Bovine Erythrocyte Superoxide Dismutase: Diazo Coupling, Subunit Interactions, and Electrophoretic Variants†

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ABSTRACT: The copper and zinc-containing superoxide dismutase of bovine erythrocytes was modified and inactivated by several diazonium reagents. Treatment of native or of zinc-only enzymes, with diazonium 1*H*-tetrazole, derivatized 1 tyrosine, 1 histidine, and 10 lysine residues per subunit. The partial inactivation, accompanying this treatment, appeared to be due to modification of the lysine residues, since acetylation caused a similar extent of lysine modification and of inactivation. Apoenzyme exhibited greater reactivity toward diazonium 1*H*-tetrazole, reflecting the exposure of two histidine residues per subunit in the active site region. Diazo coupling with diazonium 1*H*-tetrazole grossly increased the anodic mobility of the enzyme and yielded a derivative useful for studies of subunit interactions. Admixture of native enzyme with diazo-coupled, H_2O_2-inactivated enzyme, in the presence of 8.0 M urea, followed by removal of the urea, generated a new, enzymatically active, species. The new species exhibited an electrophoretic mobility nearly intermediate between that of the native and diazo-coupled proteins, but had the same molecular weight as the parental species. When isolated from polyacrylamide gels and incubated at pH 7.8 for 2 h at 25 °C, the new species gave rise to both parental species, demonstrating that it was an unstable hybrid. The specific activity of the hybrid was approximately half that of the native enzyme. A native subunit thus exhibited the same catalytic activity, whether paired with another native subunit or with a chemically modified and catalytically inactive subunit. Mutually inhibitory interactions between subunits, giving rise to half of the sites reactivity, thus seems unlikely.

X-ray crystallography (Richardson et al., 1975a,b) coupled with amino acid sequence analysis (Evans et al., 1974; Steinman et al., 1974; Abernethy et al., 1974) has generated a view of the active site of the superoxide dismutase from bovine erythrocytes. Each catalytic center of this dimeric enzyme appears to be composed of one copper and one zinc, with the Cu(II) ligated to histidines-44, -46, and -118 and joined to the Zn(II) by histidine-61, while the Zn(II) is also ligated to histidines-67 and -78 and to aspartate-81. Lippard et al. (1977) used NMR to deduce that all histidines in the apoenzyme are exposed to solvent and are reactive with diethylpyrocarbonate, whereas only histidine-19 was exposed and reactive in the holoenzyme or in the zinc-only enzyme. Reactivity of histidines in the apoenzyme and relative unreactivity in the native, zinc-only, or copper-only enzymes was also deduced from studies of photosensitized oxidation (Forman et al., 1973).

The rate of reaction of the enzyme with O_2^- and a mechanism involving alternate reduction and reoxidation of the Cu(II) were arrived at by pulse radiolysis (Klug et al., 1972; Klug-Roth et al., 1973). During catalytic turnover of the enzyme, the steady-state reduction of Cu(II) should reflect the ratio of the rate constants for the reduction of Cu(II) by O_2^- and for the oxidation of Cu(I) by O_2^-. Fielden et al. (1974), noting less than the anticipated reduction of Cu(II), proposed an inhibitory interaction between subunits. Such half-of-the-sites reactivity is difficult to envision, since the structure deduced by crystallography shows the active sites to be on opposite sides of molecule. Nevertheless, experimental evidence is necessary before it can be dismissed. In one approach to this problem Bannister et al. (1978) noted that the copper–zinc superoxide dismutase of swordfish liver, when dissociated into subunits in 8.0 M urea, was as active as the native dimer. This indicates that individual subunits exhibit the same activity, when separated by urea, as they do when associated into dimers. The bovine erythrocyte enzyme, for which half-of-the-sites reactivity was actually proposed, is not dissociated by urea, so this method cannot be applied to it. One could, however, compare the activities of subunits paired either with other active subunits or with chemically modified, catalytically inactive subunits. Enzyme hybridization has been used to demonstrate subunit independence during the catalytic turnover of several multimeric enzymes (Boettcher & Martinez-Carrion, 1975; Meighen & Schachman, 1970a,b) and interspecies hybrids of the copper–zinc superoxide dismutases have been prepared (Tegelström, 1975). Diazo coupling was explored as the means of preparing the modified subunits needed for these experiments. This approach was successful and native subunits were shown to exhibit the same activity when paired with a native partner or with a modified and inactive partner.

Materials and Methods

Superoxide dismutase was prepared from bovine erythrocytes by a modification (Abernethy et al., 1974) of the original procedure (McCord & Fridovich, 1969). Residual carbonic anhydrase was removed by passage over an affinity adsorbent. The procedure of Whitney (1974) for preparing this adsorbent was modified as follows: *p*-(aminomethyl)benzenesulfonamide was coupled to the hexanoic acid spacer arms of CH-Sepharose 4B, using a modified procedure of Belleau & Malek (1968). Swollen CH-Sepharose 4B (0.28 mmol of carboxyl groups in 20 mL) was suspended in 30 mL of H_2O, to which 0.28 mmol of *p*-(aminomethyl)benzenesulfonamide was added. The pH of the suspension was adjusted to 7.1 and 1.4 mmol of *N*-ethoxycarbonyl-2-ethoxy-1,2-hydroquinoline (EEDQ)[1] was added. The reaction mixture was then shaken at 37 °C, while 10-mL aliquots of 95% ethanol were added, at hourly intervals, until 30 mL had been added, to solubilize the EEDQ. After

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[1] Abbreviations used: EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; DHT, diazonium 1*H*-tetrazole; DSM, 4-diazobenzenesulfonamide; monoazo-, bisazo-, 5-azo-1*H*-tetrazole derivatives of histidine and tyrosine residues; BESOD, bovine erythrocyte superoxide dismutase; BLSOD, bovine liver superoxide dismutase; KP_i, potassium phosphate buffer.

