somewhat daunting at this time. Nevertheless, this type of combined approach may lead to the eventual determination of cell surface structure in intact biological membranes themselves.

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Chemical Modification of Arginine at the Active Site of the Bovine Erythrocyte Superoxide Dismutase†

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ABSTRACT: Several α,β-diketones inactivated the bovine erythrocyte superoxide dismutase, while modifying 1 arginine residue/subunit. With phenylglyoxal it was shown that the degree of inactivation was a linear function of the extent of arginine modification, to a limit of 1 arginine/subunit, and that arginine modification caused extensive changes in the visible absorbance attributed to the copper at the active site. Butanedione or cyclohexanedione plus borate causes a modification of arginine which is reversible by dialysis. With these reagents it was shown that reversal of arginine modification restored the lost activity. Since this reactivation was seen even when (ethylenedinitrilo)tetraacetic acid was present during dialysis, it follows that copper loss was not a factor in the inactivation which accompanied arginine modification. Analysis of tryptic fragments of cyanogen bromide peptides demonstrated that the essential arginine was no. 141 in the linear sequence. This residue is known, from X-ray crystallography, to lie within 6 Å of the active-site copper. Modification of arginine diminished activity to a limit of 10–20% of the native activity, as measured at pH 7.8. It appears possible that arginine may provide electrostatic attraction for incoming O_2^- or may serve in proton conduction during the second half of the catalytic cycle. Alternately, modification of this arginine may simply distort the ligand field of the Cu(II). Copper- and zinc-containing superoxide dismutases from wheat germ, chicken liver, and several mammalian sources all exhibited comparable sensitivity to arginine-modifying reagents.

Bovine erythrocyte superoxide dismutase catalyzes the reaction $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ and does so at a rate close to the diffusion limit, over the entire range of pH from 5 to 10 (Klug et al., 1972; Rotilio et al., 1972). The active site contains copper and zinc in close proximity, as determined by X-ray diffraction analysis (Richardson et al., 1975a,b), and the copper functions in catalysis by alternate reduction and reoxidation during successive encounters with O_2^- (Klug et al., 1973; Fielden et al., 1973). Several other copper proteins have been found devoid of this activity, as is the EDTA–copper complex (Asada et al., 1976; McCord & Fridovich, 1969).

Hydrated Cu(II) is an effective catalyst of O_2^- dismutation, but it loses its activity as the pH is raised to the neutral and weakly alkaline range, due to conversion to $Cu(OH)_2$ (Rabani et al., 1973; Koppenol et al., 1976). If the formation of the hydroxide could somehow be prevented, then free Cu(II) might exhibit an activity comparable to that of the Cu(II) at the active site of the enzyme over a broad range of pH. Even then, one would be hard put to explain the catalytic efficiency of the enzyme, since it has 130 times more surface area than does aquated Cu(II) and random collisions with the protein surface would render most collisions between the enzyme and O_2^- fruitless (Koppenol, 1979). Furthermore, the isoelectric point of BESOD[1] is 5.0 (Bannister et al., 1971) and the protein

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would acquire a progressively great net negative charge as the pH was increased from 5 to 10 and this would raise an electrostatic barrier to collision with O_2^-.

X-ray diffraction analysis has provided a possible explanation for the ability of BESOD to exhibit great catalytic efficiency over a broad range of pH. Thus, there is an arginine residue, no. 141 in the linear sequence, which is situated within 6 Å of the active-site copper (Richardson et al., 1975a,b). The positive charge on this arginine might serve to attract the anionic O_2^- into the active site. There is precedent for this concept, since arginine has been seen to play a role in binding anionic substrates in many enzymes including alkaline phosphatase (Daemen & Riordan, 1974), phosphoglycerate mutase (Riordan et al., 1977), creatine kinase (Borders & Riordan, 1975), lactate dehydrogenase (Yang & Schwert, 1972), ribonuclease A and lysozyme (Patthy & Smith, 1975b), glutamic dehydrogenase (Austen & Smith, 1976; Blumenthal & Smith, 1975), aspartate transcarbamylase (Kantrowitz & Lipscomb, 1976), and enolase (Elliot & Brewer, 1978). Alternatively, one might propose that this arginine residue might facilitate proton conduction during the very rapid catalytic cycle of this enzyme. α,β-Diketones have frequently been used in group-specific attack upon arginine residues, and we have employed several of these reagents in probing the essentiality of arginine-141 for the catalytic activity of BESOD. The results of these endeavors are reported below.

Materials and Methods

CuZnSOD from bovine erythrocytes and liver were prepared as previously described (McCord & Fridovich, 1969; Abernethy et al., 1974). The corresponding chicken liver enzyme was prepared as described by Weisiger & Fridovich (1973) while the wheat germ isoenzyme II was isolated by the method of Beauchamp & Fridovich (1973). MnSOD and FeSOD from *Escherichia coli* were isolated by the method of Keele et al. (1970) and Yost & Fridovich (1973), respectively. MnSOD from *Streptococcus faecalis* was prepared as described by Britton et al. (1978). CuZnSOD were generously provided by Dr. Marvin L. Salin, Department of Biochemistry, Mississippi State University.

Glyoxal, phenylglyoxal monohydrate, 2,3-butanedione, and 1,2-cyclohexanedione were from Aldrich Chemical Co. In some experiments the butanedione was distilled (bp 85–92 °C) immediately prior to use. Phenylboric acid and *m*-aminophenylboronic hemisulfate were also from Aldrich. Phenanthraquinone and mercaptoacetic acid were from Eastman, while sulfanilic acid was from J. T. Baker. TPCK-trypsin was from Worthington Biochemical Corp. Bio-Gel P-2 (100–200 mesh) was from Bio-Rad Laboratories, and thin-layer plates (20 × 20 cm, cellulose MN300) were from Brinkman Instruments, Inc. Pyridine from J. T. Baker was distilled over ninhydrin, and iodoacetic acid from Fluka was twice recrystallized from hexane and stored at 4 °C, in the dark, over a desiccant.

Assays. Superoxide dismutase was measured by its ability to inhibit the reduction of cytochrome *c* by O_2^-, generated by xanthine oxidase (McCord & Fridovich, 1969). Carry-over of small amounts of diketones did not interfere with this assay, but borate, which is known to inhibit xanthine oxidase (Rousch & Norris, 1950), was a problem which was circumvented by the inclusion, in the assay mix, of 0.1 M D-mannitol to scavenge the borate. This strategy completely prevented borate inhibition of xanthine oxidase without interfering with the SOD assay. However, elimination of borate with mannitol caused a gradual reversal of the borate-dependent inhibition of SOD by butanedione and by cyclohexanedione. This reversal did not become serious until 2 to 3 min after dilution into the assay mix. Consequently, activities for SOD samples treated with diones plus borate were deduced from rates taken within the first minute of cytochrome *c* reduction.

