–enzyme complex is estimated to be 1.4×10^{-6} M^2. The axoneme–basal apparatus preparations contain adenylate cyclase activity whose magnitude is 1–10% that of the guanylate cyclase activity.

It has been apparent that cGMP must play an important role in the function or development of vertebrate retinal rods. Rod outer segments (ROS)[1] contain high levels of a cGMP-specific phosphodiesterase (Pannbacker et al., 1972; Chader et al., 1974a,b; Robb, 1974; Pannbacker & Lovett, 1977) which is activated by bleached rhodopsin in the presence of GTP or ATP (Miki et al., 1973; Chader et al., 1974a; Bitensky et al., 1975; Manthorpe & McConnell, 1975; Sitaramayya et al., 1977). Recent experiments demonstrate that the cGMP hydrolysis is quite rapid; bleaching of a single rhodopsin molecule may result in the hydrolysis of as many as 4×10^5 cGMP molecules/s (Woodruff et al., 1977; Yee & Liebman, 1978).

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; ROS, rod outer segments; Pipes, 1,4-piperazinediethanesulfonic acid; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEI, polyethylenimine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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