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an additional 13 h at 37 °C, the resin was thoroughly washed with water and was then used as described by Whitney (1974). This affinity resin had a binding capacity of 7 mg of carbonic anhydrase per mL of bed volume. The purified enzyme, when assayed as described by McCord & Fridovich (1969), possessed a specific activity of 4000 units/mg. The apo form of the enzyme was prepared as previously described (Forman et al., 1973). It exhibited less than 2% of the native activity and could be fully activated by exposure to a 20-fold molar excess of $CuSO_4$, for several minutes, prior to dilution into the assay mixture. Under the conditions of assay $CuSO_4$, per se, exhibited no activity because of the EDTA present in the assay buffer. The potential activity retained by apo- or zinc-only enzymes, during various chemical modifications, was always measured after exposure of the enzyme to a 20-fold molar excess of $CuSO_4$.

The superoxide dismutase from bovine liver, employed in the acetylation experiments, was isolated from fresh bovine liver following the procedure described for the isolation of the bovine erythrocyte superoxide dismutase.

Protein was estimated from absorbance in the short ultraviolet (Murphy & Kies, 1960). Enzyme modified with diazonium 1*H*-tetrazole (DHT) exhibited an increased ultraviolet absorbance, which could lead to a 18% overestimation of protein concentration by this method. This was determined from quantitative amino acid analysis. Corrections were therefore made for this potential source of error. DHT, which had been quenched with azide, interfered with the assay for superoxide dismutase. Hence, prior to all enzyme assays, the azide-quenched DHT was removed by dialysis.

Amino acid analyses were performed on desalted samples of enzyme after hydrolysis in 6 N HCl, 0.1% (w/v) phenol, in vacuo, for 24 h at 110 °C. Hydrolysates were analyzed with Beckman 120B and 120C amino acid analyzers employing slight modifications (Hubbard, 1965) of the original chromatographic procedure (Moore et al., 1958). No corrections were made for loss of threonine and serine or for the slow liberation of valine and isoleucine. Total diazonium modification was quantitated by the loss of specific amino acids, since acid hydrolysis of diazolysine, diazohistidine, and diazotyrosine results in the destruction of these amino acid derivatives, without regeneration of the parent amino acid (Sokolovsky & Vallee, 1966). The monoazo and bisazo derivatives of tyrosine and histidine were quantitated spectrophotometrically (Takanaka et al., 1969).

Disc gel electrophoresis was performed as described by Davis (1964) and Maurer (1971). Electrophoresis on polyacrylamide gels at pH 8.9 was performed essentially as described by Maurer (1971) with the modification that the upper electrophoresis buffer consisted of 0.05 M Tris, 0.05 M glycine, pH 8.9, while the lower electrophoresis buffer consisted of 0.1 M Tris, 0.05 M HCl, pH 8.2, and the separating gel was polymerized with 8.8 mM rather than with 3.1 mM ammonium persulfate. Electrophoresis at pH 7.5 was performed as described by Maurer (1971) with the modifications that 0.5 M H_2SO_4 was substituted for 1 N HCl in the preparation of the separating gel and the pH of both the upper and lower electrophoresis buffers (8 mM Tris, 0.03 M barbital) was 7.5. Superoxide dismutase was localized on gel electropherograms as previously described (Beauchamp & Fridovich, 1971). Xanthine oxidase was prepared from unpasteurized cream (Waud et al., 1975). Cytochrome *c* (type III, horse heart) and *p*-aminomethylbenzenesulfonamide were obtained from the Sigma Chemical Co.; DEAE-cellulose was obtained from Whatman as DE-52; CH-Sepharose 4B from Pharmacia; sulfanilamide from Calbiochem; 5-aminotetrazole monohydrate from Aldrich; *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was from Pierce Chemical Co.

Table I: Amino Acid Composition of BESOD Modified with Diazonium Reagents

	native[a]	DSM modified[a]		DHT modified	
reagent excess:		460	35	390	38
sp act. (U/mg):	3500	<35.0	<35.0	1080	2300
amino acid	residues per subunit				
monoazo-Tyr[b]	0	0	0	1.07	ND[c]
bisazo-Tyr[b]	0	0	0	0.41	ND
bisazo-His[b]	0	0	0	0.08	ND
Lys	9.45	3.37	8.65	0	4.50
His	7.19	0.27	2.20	6.40	7.04
Arg	3.82	3.94	3.95	3.81	3.87
Asp	17.11	17.17	17.20	17.56	18.21
Thr	11.55	11.27	11.45	10.73	11.10
Ser	7.62	7.68	7.82	7.50	7.70
Glu	11.07	10.76	10.99	12.13	11.61
Pro	6.26	5.38	4.98	5.66	5.71
Gly	25.43	25.79	24.75	24.93	24.76
Ala	8.73	8.82	8.94	7.78	7.92
½-cystine	2.61	1.30	ND	ND	ND
Val	10.95	10.18	11.35	9.87	10.24
Met	0.83	0.72	0.73	0.73	0.83
Ile	6.41	5.75	5.99	5.57	5.69
Leu	7.82	7.84	7.90	7.85	8.02
Tyr	0.89	0	0.84	0	0.40
Phe	3.92	3.67	3.82	3.46	3.60

[a] Composition normalized to (Asp + Glu + Gly + Ala) = 62.0 per subunit. [b] Spectrophotometric determination. [c] Not determined.

Results

Reaction with 4-Diazobenzenesulfonamide. Sulfanilamide (1% in 1.0 N HCl) was diazotized by admixture with an equal volume of 5% $NaNO_2$ at 0 °C for 3 min. Native enzyme (0.3 mL, 1.06×10^{-4} M in 0.25 M $NaHCO_3$, pH 8.8) was treated with 0.6 mL of this diazotized sulfanilamide, followed, after 3 min at 23 °C, by 2.0 mL of 20% Na_2CO_3. After 20 min at 23 °C, 1 equiv of NaN_3 was added to quench the unreacted diazonium salt and the sample was dialyzed at 4 °C against changes of 50 mM potassium phosphate, pH 7.8, for 48 h. Table I demonstrates that this diazo coupling caused modification of 6 lysine, 7 histidine, and 0.9 tyrosine residues per subunit. The modified enzyme retained less than 1% of its native activity. Exposure of holoenzyme to a smaller molar excess of the diazo reagent (35-fold rather than 460-fold) gave similar results with respect to activity loss and color change except that only 0.8 lysine and 5 histidine residues per subunit were modified. Since six of the eight histidines per subunit are involved in liganding Cu and Zn, at the active site, it seems likely that inactivation was due to modification of histidine residues.