Arginine Modification. Reagents and buffers were prepared fresh daily. Addition of diones to borate buffer caused some acidification, necessitating readjustment with NaOH to the initial pH. Samples taken at intervals for measurement of residual activity were diluted directly into the xanthine oxidase–cytochrome *c* assay mixtures. Samples intended for amino acid analysis were delivered into 30% acetic acid to stop further reaction and to stabilize the modified arginine. Samples intended for metal analyses were separated from reagents by gel exclusion on a 0.9 × 23 cm column of Sephadex G-25, followed by exhaustive dialysis against 0.1 M Tris, pH 8.0, at 4 °C. Atomic absorption spectrophotometry was done with a Perkin-Elmer Model 107 equipped with a graphite furnace. Protein content of diketone-modified SOD was determined by the method of Lowry et al. (1951) with native BESOD as the standard protein.

The stability of arginine–diketone adducts was assessed by incubating L-arginine (0.8 mM) with 10–12 mM butanedione or phenylglyoxal in 0.2 M sodium bicarbonate or in 50 mM sodium borate, at pH 9.0, for 2 h at 25 °C. Samples (25 μL) were then diluted into 1.0 mL of cold 30% acetic acid and lyophilized after incubation at 4 °C for up to 72 h. The 72-h sample was lyophilized in duplicate. The lyophilized samples were dissolved in 2.0 mL of 10 mM HCl and 0.1% phenol and analyzed on the short column of the amino acid analyzer. The duplicate 72-h sample was hydrolyzed for 24 h at 110 °C in 6.0 N HCl and 0.1% phenol before being lyophilized and then redissolved and analyzed as for the first sample.

Preparation and Analysis of CNBr Peptides. BESOD at 0.41 mM in 50 mM sodium borate, pH 9.0, was incubated for 1 h at 25 °C with 15 mM butanedione. An aliquot was removed for residual activity, and the remainder was brought to 30% acetic acid and placed on a 1 × 24 cm column of Bio-Gel P-2 equilibrated with 20% acetic acid. Fractions of 1.0 mL were collected, and the protein peak was found by the manual ninhydrin method, applied after alkaline hydrolysis (Hirs, 1967). A sample of the protein peak was saved for amino acid analysis, and the remainder was lyophilized for peptide analysis. A second sample of BESOD was identically modified with butanedione, but its activity was regenerated by a 24-h dialysis vs. 50 mM potassium phosphate at pH 7.8 and 4 °C before it was desalted and lyophilized. Samples (0.8-μmol subunit) of the butanedione-modified and the modified-regenerated enzyme were dissolved in 6.0 M guanidinium chloride, 10 mM EDTA, and 50 mM sodium borate, pH 8.0, under N_2 and were reduced with 2-mercaptoethanol and carboxymethylated with iodoacetate, essentially as described by Crestfield et al. (1963). Following desalting over Bio-Gel P-2 in 20% acetic acid in the dark, aliquots were taken for amino acid analyses and the remainders were lyophilized. These dry samples were treated with 0.1 M CNBr in 70% formic acid for 24 h at 23 °C. Aliquots were taken for amino acid analysis, and the remainders were lyophilized. CNBr cleavage products were separated by gel exclusion chromatography on a 0.9 × 118 cm column of Sephadex G-75 in 20%

[1] Abbreviations used: BESOD, bovine erythrocyte superoxide dismutase; SOD, superoxide dismutase; CuZnSOD, copper- and zinc-containing superoxide dismutases; FeSOD, iron-containing superoxide dismutase; MnSOD, manganese-containing superoxide dismutase; PGO, phenylglyoxal; BDO, butanedione.

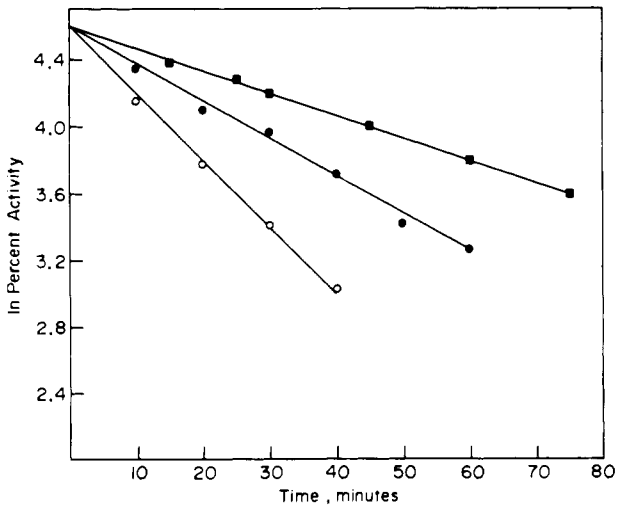

FIGURE 1: Inactivation of bovine erythrocyte superoxide dismutase by phenylglyoxal. Native BESOD (1.57 μM final concentration) was incubated in 1.0 mL of 0.125 M sodium bicarbonate, pH 8.0, at 25 °C with the following concentrations of phenylglyoxal: 5.1 (■); 10.2 (●); 15.4 (○) mM. The natural logarithm of residual activity is here presented as a function of time of incubation of the enzyme with phenylglyoxal.

acetic acid at a flow rate of 4 mL/h. Fractions of 1.0 mL were collected, and the peptide peaks were located by manual ninhydrin analysis. CNBr peptides B1 and B2 (Evans et al., 1974; Steinman et al., 1974) were sampled for amino acid analysis, and the remainders were lyophilized. CNBr peptide B2 derived from the native, butanedione-modified and modified-regenerated samples was dissolved in 0.1 M sodium borate, pH 8.0, to a concentration of 0.39 μmol/2.0 mL. TPCK-trypsin was added to 47 μg/mL, and digestion was allowed at 37 °C for 4 h. Following lyophilization, digests were taken up in 0.5 mL of 20% acetic acid and analyzed by two-dimensional mapping on thin-layer cellulose plates essentially as described by Evans et al. (1974). Plates were sprayed with ninhydrin to detect all peptides, with phenanthraquinone to detect arginine-bearing peptides, and with the Pauly reagent to detect histidine-bearing peptides (Easley, 1965; Easley et al., 1969).

Amino Acid Analysis. Amino acid analysis was performed on desalted samples containing 0.1–0.2 mg of protein after 24-h hydrolysis in 6.0 N HCl and 0.1% phenol at 110 °C. Hydrolysates were analyzed with Beckman 120C and 119 automatic amino acid analyzers employing slight modifications (Hubbard, 1965) of the original procedure (Moore et al., 1958; Moore & Stein, 1963). The cyclohexanedione-modified enzyme was hydrolyzed in 2.0 mL of 6.0 N HCl with 20 μL of mercaptoacetic acid as previously described by Patthy & Smith (1975a).