Since this procedure entailed exposure of the enzyme first to acid and then to alkali, it is not surprising that no differences were observed between apo- and holoenzymes, with respect to reactivity with the diazo reagent. In contrast, when the diazotized sulfanilamide was adjusted to pH 7.0, by cautious addition of 20% Na_2CO_3, prior to addition of the enzyme, the apoenzyme lost 75% activity while the holoenzyme lost only 20%. The holoenzyme was modified, as indicated by the generation of a family of six bands of increasing anodic mobility on polyacrylamide gel electropherograms, which probably represent different degrees of coupling with lysine residues. This reagent was not further explored since it self-couples in neutral solution, so that the actual concentration

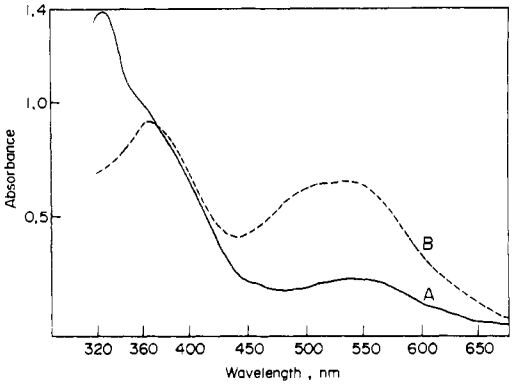

FIGURE 1: Effect of copper on the absorption spectra of native BESOD modified with 100-fold molar excess of diazonium 1*H*-tetrazole. (Curve A) One milliliter of 5.4×10^{-5} M BESOD was reacted with 50 μL of 0.15 M DHT in 0.5 M $NaHCO_3$, pH 8.8, at 0 °C for 60 min. After addition of N_3^- to stop the reaction, the sample was dialyzed against 0.56 M sodium borate, pH 8.8. pH of sample was adjusted to 8.0 prior to recording the spectrum. Protein concentration: 1.1 mg/mL, 3.5×10^{-5} M. (Curve B) Addition of 7.5 μL of 0.2 M $CuSO_4$ to 1.0 mL of above sample and buffer reference solution. Spectrum recorded after approximately 5 min at 23 °C.

remaining for reaction with the enzyme was uncertain.

Diazonium 1H-Tetrazole. This reagent is useful in protein modification, since it can be applied at controlled pH without self-coupling and allows spectrophotometric monitoring of histidine and tyrosine modification (Sokolovsky & Vallee, 1966; Takenaka et al., 1969). DHT was prepared and assayed according to Sokolovsky & Vallee (1966) and was used essentially as described by Riordan & Vallee (1972a). Native enzyme (5.0 mL, 5.3×10^{-5} M) in 0.50 M $NaHCO_3$, pH 8.8, was treated with 1.0 mL of 0.11 M DHT at 0 °C for 60 min. The pH was maintained at 8.8 by cautious addition of 0.1 N NaOH. NaN_3 (1.0 mL, 0.46 M) was added to terminate the reaction and the optical spectrum was recorded following dialysis against 0.56 M sodium borate, pH 8.8, at 4 °C. The spectrum, Figure 1, curve A, revealed that one tyrosine per subunit had been converted to the monoazo adduct. Amino acid analysis indicated modification of 9.5 lysine, 0.9 tyrosine, and 0.8 histidine residues per subunit. This enzyme retained 31% of native activity. Similar treatment of native enzyme with only a 38-fold molar excess of DHT modified 5 lysine and 0.5 tyrosine residues per subunit and allowed retention of 66% of the native activity. Table I summarizes these results.

Native, apo- and zinc-only enzymes were compared for their reactivities with a 100-fold molar excess of DHT, applied for 1 h at 0 °C, pH 8.8. The zinc-only enzyme was prepared from the apoenzyme by addition of one Zn^{2+} (as $ZnSO_4$) per subunit, followed sequentially by incubation at 0 °C for 24 h, and dialysis against 0.5 M $NaHCO_3$, pH 8.8, at 4 °C. Both the holo- and zinc-only enzymes revealed similar extents of amino acid modification: 9.4 lysine, 0.4 histidine, and 0.9 tyrosine residues per subunit were modified (Table II). Despite the similarity of modification, a difference in the degree of inactivation was apparent (Table II). The native enzyme lost 50% of its activity, while the zinc-only enzyme lost 80% of its activity. With the apoenzyme, 9.3 lysine, 2.5 histidine, and 0.9 tyrosine residues per subunit were modified with 95% loss of activity. The differences in degree of derivatization between the holo- and apoenzymes were greater than revealed by quantitative amino acid analysis. In the native enzyme, the single tyrosine residue was largely converted to the monazo derivative with only a 30% conversion to the bisazo derivative and the histidine modified was converted to the monoazo adduct. In the case of apoenzyme, in contrast,

Table II: Amino Acid Composition of Native, Zn Only, and Apo-BESOD Modified with Diazonium 1*H*-Tetrazole

	native I	native II	Zn only	apo
reagent excess:	108	135	103	126
sp act. (U/mg):	2080	1825	701	184[a]
amino acid	residues per subunit[b]			
monoazo-Tyr[c]	0.55	0.74	0.81	0.39
bisazo-Tyr[c]	0.29	0.32	0.25	0.50
bisazo-His[c]	0.03	0.00	0.05	0.52
Lys	0.18	0	0.13	0.12
His	6.73	6.86	6.67	4.68
Arg	3.92	3.95	3.98	3.98
Asp	16.05	16.05	16.11	16.17
Thr	11.48	11.35	11.25	11.16
Ser	7.43	7.68	7.84	7.74
Glu	11.58	11.47	11.41	11.37
Pro	6.52	6.09	6.09	6.71
Gly	25.41	25.33	25.41	25.88
Ala	8.88	8.99	8.86	8.83
½-cystine	1.58	1.24	1.19	1.17
Val	10.56	10.70	9.62	9.59
Met	0.74	0.62	0.71	0.75
Ile	5.96	5.87	5.42	5.34
Leu	7.78	7.57	7.52	7.41
Tyr	0	0	0	0
Phe	3.93	3.82	3.91	3.89

[a] The apoenzyme, without modification by DHT, could be reconstituted to a specific activity of 2153 U/mg. [b] Composition normalized to (Asp + Glu + Gly + Ala) = 62.0 per subunit. [c] Spectrophotometric determination.

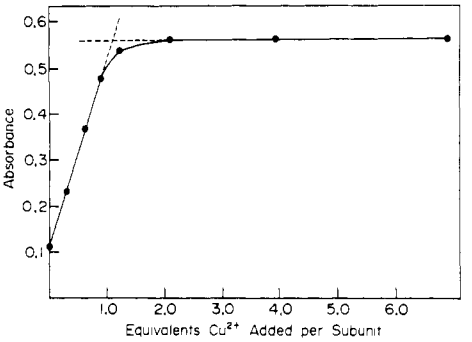

FIGURE 2: Increase in 545-nm absorption of DHT modified BESOD by titration with Cu^{2+}. Two-tenths of a milliliter of 1.5 mg/mol of BESOD (4.8×10^{-5} M) in 0.5 M $NaHCO_3$, pH 8.8, was reacted for 30 min at 0 °C with 85-fold molar excess of DHT (4.98 mM). After addition of N_3^-, to stop the reaction, the sample was dialyzed against 0.56 M sodium borate, pH 8.8. Protein concentration = 1.09 mg/mL (3.45×10^{-5} M) initially. Visible spectrum was recorded against above buffer. At 5-min intervals, the following volumes in microliters of 0.02 M $CuSO_4$ were added to 1.0 mL of both protein and buffer blank and the spectra recorded: 1.0; 1.0; 1.1; 3.0; 10.0; 21.0. The increase in absorbance which occurred at 545 nm was plotted as a function of the equivalents of Cu^{2+} added per subunit.

both tyrosine and histidines were largely converted to bisazo derivatives. The results, shown in Table II, reveal that the apoenzyme was more reactive toward DHT than either the holo or the zinc-only enzymes.

Ligation of Cu(II) to DHT-Modified Enzyme. Cu(II), added to DHT-modified enzyme, engendered a purple color (Figure 1, curve B) which was not lost during subsequent dialysis or gel filtration. Spectrophotometric titration of DHT-modified holoenzyme (Figure 2) indicated the binding of 1.0 Cu(II) per subunit. The activity of this modified enzyme, which was 40% of the native activity, was not affected by Cu(II). Azophenols are known to be chromogenic chelating agents (Anderson & Nickless, 1964). Monoazo-DHT tyrosine was prepared as described by Sokolovsky & Vallee (1966). When exposed to Cu(II), it exhibited the same spectral

Table III: Acetylation of Bovine Erythrocyte and Bovine Liver Superoxide Dismutases

protein	sp act. (U/mg)	sp act. after hydroxylamine[a] (U/mg)	Leu (equiv[b] per mol of enzyme)	*O*-acetylhydroxyamino acid[c] (residues per mol of enzyme)	*O*-acetyl-Tyr[d] (residues per mol of enzyme)
BESOD	3892	3969	17.1		
acetylated BESOD	1748	1839	0.1	4.7	ND[e]
BLSOD	3458	3465	21.8		
acetylated BLSOD	1766	1760	0.1	5.9	1.99[f]

[a] Enzyme treated with 1 M hydroxylamine at pH 7.3 for 10 min, 23 °C, and then dialyzed against 0.05 M KP_i, pH 7.8. [b] Determined from ninhydrin reaction employing L-leucine as the standard. [c] Treatment of enzyme with 0.7 M hydroxylamine, pH 11.4, for 10 min followed by quantitation of hydroxamate formation as described in the text. Values refer to acetylated tyrosine + serine + threonine. [d] Treatment of enzyme with 0.7 M hydroxylamine, pH 7.1, for 10 min followed by quantitation of hydroxamate formation as described in the text. [e] Not determined. [f] Bovine liver superoxide dismutase contains 1 tyrosine, 11-12 threonine, and 8 serine residues per subunit as determined from the amino acid composition.

changes as did the DHT-modified enzyme (data not shown). Johansen & Vallee (1971) noted a similar binding of Zn(II) to an arsanilocarboxypeptidase.

Acetic Anhydride. DHT treatment of the holoenzyme caused 50% inactivation, concomitant to the modification of 9.4 lysine, 0.4 histidine, and 0.9 tyrosine residues per subunit. The 1 tyrosine per subunit can be modified without loss of activity (Agro et al., 1977). It therefore appeared possible that the activity loss might have been associated with modification of the lysine residues. To test this possibility, 1.0 mL of 6.6×10^{-5} M holoenzyme in 50 mM potassium phosphate, pH 7.8, was treated with 4 μL of acetic anhydride with stirring at 0 °C. The pH was maintained between 7.5 and 8.5 by the addition of 1.0 N NaOH under a glass electrode. After 30-min reaction, the sample was dialyzed against 50 mM potassium phosphate, pH 7.5, at 4 °C. In addition, a sample of bovine liver superoxide dismutase (1 mL of 3.2×10^{-4} M) in 50 mM potassium phosphate, pH 7.8, was treated with 2-μL aliquots of acetic anhydride added at 10-min intervals under stirring at 0 °C. The pH was maintained as described above. After 74 min of reaction, and a total of 14 μL of acetic anhydride, the sample was dialyzed against 50 mM potassium phosphate, pH 7.5, at 4 °C. The degree of lysine acetylation in both of these samples of superoxide dismutase was determined by reaction with ninhydrin (Moore & Stein, 1948). The extent of tyrosine O-acetylation was determined by treatment of the acetylated enzyme with 1.0 M hydroxylamine for 10 min at pH 7.3 and 23 °C (Riordan & Vallee, 1972b) followed by quantitation of hydroxamate formation (Hestrin, 1949; Balls & Wood, 1956). Total hydroxyamino acid O-acetylation, including tyrosine, serine, and threonine, was determined by treatment with 0.7 M hydroxylamine at pH 11.4 (Gounaris & Pearlmann, 1967) followed by quantitation of hydroxamate formation. Quantitation in all cases was referred to a standard curve prepared with ethyl acetate and alkaline hydroxylamine as described above. Under the conditions employed, the deacetylation of ϵ-acetyllysine residues should be minor in comparison to the deacetylation of the hydroxyamino acids (Hestrin, 1949; Gounaris & Pearlmann, 1967). The results are shown in Table III. Acetylation of bovine erythrocyte superoxide dismutase blocked most of the lysine residues and no more than 5 hydroxyamino acids, which presumably included the 2 tyrosine residues and 3 serine plus threonine residues out of 40 per molecule of enzyme. Acetylation of the bovine liver enzyme also blocked most of the lysine residues, the 2 tyrosine residues per molecule and 4 serine plus threonine residues out of 40 per molecule. Acetylation of both enzymes resulted in 45–50% loss of enzymatic activity. The activity of the acetylated enzymes was not changed by deacetylation of the tyrosine residues with hydroxylamine at pH 7.3. Thus acetylation of the tyrosine residues in the bovine enzymes is not responsible for the inactivation observed. We conclude that extensive lysine modification causes partial loss of activity and that the activity loss seen during DHT treatment was probably due to lysine modification. There is some uncertainty in the acetylation results due to the possible effects on activity of the small extent of modification of serine plus threonine. We did attempt to examine the effects of deblocking these residues on catalytic activity. Unfortunately exposure of native enzyme to the conditions needed for removal of serine plus threonine acetylation (Table III) caused 70% inactivation which could not be reversed by dialysis against 50 μM Cu(II) plus Zn(II) in a neutral Tris buffer.