Results

Glyoxal. We began our studies with this reagent since it had already been seen, by J. M. McCord (personal communication), to inactivate BESOD. Incubation of BESOD with 40 mM glyoxal caused a progressive inactivation to a limit of 15–20% residual activity. The rate constant for this inactivation at pH 8.0 and 25 °C was 1.5 M^{-1} min^{-1}, and the limit of 80–85% of inactivation was seen at all concentrations of glyoxal tested. Amino acid analysis of BESOD inactivated with 50 mM glyoxal to the limit of 20% residual activity demonstrated a loss of close to 1 arginine and 1 lysine/subunit. Because glyoxal modified lysine as well as arginine, its use was discontinued and other diketones were tested.

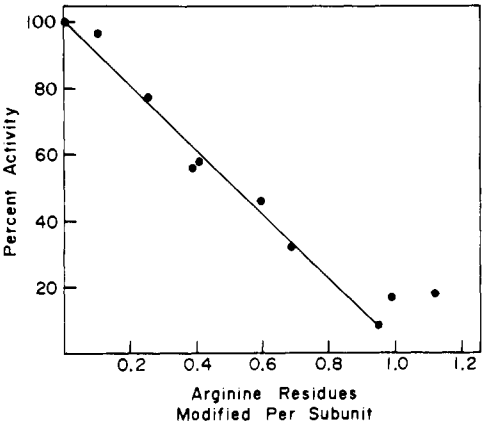

FIGURE 2: Correlation of inactivation of BESOD with arginine modification by phenylglyoxal. The data represent the results of three separate experiments. In the first experiment, native BESOD (1.99 mg/mL) was incubated in 19.5 mM phenylglyoxal and 0.12 M sodium bicarbonate, pH 8.0, 25 °C. At 10-min intervals, 5 μL was removed, diluted into 0.2 mL of 50 mM potassium phosphate, pH 7.8, and assayed for residual activity. In addition, 100 μL was removed, diluted with an equal volume of 60% acetic acid, and then dialyzed against 30% acetic acid for amino acid analysis. In the second experiment, native BESOD (2 mg/mL) was incubated in 9.5 mM phenylglyoxal and 0.125 M sodium bicarbonate, pH 8.0, 25 °C. At 13 and 26 min, 10 μL was diluted into 0.4 mL of 50 mM potassium phosphate, pH 7.8, and assayed for residual activity. An additional aliquot (50 μL) was removed, diluted into 1 mL of 30% acetic acid, and dialyzed against the same solution. In the third experiment, native BESOD (1 mg/mL) was incubated in 10.2 mM phenylglyoxal and 0.125 M sodium bicarbonate, pH 8.0, 25 °C. At 6.5 and 35 min, 10 and 15 μL, respectively, were diluted into 0.2 mL of 50 mM potassium phosphate, pH 7.8, and assayed for residual activity. An additional aliquot (100 μL) was removed, diluted into 1.0 mL of 30% acetic acid, and dialyzed against the same solution at 4 °C. Arginine modification was quantitated on the basis of 4.0 arginine residues/subunit in native BESOD.

Phenylglyoxal. This reagent also inactivated BESOD to a limiting residual activity, in this case ~10% of the initial activity. The time course of this inactivation at three different concentrations of phenylglyoxal at pH 8.0 is shown in Figure 1. The rate of inactivation was 2.5 M^{-1} min^{-1} at pH 8.0 and 4.0 M^{-1} min^{-1} at pH 9.0. The phenylglyoxal derivative of arginine has been reported to be stable in acidic media but not in neutral or alkaline media (Takahashi, 1968), and both 1:1 (Borders & Riordan, 1975) and 2:1 (Takahashi, 1968) complexes of the reagent with this amino acid have been proposed. We noted that the arginine–phenylglyoxal product could be diluted into 30% acetic acid and then incubated for 72 h at 4 °C in this medium without regenerating detectable free arginine. This provided confidence that enzyme modified with phenylglyoxal could safely be dialyzed against 30% acetic acid prior to amino acid analysis.

BESOD was incubated with 19.7 mM phenylglyoxal for 1 h at pH 8.0 and 25 °C. This enzyme, which exhibited 10% residual activity, was dialyzed against 30% acetic acid prior to lyophilization and amino acid analysis. The results of this analysis revealed the loss of ~1 arginine residue/subunit and no significant changes in the recovery of any other amino acids. Inactivation by phenylglyoxal thus appears to be due to modification of a single arginine residue per subunit. This conclusion was strengthened by inactivating BESOD to different degrees and then analyzing for the extent of arginine modification. It is clear from the results in Figure 2 that loss of activity was a linear function of arginine modification with a limit at 1.0 arginine modified/subunit.

The visible color of BESOD is due to its content of Cu(II). Modification of the enzyme with phenylglyoxal was accom-

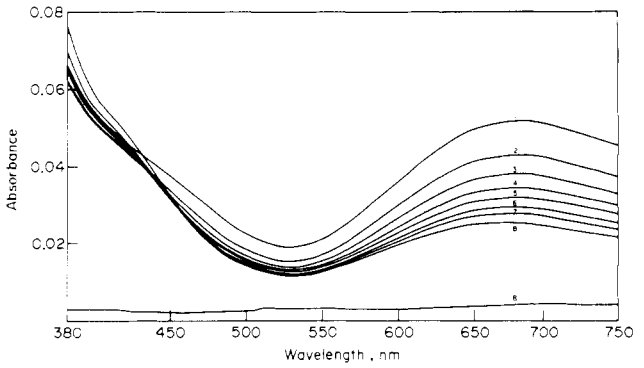

FIGURE 3: Effect of phenylglyoxal modification on the visible absorption spectrum of bovine erythrocyte superoxide dismutase. Native BESOD (0.2 mL of 24.8 mg/mL) was added to 0.8 mL of 15 mM phenylglyoxal in 0.156 M sodium bicarbonate, pH 8.0 (final concentration 1.6×10^{-4} M BESOD and 12 mM phenylglyoxal). The sample was incubated at 23 °C, and the spectrum was recorded at the following times: scan 1, 0 min; scan 2, 5 min; scan 3, 10 min; scan 4, 16 min; scan 5, 22.5 min; scan 6, 30 min; scan 7, 40 min; scan 8, 60 min. The reference solution was 0.156 M sodium bicarbonate, pH 8.0. The baseline (B) was recorded with 0.156 M sodium bicarbonate, pH 8.0, in both cuvettes.

panied by bleaching of this color. The effects of incubation with 12 mM phenylglyoxal on the optical spectrum of BESOD, in the visible range, are shown in Figure 3. This raised the possibility that modification of arginine with phenylglyoxal caused secondary loss of copper, which was then actually responsible for the loss of activity and of color. This was tested by copper analyses on samples of enzyme treated with phenylglyoxal and then freed of small molecules by gel exclusion chromatography on a 0.9 × 23 cm column of fine Sephadex G-25 equilibrated with 50 mM potassium phosphate, pH 7.8. Native BESOD had 1.97 g-atoms of Cu per mol. This was decreased by 13% when the enzyme was 40% inactivated by phenylglyoxal and by 23% when the enzyme was 63% inactivated by this reagent. It is clear that modification of one arginine by phenylglyoxal did decrease the affinity of the active site for Cu(II) and did result in some loss of Cu, but not nearly enough to account for the loss of activity or of absorption in the visible region. These results strongly suggest that the arginine modified was close to the Cu, since modification of the arginine decreased both the catalytic activity and the color associated with that Cu and moreover decreased the tightness of binding of that Cu. It nevertheless seemed desirable to seek arginine-specific reagents which did not cause loss of Cu from the enzyme.