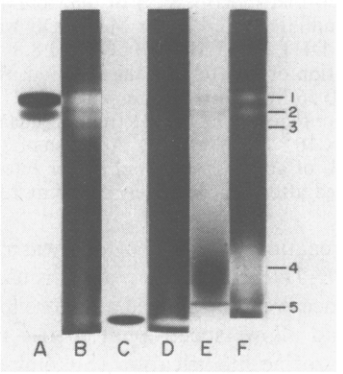

FIGURE 3: Disc gel electrophoresis of native, DHT-modified H_2O_2-inactivated and isolated hybrid superoxide dismutase. Electrophoresis was performed at pH 8.9. (A) Native enzyme, 40 μg. (B) Activity stain of native enzyme, 2 μg. (C) DHT-modified H_2O_2-inactivated holoenzyme, 39 μg. (D) Activity stain of DHT-modified H_2O_2-inactivated holoenzyme, 3.9 μg. The hybrid superoxide dismutase was prepared from 3.3 mg of DHT-modified H_2O_2-inactivated enzyme (specific activity, 22 U/mg) and an equal amount of native enzyme (both in 0.05 M KP_i, pH 7.8) incubated in 8.0 M urea for 2 h at 23 °C. Following dialysis, against 0.05 M potassium phosphate, pH 7.8 at 4 °C, the hybrid was isolated by electrophoresis from gel tubes as described in the text. (E) Seventy-three micrograms of isolated hybrid. (F) Activity stain of hybrid, 3.7 μg. Bands 1, 2, and 3 represent the three major bands of native superoxide dismutase. Band 4 represents the hybrid superoxide dismutase. Band 5 represents the DHT-modified H_2O_2-inactivated enzyme.

Hybridization of Native and DHT-Modified Enzymes. Polyacrylamide gel electrophoresis of the purified native enzyme reveals a family of active bands (gels A and B, Figure 3). An identical pattern of active bands can be seen with crude hemolysates, so they are not artifacts of preparation and their copurification reveals great similarity. By the criterion of retardation with increasing gel density (Ferguson, 1964; Hedrick & Smith, 1968) the three most prominent of these bands are of identical molecular weight, lines 1–3 of Figure 4. These bands do not represent enzymes with differing fractions of the full complement of Cu(II) and Zn(II). This can be shown by removal and by treatment with an excess of

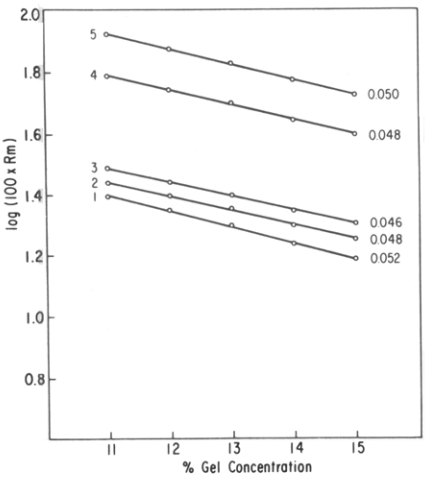

FIGURE 4: Ferguson plot of native, DHT-modified, H_2O_2-inactivated, and hybrid superoxide dismutases. The relative mobilities (R_m) of the various enzyme species obtained from the mixture used in Figure 6 (A) were determined on polyacrylamide gels of increasing acrylamide concentration. Plots 1 through 3 refer to the three major isozymes of native BESOD shown in Figure 6. Plot 4 refers to the hybrid band and plot 5 refers to the DHT-modified, H_2O_2-inactivated enzyme, also shown in Figure 6. The negative slope of each line, as determined through a least-squares linear regression analysis, is shown to the right of each respective plot.

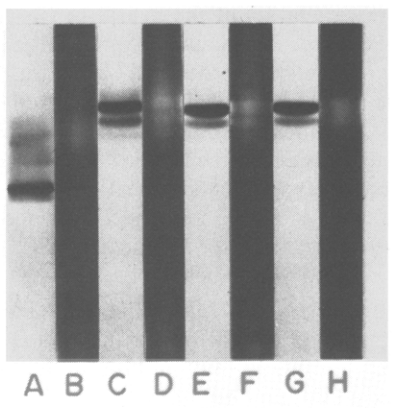

FIGURE 5: Effect of copper and zinc on mobilities of apo- and native erythrocyte superoxide dismutase. One-half of a milliliter of 1×10^{-5} M apoenzyme (specific activity, 132 U/mg) and 0.5 mL of 1×10^{-5} M native enzyme (specific activity, 4100 U/mg) were treated with 4 equiv of Cu^{2+} and 4 equiv of Zn^{2+} (as acetate salts) in 0.05 M sodium acetate, pH 5.0, for 24 h at room temperature. This is referred to as reconstitution. (A) Apo-BESOD, 31.5 μg; (B) 3.15 μg of apo-BESOD stained for activity; (C) 31.5 μg of reconstituted apo-BESOD; (D) 3.15 μg of reconstituted apo-BESOD stained for activity; (E) 31.5 μg of native BESOD; (F) 3.15 μg of native BESOD stained for activity; (G) 31.5 μg of reconstituted native BESOD; (H) 3.15 μg of reconstituted native BESOD stained for activity.

these metals. Thus as shown in Figure 5 the apoenzyme shows greater anodic mobility than any component of the native pattern of bands, while restoration of the Zn(II) and Cu(II) or treatment of the holoenzyme with an excess of these metals restores the native pattern, but does not simplify it.