Butanedione and Cyclohexanedione Plus Borate. These reagents offer the great advantage of reversibility. Butanedione did inactivate BESOD when incubated with it at pH 9.0 in 50 mM borate, and once again there was a limiting residual activity of ~10%. The rate of inactivation by this reagent at 25 °C was 10.9 M^{-1} min^{-1}. Borate, which was itself without effect on BESOD, was needed to support the inactivation by butanedione, and no inactivation was seen when the reagent was applied in a bicarbonate buffer.

Cyclohexanedione, whose behavior is similar to that of butanedione (Riordan, 1973; Patthy & Smith, 1975a,b), also inactivated BESOD when applied in 50 mM borate at pH 9.0, and the rate was 6.6 M^{-1} min^{-1} at 25 °C. In this case the residual limiting activity was 18%. It has been shown by Patthy & Smith (1975a) that the arginine derivative generated with cyclohexanedione plus borate is stable in 30% acetic acid and is destroyed without regenerating arginine upon hydrolysis in 6 N HCl in the presence of mercaptoacetic acid. Riordan (1973) similarly noted that the comparable butanedione adduct

Table I: Amino Acid Composition (Residues Per Subunit) of Bovine Erythrocyte Superoxide Dismutase Inactivated by Butanedione and Cyclohexanedione[a]

	sample (sp act., units/mg)				
amino acid	native (3500)	BDO–borate[b] (801)	BDO–$NaHCO_3$[c] (3500)	CHD–borate[d] (890)	CHD–$NaHCO_3$[e] (3070)
Lys	9.86	9.89	9.97	9.93	9.95
His	7.47	7.41	7.54	7.34	7.48
Arg	4.03	2.75	3.63	3.04	3.65
Asp	17.13	17.21	16.83	17.06	17.27
Thr	11.69	11.33	11.90	11.40	11.39
Ser	7.55	7.59	7.61	7.61	7.63
Glu	11.04	11.08	11.22	11.13	11.12
Pro	6.38	5.38	5.81	6.30	5.85
Gly	24.84	24.56	24.51	24.52	24.47
Ala	8.96	8.98	9.08	9.03	8.95
1/2-Cys	2.89	0.80	2.45	2.77	1.80
Val	11.65	11.41	11.95	11.20	11.28
Met	0.82	0.76	0.56	0.75	0.60
Ile	6.05	6.29	6.47	6.02	6.08
Leu	8.12	8.06	8.15	7.94	7.95
Tyr	0.93	0.95	1.04	0.91	0.92
Phe	3.97	3.98	3.94	4.01	3.87

[a] All samples were hydrolyzed in 6 N HCl and 0.1% phenol for 24 h, in vacuo, at 110 °C. Compositions were normalized to Asp + Glu + Ala = 62.0/subunit. No significant difference was observed between hydrolysis of cyclohexanedione-modified BESOD in 6 N HCl and 0.1% phenol and 6 N HCl and 1% mercaptoacetic acid. [b] Native BESOD (6.3 μM) was incubated in 9.5 mM butanedione (BDO) and 50 mM sodium borate, pH 9.0, for 45 min at 25 °C prior to activity measurements and termination of the reaction. [c] Reaction conditions were identical with those of *b* except that the buffer was 50 mM sodium bicarbonate, pH 9.0. [d] Native BESOD (6.3 μM) was incubated in 20 mM cyclohexane (CHD) and 50 mM sodium borate, pH 9.0, for 45 min at 25 °C prior to activity measurements and termination of the reaction. [e] Reaction conditions were identical with those of *d* except that the buffer was 50 mM sodium bicarbonate, pH 9.0.

was stable at pH 2.2 and was destroyed in 1.0 N HCl. We found the butanedione–borate adduct of arginine stable in 30% acetic acid at 4 °C for 72 h and noted no regeneration of arginine during subsequent acid hydrolysis. Amino acid analysis of BESOD modified with these reagents could therefore be undertaken without concern for the stability of the arginine adducts.

BESOD was not inactivated by 9.5 mM butanedione when incubated for 45 min in 50 mM bicarbonate at 25 °C and pH 9.0, but it did suffer modification of 0.4 arginine/subunit. In contrast, when borate buffer was used there was 77% inactivation and loss of 1.3 arginines/subunit. Essentially similar results were obtained with cyclohexanedione, and these data are presented in Table I. The half-cystine data shown in this table are not reliable since the protein was neither reduced and carboxymethylated nor treated with performic acid prior to hydrolysis (Moore & Stein, 1963). The nonintegral values for arginine seen with the modified proteins were not a reflection of the use of 30% acetic acid to terminate the reaction, since similar results were obtained when 1.7 N HCl was used in place of the acetic acid. Oligomerization of the diketones, described by Yankeelov (1970), was also not an apparent problem, since the use of freshly distilled butanedione did not change the results.

Reversibility. The correlation between loss of activity and modification of arginine would gain strength if it could be followed in the reverse. BESOD, which had lost 80% of its activity and 1.3 arginines/subunit as a consequence of exposure to butanedione plus borate, was restored to 93% of its original

Table II: Reversibility of BESOD Inactivation and Arginine Modification by Butanedione and Cyclohexanedione[c]

	sample (sp act., units/mg)					
amino acid	BDO-nondialyzed[a] (607)	BDO-dialyzed, KP_i[a] (3300)	BDO-dialyzed, KP_i, EDTA[a] (3475)	CHD-nondialyzed[b] (1197)	CHD-dialyzed, KP_i[b] (3565)	CHD-dialyzed, KP_i, EDTA[b] (3238)
Lys	10.00	10.00	10.00	10.00	10.00	10.00
His	7.40 ± 0.02	7.61 ± 0.02	7.56 ± 0.00	7.52 ± 0.06	7.56 ± 0.01	7.56 ± 0.03
Arg	2.69 ± 0.09	3.65 ± 0.04	3.66 ± 0.02	3.16 ± 0.03	4.00 ± 0.03	4.01 ± 0.06

[a] Native BESOD (2.3×10^{-5} M) was incubated in 9.9 mM butanedione and 50 mM sodium borate, pH 9.0, for 45 min at 25 °C. An aliquot (20 μL) was diluted into 0.2 mL of 50 mM sodium borate, pH 9.0, and assayed for residual activity. A second aliquot (0.25 mL) was diluted into 0.75 mL of 40% acetic acid and dialyzed against 30% acetic acid at 4 °C prior to amino acid analysis. A third and fourth aliquot (1 mL each) were dialyzed against 50 mM potassium phosphate, pH 7.8, and 50 mM potassium phosphate and 10^{-4} M EDTA, pH 7.8, at 4 °C for 24 h. The samples were assayed for activity and dialyzed against 30% acetic acid for amino acid analysis. [b] Reaction conditions were identical with those of *a* except that 9.8 mM cyclohexanedione was employed. [c] Values are given in residues per subunit. Compositions were normalized to Lys = 10.0/subunit. Values represent the average of duplicate analyses ± 1 SD.