When native enzyme was treated with a 400-fold molar excess of DHT, as described above, the majority of protein migrated with the dye front as a single sharp band, as shown by gels C and D in Figure 3. The residual activity of the DHT-modified enzyme could be largely eliminated by treatment with a 55 M excess of H_2O_2, in 0.5 M $NaHCO_3$ at pH 10.0 and 23 °C, for 40 min (Bray et al., 1974; Hodgson & Fridovich, 1975), followed by dialysis against 50 mM potassium phosphate, pH 7.8 (Table IV). This inactivation with H_2O_2 did not change the electrophoretic mobility of the

Table IV: Effect of Hybridization on Specific Activity

sample (mg)	sp act. (U/mg) before urea	sp act. (U/mg) after urea[a]
native BESOD (0.78)	4312	4034
DHT-H_2O_2 inactivated BESOD (0.45)	12	31
native BESOD (1.07) + DHT-H_2O_2 BESOD (0.99)[b]	2410	2402[b]

[a] Samples treated with 8 M urea for 2 h at 23 °C, followed by dialysis to remove the urea. [b] Treatment with urea generated a hybrid species detected by electrophoresis on polyacrylamide gels (Figures 3 and 6).

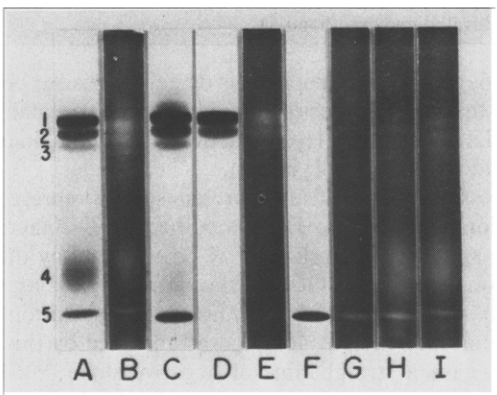

FIGURE 6: Disc gel electrophoresis of hybrid superoxide dismutase in pH 8.9 gel system. Two-tenths of a milliliter of 1.13×10^{-4} M native enzyme was mixed with 2 mL of 1.25×10^{-5} M DHT-modified, H_2O_2-inactivated enzyme (0.8 mg of each in 0.05 M KP_i, pH 7.8). The mixture was made to 8 M urea by addition of 10 M deionized urea and allowed to react for 2 h at 23 °C. (A) Following dialysis and ultrafiltration, 71 μg of the mixture was applied to the gel; (B) activity stain for 4.3 μg of the mixture of gel A; (C) admixture of native BESOD (16 μL of 1.13×10^{-4} M) with DHT-modified, H_2O_2-inactivated enzyme (40 μL of 4.8×10^{-5} M) without urea for 5 days at 0 °C; 104 μg was applied to the gel; (D) 0.3 mL of 3.18×10^{-5} M native enzyme treated with 8 M urea as described for (A); 50 μg applied to the gel; (E) activity stain for gel D, 4 μg; (F) 0.43 mL of 2.23×10^{-5} M DHT-modified, H_2O_2-inactivated enzyme treated with 8 M urea as described for A, 22 μg; (G) activity stain for gel F, 5.9 μg; (H) extracted hybrid band obtained from electrophoresis of 71 μg of mixture prepared for gel A; (I) extracted hybrid band isolated as in H but treated with 8 M urea for 2 h prior to electrophoresis.

DHT-modified enzyme. Increasing gel density retarded the DHT-modified, H_2O_2-inactivated enzyme to the same extent as native enzyme, indicating no change in molecular weight, line 5 in Figure 4. Native enzyme was mixed with an equal amount of the DHT-modified, H_2O_2-inactivated enzyme in 50 mM potassium phosphate, pH 7.8, and deionized 10 M urea was added to a final concentration of 8.0 M. The resulting pH of the solution was 9.9. The mixture was incubated for 2 h at 23 °C. Urea was then removed by dialysis against cold buffer. The specific activity of the mixture of native and modified enzymes was not affected by this urea treatment, as shown in Table IV, and thus gave no indication of chemical change. However, polyacrylamide gel electrophoresis revealed that a new and diffuse active band had been generated (Figure 6, gels A and B). In a separate experiment, readjustment of the pH back to 7.2 upon the addition of the urea produced results identical with those obtained at pH 9.9. Comparable urea treatment of native enzyme or of DHT-modified, H_2O_2-inactivated enzyme, separately, did not generate any new bands (Figure 6, gels D through G). Incubation of the native plus DHT-modified H_2O_2-inactivated enzyme, in the absence of urea, also gave rise to the new band, but much more slowly

Table V: Relative Mobilities of Superoxide Dismutase Species in Different Polyacrylamide Gel Systems

BESOD species[a]	R_m pH 8.9	R_m pH 7.5
native 1	0.32	0.18
native 2	0.36	0.22
native 3	0.41	0.26
hybrid 4	0.86 (0.68)[b]	0.58 (0.52)[b]
DHT-H_2O_2 5	1.00	0.83
DHT 5	1.00	0.78

[a] Bovine erythrocyte SOD species refer to protein bands labeled in Figures 3 and 6. [b] Number in parentheses refers to the relative mobility of a protein with a net charge intermediate between the second native BESOD isozyme (band 2) and the DHT-modified, H_2O_2-inactivated enzyme (band 5).

(Figure 6, gel C). Electrophoresis on gels of graded porosity revealed that the new species differed from the parental native and the DHT-modified, H_2O_2-inactivated enzymes, in charge but not in size (Figure 4, line 4).

The possibility that this new protein species represented a trimer composed of two DHT-modified, H_2O_2-inactivated subunits and one native subunit was discounted by the Ferguson plot. A trimeric SOD would possess a molecular weight of 48 000. When *Streptococcus faecalis* SOD (Britton et al., 1978), molecular weight 45 000, was analyzed by this technique, it gave a straight line of negative slope 0.0635[2] as opposed to the slopes obtained for the native and hybrid SOD, i.e., 0.050. Clearly if the new protein species had differed from the parental proteins by molecular weight as well as net charge, this difference would have been revealed by the Ferguson plot. This conclusion was verified by gel exclusion chromatography, in which the native, DHT-modified and new band all eluted together at a position consistent with a molecular weight of 32 000 (data not shown).

At pH 8.9 the DHT-modified enzyme migrated with the dye front and inactivation with H_2O_2 did not modify this mobility. The presumed hybrid, generated by treatment of native plus DHT–enzyme with urea, did not migrate midway between the two parental species, as shown in Figure 6. However, at pH 7.5, under which condition the DHT–enzyme did not migrate with the gel front, H_2O_2 treatment did modify the mobility, and the mobility of the hybrid was very close to that expected. These results are presented in Table V. It thus appears that at pH 8.9 the mobility of the DHT–enzyme was limited by the buffer discontinuity, marked by the dye front, and the hybrid could then not be expected to exhibit the anticipated intermediate mobility (0.68). However, at pH 7.5 when the DHT–enzyme was not trapped in the dye front, the mobility of the hybrid (0.58) was very close to that expected (0.52).

The new diffuse band appeared to be a hybrid, generated by subunit exchange between native and DHT-modified enzymes. This was verified by observing its ability to regenerate both parental species, by resegregation of subunits. Thus, the portion of the unstained gel electropherogram bearing the hybrid was cut out, homogenized gently in 50 mM potassium phosphate, pH 7.8, and was then soaked for 12 h at 4 °C in this buffer. Gel fragments were removed by centrifugation and the extract was concentrated by lyophilization, dialyzed against water at 4 °C for 18 h, and was again lyophilized. The isolated, presumed hybrid was dissolved in 50 mM potassium phosphate, pH 7.8, and was incubated at 23 °C for 2 h in the absence and the presence of 8 M urea. Subsequent electrophoresis and staining demonstrated that the hybrid had resegregated both parental species and that urea was not necessary for the resegregation of subunits (Figure 6, gels H and I). Those gel portions not used for homogenization and extraction of the hybrid were stained for protein to verify that the native enzyme and the DHT-modified, H_2O_2 inactivated enzyme had not been eluted from the gels along with the hybrid.

In further experiments, the hybrid enzyme was isolated by elution from the polyacrylamide gel during electrophoresis (vide infra). It was found that heating the isolated hybrid at 55 °C increased the extent of resegregation. When the hybrid, which had been stored for approximately 1 month at 0 °C, was subjected to electrophoresis and then stained for activity, densitometric scans of the gel revealed that approximately 25% of the total activity applied to the gel (2.3 units) represented native, resegregated BESOD. Seventy three percent of the activity represented the hybrid enzyme and 2% represented the DHT-modified, H_2O_2-inactivated enzyme. Upon heating the hybrid to 55 °C for 20 min, which had no effect on its specific activity, densitometric analysis after electrophoresis and activity staining revealed that 40% of the applied activity (2.3 units) represented native BESOD, 57% of the activity represented the hybrid, and 3% represented the DHT-modified, H_2O_2-inactivated enzyme. This result revealed that the amount of resegregation of the hybrid to yield parental enzymes was increased. The amount of native BESOD produced during resegregation nearly doubled during this heating step. The increase in the DHT-modified, H_2O_2-inactivated enzyme during heat treatment was not readily apparent on these activity stained gels due to the very low specific activity of this protein. Control experiments of heating the native enzyme alone for 20 min at 55 °C and the DHT-modified, H_2O_2-inactivated enzyme, alone for 20 min at 55 °C, had no effect upon their electrophoretic behavior.

Specific Activity of the Hybrid Enzyme. The observation that hybridization of the native enzyme with DHT-modified H_2O_2-inactivated enzyme did not change the activity of the mixture, Table IV, suggested that a native subunit exhibited the same catalytic activity when associated with another native subunit, as it did when associated with a chemically modified and catalytically inactive subunit. In that case the specific activity of the hybrid enzyme should be one-half that of the native enzyme. The hybrid band was isolated by attaching small dialysis sacs to the anodic end of the polyacrylamide gel cylinders, after the rapidly moving DHT-modified enzyme had migrated out of the end of the gel. Potential was then reapplied long enough (50 min) to move the diffuse hybrid band out of the anodic end of the gel, into the dialysis sac. The hybrid band, isolated in this way, was assayed for superoxide dismutase activity and was analyzed by polyacrylamide gel electrophoresis. As shown in Figure 3, gels E and F, the hybrid had regenerated some of the parental species, but most of the protein remained in the hybrid form. This was the case because it had been kept cold to minimize resegregation of subunits. Staining of the polyacrylamide gels from which the hybrid had been eluted revealed that the native enzyme was still present on the gel and had not eluted into the dialysis sac along with the hybrid. The amount of native enzyme which had been generated by resegregation was estimated to be approximately 17% of the total activity applied to gel F, Figure 3 (2.6 units), as determined from densitometric scanning.

Determination of the protein concentration of the isolated hybrid was based upon analysis for lysine with the amino acid analyzer. This was necessary for the following reason. Present

[2] L. N. Britton, personal communication.

in the DHT–H_2O_2-inactivated enzyme sample was a broad, diffuse band of protein which possessed a mobility on gel electropherograms similar to that possessed by the hybrid. This material, which was presumably unfolded DHT-modified enzyme, possessed no enzymatic activity and stained poorly with amido black. Consequently, this material is not evident in the photograph shown in Figure 3, gels C and D, but was visible to the eye. This material, when eluted out with the hybrid, would contribute to protein (if determined spectrophotometrically) but not to activity, thus yielding an erroneously low value for the specific activity of the hybrid. The solution of hybrid recovered from gel electropherograms was subjected to quantitative amino acid analysis to determine the total amount of protein present. The concentration of hybrid present in the solution was calculated from the amino acid analysis on the basis that the native enzyme contains 10 lysine residues per subunit while the DHT-modified enzyme has none. For the hybrid, the lysine content averages out to 5 residues per subunit. On this basis, 60% of the protein recovered from the gels was the unfolded DHT-modified, H_2O_2-inactivated protein, while the remaining protein was the hybrid. This yielded a concentration of 1.4 μM hybrid superoxide dismutase and, when this concentration was used to calculate the specific activity of the hybrid, a value of 1800 U/mg was obtained, half that of the native enzyme.