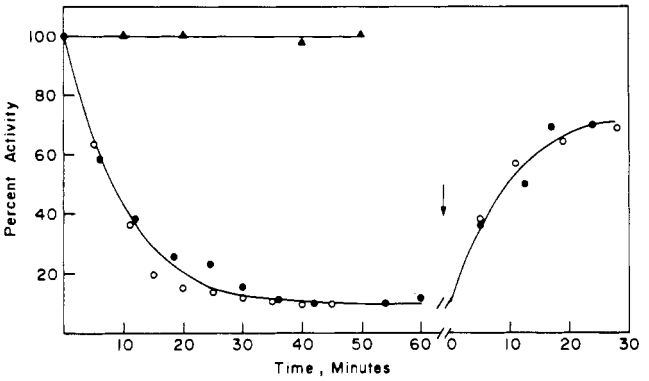

FIGURE 4: Inactivation of bovine erythrocyte superoxide dismutase by butanedione in phenylborate and *m*-aminophenylborate buffer. Native BESOD (2.1 μM) was incubated in 1.0 mL of the following solutions at 25 °C: 50 mM phenylborate, pH 9.0 (▲); 11.3 mM butanedione and 50 mM phenylborate, pH 9.0 (●); 11.3 mM butanedione and 25 mM *m*-aminophenylborate, pH 9.0 (○). At the arrow, aliquots of the inactivated samples were diluted into 3.0 mL of the cytochrome *c* assay mixture containing 0.1 M mannitol and incubated for the indicated times at 25 °C prior to the addition of xanthine oxidase and determination of residual activity.

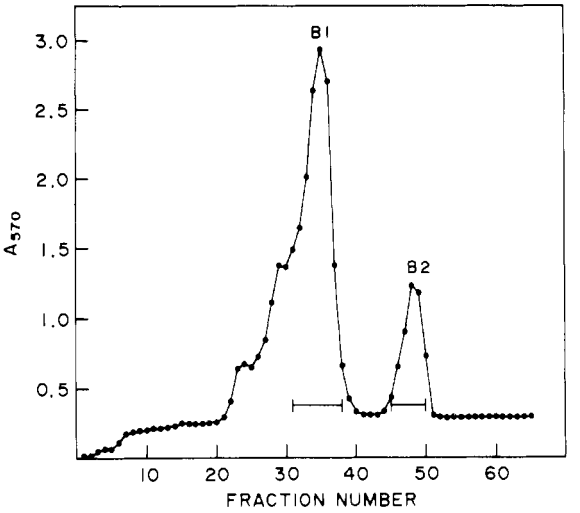

FIGURE 5: Separation of cyanogen bromide peptides from reduced and carboxymethylated butanedione-modified BESOD. The sample was treated with cyanogen bromide, and the resulting peptides were fractionated on a column of Sephadex G-75 equilibrated in 20% acetic acid as described under Materials and Methods. Fractions 31–38 were pooled and identified as cyanogen bromide peptide B1 based upon amino acid analysis (Evans et al., 1974). Fractions 45–50 were pooled and identified as cyanogen bromide peptide B2 based upon amino acid analysis (Evans et al., 1974).

activity when dialyzed against 50 mM potassium phosphate plus or minus 0.1 mM EDTA at pH 7.8 and 4 °C. This dialysis also caused the reappearance of 1 arginine/subunit. Similar reversibility of the effects of cyclohexanedione plus borate was also achieved, and these results are presented in Table II. The correlation between activity and 1 arginine/subunit thus holds both when the arginine is blocked by group-specific attack and when it is deblocked by dialysis. The fact that 0.1 mM EDTA, present during deblocking, did not prevent restoration of catalytic activity establishes that the effects of butanedione and cyclohexanedione did not depend upon labilization of copper. Borate, which is essential for the arginine-specific attack of butanedione and cyclohexanedione (Riordan, 1973; Daemen & Riordan, 1974), could be replaced by phenylborate and by *m*-aminophenylborate and once again reversibility could be demonstrated, as shown in Figure 4.

Identification of the Essential Arginine Residue. Modification of 1 arginine/subunit of BESOD markedly reduced catalytic activity. When the modifying reagent was butanedione or cyclohexanedione plus borate, the effects were reversible, and when phenylglyoxal was used, there was a bleaching of the Cu(II) spectrum and some labilization of copper. All of this suggests that the arginine attacked was residue no. 141, which lies within 6 Å of the copper in the native enzyme. We set out to gain additional support for this conclusion by peptide analysis.

BESOD treated with phenylglyoxal showed modification of 1.2 arginines/subunit. Reduction and carboxymethylation reduced this to 0.8/subunit. Partial reversal of phenylglyoxal modification of arginine during this treatment has also been noted by Weng et al. (1978). Modification of arginine with butanedione plus borate proved more resistant to the conditions of reduction and carboxymethylation. Thus, BESOD treated with butanedione plus borate, to the limit of 13% residual activity, showed 1.6 arginines modified/subunit prior to reduction and carboxymethylation and 1.4 afterwards. Borate was maintained at 50 mM throughout to gain maximal stability. A sample similarly inactivated with butanedione plus borate was subsequently restored to 90% of its initial activity by dialysis against 50 mM potassium phosphate for 48 h at pH 7.8 and 4 °C, and it then showed only 0.7 modified arginine/subunit. Both the modified and the modified-regenerated enzymes were treated with cyanogen bromide after reductive carboxymethylation. The two CNBr peptides, B1 and B2, were isolated by chromatography, as shown in Figure 5. Identical elution profiles were obtained with CNBr peptides derived from native BESOD, modified BESOD, and modified-regenerated BESOD.

Peptide B1 represents amino acid residues 1–114, and it contains two arginines, at positions 77 and 113. Peptide B2 represents residues 116–151 and also contains two arginines, these at positions 126 and 141 (Evans et al., 1974; Steinman

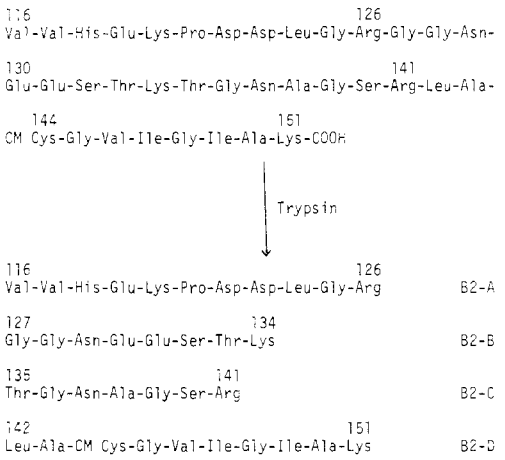

FIGURE 6: Amino acid sequence of the cyanogen bromide peptide B2 of bovine erythrocyte superoxide dismutase. Also indicated are the expected tryptic peptides of B2. CM Cys refers to *S*-(carboxymethyl)cysteine. [Taken from Steinman et al. (1974)].

et al., 1974). Amino acid analysis of peptides B1 from native, modified, and modified-regenerated samples of BESOD showed that all contained two arginines. This eliminates the possibility that the essential dione-modifiable arginine was either no. 77 or 113. In contrast, peptide B2 from modified BESOD had only 0.5 arginine, whereas the corresponding peptide from native BESOD had 2 arginines and the peptide from the modified-regenerated enzyme had 1.4 arginines. This establishes that the essential arginine was either no. 126 or no. 141.

The sequences of the CNBr peptide B2 of carboxymethylated BESOD and of its tryptic fragments are given in Figure 6. Peptides B2 from the native, modified, and modified-regenerated enzymes were digested with trypsin, and the peptide fragments so produced were analyzed by peptide mapping. Peptide spots were located with ninhydrin, arginine-containing spots with phenanthraquinone, and histidine-containing spots with diazotized sulfanilic acid. The results are summarized in Figures 7A–C. As shown in Figure 6, four tryptic peptides are expected. Of these, B2-A is the only one containing both histidine and arginine and it must therefore correspond to spot 2 in Figure 7A. B2-B contains neither arginine nor histidine, and at pH 6.5 it would have a negative charge due to an excess of glutamate over lysine. Spot 5 therefore corresponds to B2-B. Fragment B2-C contains arginine-141 and would be cationic. Spot 4 in Figure 7A therefore corresponds to B2-C. Fragment B2-D is the most hydrophobic of the tryptic fragments and would be neutral, because the positive charge on lysine-151 would be offset by the negative charge on the (carboxymethyl)cysteine-144. Spot 1 in Figure 7A therefore corresponds to B2-D. Spot 3 cannot be unequivocally identified but was probably due to an undigested fragment containing residues 127–151 since it contained arginine but not histidine.

The peptide map obtained from CNBr peptide B2 from modified enzyme (Figure 7B) showed smaller spots 1, 2, and 5, indicating that tryptic cleavage at arginine-126 was restricted, and it lacked spot 4 entirely, showing that tryptic cleavage at arginine-141 had been inhibited. This, in turn, means that arginine-141 had been the predominant site of modification when the enzyme was treated with butanedione plus borate. Spot 3 in Figure 7B probably represents undigested B2 or a composite of B2-A and B2-B plus B2-C and B2-D. In the case of modified-regenerated enzyme (Figure 7C), spot 4 was again evident with increased production of B2-D, demonstrating deblocking of arginine-141, concomitant

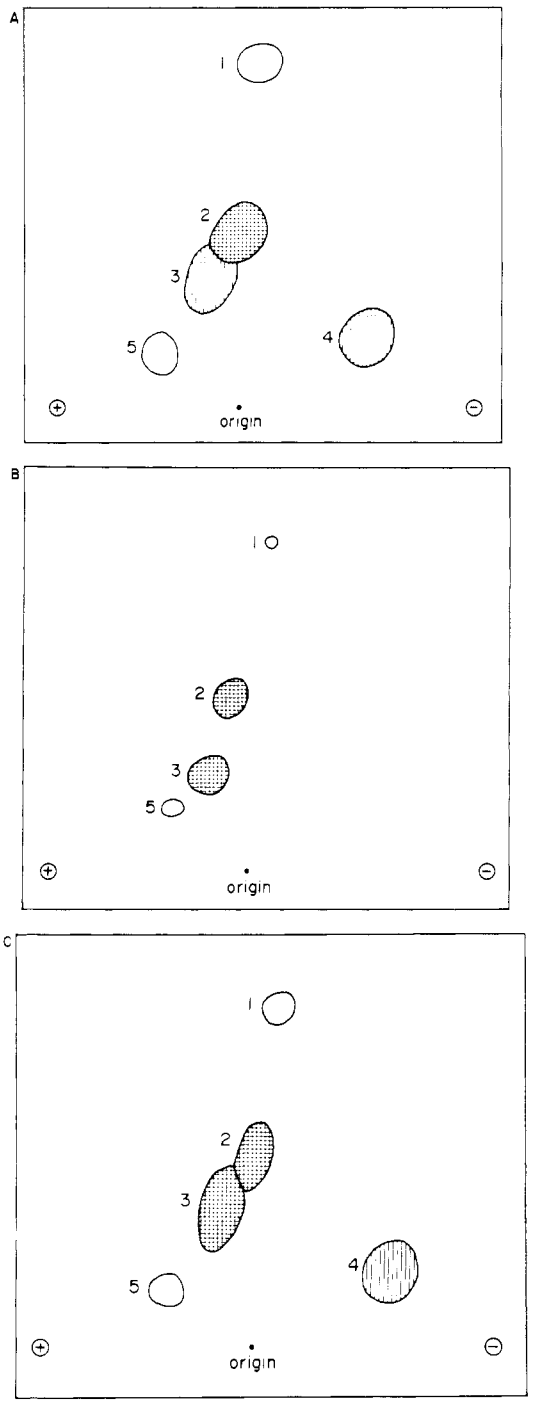

FIGURE 7: Tryptic peptide maps of the cyanogen bromide peptide B2 from native and butanedione-modified bovine erythrocyte superoxide dismutases. Digestion of the peptides by trypsin and subsequent mapping were performed as described under Materials and Methods. Ninhydrin-positive spots are enclosed by solid lines. Phenanthraquinone-positive spots are cross-hatched vertically, and diazosulfanilic acid positive spots are cross-hatched horizontally. Tryptic digests of CNBr peptides B2 from reduced and carboxymethylated (A) native BESOD, (B) butanedione-modified BESOD, and (C) butanedione-modified and regenerated BESOD.

with the restoration of activity during dialysis of the enzyme against phosphate buffer. Spot 3 in Figure 7C again probably represents undigested B2, while the other spots have the identities already discussed for the case of the native enzyme in Figure 7A.