As a control, the native enzyme and the DHT-modified, H_2O_2-inactivated enzyme were also subjected to this preparative electrophoretic procedure to determine the effect upon their specific activities. As expected, this procedure was found to have no effect upon the enzymatic activity of the native enzyme and did not cause a reactivation of the DHT-modified, H_2O_2-inactivated enzyme.

Specificity of Hybridization. The ability of the native bovine erythrocyte superoxide dismutase to form hybrids with the following proteins was tested: 4-diazobenzenesulfonamide (DSM) modified BESOD, DHT-modified apo-BESOD, and the manganese containing superoxide dismutase from *Streptococcus faecalis*. Incubation of native BESOD with DHT-modified apo-BESOD in the presence of 8.0 M urea for 2 h at 23 °C, followed by dialysis against 0.05 M potassium phosphate, pH 7.8, at 4 °C, revealed only minute amounts of enzymatically active hybrid after polyacrylamide gel electrophoresis at pH 8.9. In the case of the DSM-modified BESOD and the MnSOD from *Streptococcus faecalis* attempted hybridization with native BESOD by the urea treatment described above produced no enzymatically active hybrids. These results demonstrate that the hybridization process shows subunit specificity.

Discussion

Native bovine erythrocyte superoxide dismutase does react with 4-diazosulfanilamide, under the conditions previously described (Forman et al., 1973), as evidenced by loss of activity, change in color, and amino acid modification. The earlier report that apoenzyme was reactive, but the native enzyme was not, under these conditions, is now seen to have been incorrect. However, when the diazotized sulfanilamide was adjusted to pH 7, prior to addition of enzyme, a clear difference in reactivities between apo- and holoenzymes was seen. It now appears that this was the procedure actually used.[3] The extensive self-coupling of 4-diazobenzenesulfonamide indicated that it was not the reagent of choice and its use was discontinued. When DHT was used as the attacking reagent, under conditions of controlled pH, the apoenzyme was more reactive than the holoenzyme. Thus 2.5 histidines per subunit were derivatized in the apoenzyme, with 93% loss of activity, while 0.4→0.8 histidine residues per subunit reacted in the holoenzyme and 60% inactivation was seen. The extensive lysine modification, which was observed with both apo- and holoenzymes, was probably responsible for the partial inactivation seen with the holoenzyme, since acetylation of these lysines similarly resulted in 50% inactivation. It is to be expected that histidine residues would be more reactive toward coupling reagents, such as DHT, in the apo than in the holo forms, since 6, out of a total of 8, histidines per subunit are involved in ligation to the metals at the active site (Richardson et al., 1975a,b). It is surprising, however, that only 2 histidines per subunit were exposed to DHT by removal of the metals. This suggests that the apoenzyme retains much of the configuration of the holoenzyme, a conclusion consistent with the results of NMR studies and CD studies (Stokes et al., 1973; Wood et al., 1971). The zinc-only enzyme was as resistant to DHT as was the holoenzyme, suggesting either that zinc alone is sufficient to organize the structure at the active site, as suggested by Lippard et al. (1977), or that zinc can bind into the copper site which is more exposed to solvent, and thus block access of solutes to the zinc site.

Although amino acid analysis indicated similar reactivities of holo- and of zinc-only enzymes with DHT, the specific activity of DHT-modified holoenzyme was three times greater than that of DHT-modified zinc-only enzyme. Since the activity of the zinc-only enzyme was assessed after reconstitution with Cu(II), it is possible that DHT modification led to a conformational change which prevented this reconstitution. Alternately DHT may have reacted with an amino acid residue, whose modification prevented reconstitution of the zinc-only enzyme. This residue would have to react with DHT, without spectroscopic change, and to give a derivative which was restored to the parent amino acid upon acid hydrolysis. The reaction of arginine with DHT is known to satisfy these criteria (Higgins & Harrington, 1959) and there is an arginine residue close to the active site of this enzyme (Richardson et al., 1975a,b).

Simple admixture of native and of diazo-coupled, H_2O_2-inactivated enzymes in neutral buffer was capable of forming the hybrid enzyme very slowly. The formation of this hybrid species was greatly facilitated by the presence of urea. This hybrid protein was found to be catalytically active and to possess approximately half the specific activity of the fully native enzyme. Since the hybrid was a dimeric structure, like the native enzyme, it follows that a native subunit is as active when paired with another native subunit as it is when paired with a chemically modified and catalytically inactive subunit. The mutually inhibitory subunit interactions, proposed by Fielden et al. (1974), would therefore seem to be unlikely. Thus, to account for the present results one would have to suppose that a chemically modified subunit, incapable of the usual catalytic cycle, was nevertheless fully effective in suppressing the activity of a paired, active, subunit.

The hybrid gave a band, upon polyacrylamide gel electropherograms, which was diffuse and whose mobility was midway between that of the parental species. The diffuseness of the hybrid band can be related to the electrophoretic properties of the two parental species. The DHT-modified, H_2O_2-inactivated enzyme, when electrophoresed on polyacrylamide gels at pH 7.5, migrated as a moderately diffuse band with a relative mobility of 0.83. The native enzyme

[3] H. J. Forman, personal communication.

migrates as a family of three closely spaced bands and hybridization of these with the DHT-modified, H_2O_2-inactivated enzyme should give rise to a family of closely spaced, relatively diffuse hybrid bands, which would, because of overlaps, certainly give the appearance of a single, very diffuse band. In accord with this interpretation was the reappearance of the three native bands from the hybrid band, by resegregation of subunits.

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