It seems probable from these results that the essential arginine residue whose chemical modification leads to loss of catalytic activity is arginine-141, already known, from X-ray

Table III: Comparative Arginine Modification of Superoxide Dismutases

sample	% act. PGO[a]	% act. BDO[b]	% act. BDO + dialysis[c]	K_2 (M^{-1} min^{-1}) PGO	K_2 (M^{-1} min^{-1}) BDO	arg residues modified/subunit PGO	arg residues modified/subunit BDO
CuZnSOD							
bovine erythrocyte	10.2	12.0	95	2.4	10.8	1.0	1.6
apo bovine erythrocyte						3.6	
bovine liver	12.9	15.0	89.9	2.0	9.2	1.0	
chicken liver	16.0	31.5	86.1	5.7	5.0	1.1	1.5
porcine liver	16.3	14.1	100	1.9	9.2	1.3	
wheat germ	6.2	4.6	94.3		11.5	1.1	
MnSOD							
E. coli	64	65.6	80.1	0.4	0.7	2.3	
S. faecalis	63	100	92.4	0.3		1.7	
FeSOD							
E. coli	100	93	100			0.4	0.8

[a] Samples of superoxide dismutase were incubated in 15 mM phenylglyoxal (PGO) and 0.125 M sodium bicarbonate, pH 8.0, at 25 °C for 60 min. Aliquots were removed at 5-min intervals and assayed for residual activity. Second-order rate constants were calculated without correction for residual activity of the samples, except in the case of the chicken liver enzyme where the second-order rate constant was corrected for the 16% residual activity of the sample. After 60-min incubation, acetic acid was added to 30% (v/v final concentration) and the samples were dialyzed against 30% acetic acid at 4 °C prior to amino acid analysis. [b] Samples of superoxide dismutase were incubated in 10 mM butanedione and 50 mM sodium borate, pH 9.0, at 25 °C for 60 min. Aliquots were removed at 5-min intervals and assayed for residual activity. Second-order rate constants were determined after correcting for the residual activity of each sample. When indicated, acetic acid was added to the modified samples after 60-min incubation to 30% final concentration and the samples were dialyzed against 30% acetic acid at 4 °C prior to amino acid analysis. [c] Samples of superoxide dismutase were incubated as described in *b* and then dialyzed against 50 mM potassium phosphate, pH 7.8, at 4 °C for 24 h.

crystallography, to lie in the active-site region.

Survey of Superoxide Dismutases. Structural information, from X-ray crystallography, is thus far available only for one representative of the copper- and zinc-containing superoxide dismutases, and that is for BESOD (Richardson et al., 1975a,b). If the arginine in the active-site region does indeed serve an important function in the catalytic process, one might expect this structural feature to be conserved during the evolution of CuZnSOD. In effect, one would expect CuZnSOD from a variety of sources to show comparable sensitivity to arginine-specific reagents. MnSOD and FeSOD from a variety of sources show considerable sequence homology but appear unrelated to the CuZnSOD (Steinman & Hill, 1973; Bridgen et al., 1975). Furthermore, MnSOD and FeSOD, unlike CuZnSOD, show diminished catalytic activity at elevated pH (Forman & Fridovich, 1973). One might therefore venture to guess that MnSOD and FeSOD would probably not have an arginine residue in their active sites.

This reasoning prompted a survey of the effects of phenylglyoxal and butanedione upon a wide range of superoxide dismutases. The results are shown in Table III. It is clear that CuZnSOD from bovine liver, porcine liver, chicken liver, and wheat germ (isoenzyme II) were all susceptible to inhibition by phenylglyoxal and by butanedione plus borate. In each case the inhibition by butanedione plus borate was reversible and ~1 arginine/subunit was modified. In the case of the apo-BESOD, essentially all four of the arginines per subunit were modified by phenylglyoxal, indicating that the native structure somehow masked all of the arginines, save the one in the active-site region.

The possibility that the diketones might have exerted their effect by modifying the single thiol residue of BESOD can be eliminated since the crystal structure shows this thiol to be buried within the β barrel (Richardson et al., 1975a,b). Furthermore, the activity of BESOD is unaffected by *p*-(chloromercuri)phenylsulfonate, much as we previously noted it to be resistant to iodoacetate (D. P. Malinowski and I. Fridovich, unpublished experiments). In the case of the chicken liver CuZnSOD, which have a reactive thiol, modification of this thiol with the mercurial had no effect on enzymatic activity (Weisiger & Fridovich, 1973). We have also tested *p*-(chloromercuri)phenylsulfonate on the porcine and wheat germ enzymes and found it to have no effect. In the cases of MnSOD or FeSOD, there was some arginine modification, but little or no loss of activity, suggesting that these enzymes, unlike the CuZnSOD, lack an essential arginine.

Discussion

In toto, the evidence presented above suggests that arginine-141 of BESOD, which lies within the solvent access channel to the active site and whose guanidinium group is positioned within 6 Å of the copper, is important for the catalytic activity of this enzyme. Thus, several α,β-diketones known to exert group-specific attack upon arginine caused loss of activity. None of the CuZnSOD enzymes studied were affected by thiol-specific reagents, thus eliminating the possibility that the diketones exerted their effects by modifying thiols. In the case of glyoxal, 1 arginine and 1 lysine were modified per subunit, but with phenylglyoxal, butanedione, and cyclohexanedione, only arginine was modified. With phenylglyoxal the degree of inactivation was proportional to the extent of arginine modification, to a limit of 1 arginine/subunit at maximal inactivation. Both butanedione and cyclohexanedione give unstable diol adducts with the guanidine portion of arginine which can be stabilized with borate, which acts by adding onto adjacent hydroxyl groups. With these reagents, borate was essential for inactivation to occur and reactivation could be achieved by dialysis against phosphate buffer. Inactivation by butanedione or cyclohexanedione plus borate correlated with modification of arginine, and reactivation by dialysis correlated with deblocking 1 arginine/subunit.

There were several indications that the arginine, whose chemical modification caused inactivation, must lie close to the active-site copper. The first of these was, of course, the inactivation itself. The second was the effect of arginine modification upon the visible absorption of the enzyme, which is entirely due to the Cu(II). This spectral modification was also seen with butanedione plus borate but was most profound with phenylglyoxal. The third indication was a modest la-

bilization of copper, seen only with the phenylglyoxal. Modification of the essential arginine with this reagent apparently perturbed the active-site region more than did corresponding modification with glyoxal, butanedione, or cyclohexanedione.

The most powerful evidence that the essential arginine was no. 141 was obtained by peptide analysis of native, modified, and modified-regenerated BESOD. Cyanogen bromide B2 alone bore the modified arginine, and this immediately halved the range of possibilities from 77, 113, 126, and 141 to only 126 or 141. Then, tryptic digestion of B2 showed that the site of greatest modification was arginine-141. There was evidence of some modification also at arginine-126, and this was to be expected since butanedione plus borate modified somewhat more than 1 arginine/subunit. It appears that arginine-126 could to some extent be modified by butanedione, in the absence of borate, and that this could not be reversed by dialysis and was not associated with loss of catalytic activity.

BESOD retained some activity even after modification with arginine-specific reagents. The extent of this residual activity depended upon the specific reagent used but never exceeded 20% of the native activity. It may be that the active-site arginine is important in the electrostatic guidance of the incoming O_2^- to the catalytic center but that such electrostatic guidance is not entirely essential for activity. If we may judge from the effect of arginine-modifying reagents, we would estimate that this arginine increased catalytic efficiency by $\sim$1 order of magnitude. It is apparently a sufficiently important factor in this catalysis to have been retained during long periods of evolutionary change, as evidenced by the similar behavior of CuZnSOD from chicken, cow, pig, and wheat.

Another possible role for the active-site arginine involves proton conduction, which could be a limiting factor in catalysis were a special facilitating mechanism not provided. We envision O_2^- binding, in a bridging position, between Cu(I) and the guanidinium group of arginine-141 during the second half of the reaction of the catalytic cycle. Transfer of an electron from the Cu(I) and of a proton from the arginine would yield the leaving group, i.e., HO_2^-, which would protonate to H_2O_2 in free solution. The guanidine group of arginine-141 would then recover a proton from the solvent and be ready for another cycle. A less interesting possibility is that modification of an arginine residue within 6 Å of the Cu(II) sterically perturbs the active-site region and so diminishes its catalytic activity.

Superoxide dismutases based upon manganese or iron appeared not to possess an arginine uniquely important for activity. In these enzymes, the metal cycles between the trivalent and divalent states during catalysis and the charge on the metal itself may provide a sufficient electrostatic effect to attract O_2^-. In contrast, the copper of CuZnSOD cycles between the divalent and monovalent states and the presence of an imidazolate ligand bridging the copper to the zinc and an aspartate ligand to the zinc would practically eliminate residual positive charge when the copper was in the univalent half of its catalytic cycle. It is tempting to speculate that the ligands to the manganese and to the iron in MnSOD and FeSOD, when identified, will be found not to diminish the charge on these metals. We might also anticipate that a lysine residue lies within the active-site region of these enzymes and functions in catalysis as does the arginine in CuZnSOD.

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Subunit-Selective Chemical Modifications of Creatine Kinase. Evidence for Asymmetrical Association of the Subunits†

Yair Degani* and Chemda Degani*

ABSTRACT: The two reactive thiol groups in the dimeric enzyme creatine kinase (CK) react nonidentically with the cyanylating reagent 2-nitro-5-thiocyanobenzoic acid (NTCB). While in one subunit the thiol undergoes cyanylation, the other subunit thiol abnormally forms a mercaptonitrobenzoate (TNB) mixed disulfide. The resulting derivative, *S*-CN-*S'*-TNB-CK, is catalytically inactive. Cyanolysis of this derivative with KCN rapidly produces the dicyano enzyme *S,S'*-di-CN-CK, which possesses 75% of the original enzymic activity. The same active derivative is also formed by total cyanolysis of the inactive derivative *S,S'*-di-TNB-CK, produced by a previous reaction of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). During the cyanolysis of *S,S'*-di-TNB-CK, one TNB group is displaced much more rapidly than the other. The regeneration of enzymic activity coincides solely with the faster of the two reactions. The 75%-active half-cyanolysis product, *S*-TNB-*S'*-CN-CK, is the isomer of the inactive *S*-CN-*S'*-TNB-CK produced by NTCB. These results suggest that the subunits of CK are asymmetrically associated. Difference spectrophotometry measurements have shown that the inactive derivative *S*-CN-*S'*-TNB-CK is capable of forming the ternary complex E·MgADP·creatine at both of its subunits but fails to form the quaternary transition-state analogue (t.s.a.) complex E·MgADP·NO_3^-·creatine at either subunit. In contrast, the 75% active *S,S'*-di-CN-CK is capable of forming the t.s.a. complex at each of the subunits. Hence, the single TNB blocking group in *S*-CN-*S'*-TNB-CK which prevents catalysis in the neighboring cyanylated subunit also eliminates the ability of that subunit to form the t.s.a. complex.

In almost all of the oligomeric proteins whose quaternary structure has been determined by X-ray crystallography, the protomers were found to be arranged in a strictly symmetrical manner, as had been postulated by Monod et al. (1965). In three exceptional cases, insulin (Adams et al., 1969), α-chymotrypsin (Birktoft & Blow, 1972; Tulinsky et al., 1973), and yeast hexokinase B (Steitz et al., 1973; Anderson et al., 1974; Anderson & Steitz, 1975), asymmetry in the apoprotein arrangement in the crystal has been observed. However, the relevance of these crystallographic observations to the state of these proteins in solution has been questioned (Levitzki & Koshland, 1976; Hoggett & Kellett, 1976). Thus far, there has been no evidence to show that identical protomers can combine in an asymmetrical manner in solution phase.

In this paper we present results of chemical modification studies on the dimeric enzyme creatine kinase, suggesting that the subunits in this enzyme are associated asymmetrically in solution. The enzyme (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) contains two catalytic sites and two reactive thiol groups per dimer of molecular weight 82 600 [for a review, see Watts (1973)]. In denaturating media the enzyme dissociates into two subunits, each consisting of a single polypeptide chain. Detailed analyses of the tryptic peptides, coupled with quantitative end group analyses (Yue et al., 1967) as well as peptide mapping in a variety of different systems (Dance & Watts, 1962; Thomson et al., 1968; Gosselin-Rey & Gerady, 1970; Kumudavalli et al., 1970; Atherton & Thomson, 1969; Focant, 1970), point heavily to an identity of the two polypeptide chains. In particular, the two reactive thiol groups were shown to be identical, in that they both formed part of a unique 25-residue tryptic peptide sequence (Thomson et al., 1964; Atherton et al., 1970; Mahowald, 1965). In this paper we report that these two sequentially identical thiol groups react in a nonidentical manner with the cyanylating reagent 2-nitro-5-thiocyanobenzoic acid to form a catalytically inactive enzyme derivative, differentially modified at its two subunits. By demonstrating the formation of the highly active isomer of this derivative, we provide evidence to suggest that the subunits in this enzyme are asymmetrically associated.

Materials and Methods

Materials. Rabbit muscle creatine kinase (CK)[1] was obtained from Sigma. Most of the work described herein was done with enzyme from lot 115C-9580. The enzyme was found homogeneous in the following systems: (1) electrophoresis on 7% polyacrylamide gel at pH 8.3 (Davis, 1964);

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[1] Abbreviations used: CK, creatine kinase; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); NTCB, 2-nitro-5-thiocyanobenzoic acid; TNB, 2-mercapto-5-nitrobenzoic acid or its residue in mixed disulfide linkage; t.s.a., transition-state analogue.

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