Succinylated Copper, Zinc Superoxide Dismutase. A Novel Approach to the Problem of Active Subunits†

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ABSTRACT: Bovine erythrocyte superoxide dismutase (BESOD) has been extensively succinylated with succinic anhydride. Succinylated BESOD has an identical electron paramagnetic resonance (EPR) spectrum but only 10% as much activity as the native enzyme, showing that an increase of the negative charge of the protein surface lowers the activity without alteration of the active site structure. On the other hand, sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis indicates that interaction between subunits is strongly weakened by succinylation. $NaDodSO_4$ has no effect on either the activity or EPR spectrum of the protein. BESOD was immobilized by coupling to a Sepharose matrix with no alteration of the EPR spectrum. Succinylation of the immobilized protein led to detachment from the gel of approximately 50% of the molecules, as estimated by parallel EPR measurements of the gel and activity determinations on the eluate. It is concluded the succinylation leads to dissociation of BESOD into nondenatured subunits, having lower activity than the native protein possibly because of charge effects on the enzyme–O_2^- interaction.

A recent line of research on copper, zinc superoxide dismutase (SOD)[1] concerns the dissociation of the active dimeric protein to nondenatured monomers. All proteins of this class are made of two identical subunits (16 000 daltons), each carrying a dimetal cluster at the active site, consisting of one copper and one zinc ion. The interest in this object arises from the question of whether cooperative interactions actually occur between the two subunits in the catalytic mechanism (Fielden et al., 1974) as well as in other functional situations (Rotilio et al., 1977). No clear solution has yet been provided to the problem of obtaining nondenatured subunits of these enzymes to measure their activity as compared to that of the native enzyme. Up to date results related to this problem can be summarized as follows. Rigo et al. (1978) reported in detail the activity changes of the SOD isoenzyme from wheat germ that, contrary to most CuZnSOD, is dissociated by $NaDodSO_4$ (Beauchamp & Fridovich, 1973). In the presence of 4% $NaDodSO_4$, which has no effect on the activity of the CuZnSOD's that maintain the dimeric structure in $NaDodSO_4$, the monomer from the wheat germ isoenzyme was practically inactive and was found to be denatured as monitored by drastic changes of the copper EPR signal. After removal of the detergent, the activity was fully restored in a biphasic process, the first step of which involved the refolding of monomers and accounted for the complete recovery of the native EPR spectrum and 50% recovery of the activity. By this approach it appears that at least partially active monomers could transiently be observed. Recovery of activity only to a partial extent could be related to residual binding of $NaDodSO_4$ to the protein, which, although not directly occurring at the active site, as suggested by EPR spectra, could still be influencing the interaction of the enzyme with O_2^-. Further work reported the effect of 8 M urea on swordfish CuZnSOD (Bannister et al., 1978). Evidence from gel exclusion chromatography suggested dissociation into subunits, while activity was fully retained. Marmocchi et al. (1978) reported similar results with bovine and yeast CuZnSOD, indicating that the effect of urea was general for all enzymes of the same class. However, the same authors pointed out that determinations of molecular weight from gel exclusion chromatography in the presence of chaotropic agents "rely on the assumption of complete denaturation of the molecule under study. Therefore conclusions are uncertain especially when a molecule that does not apparently change any typical property in 8 M urea, such as bovine superoxide dismutase, is compared to proteins routinely used as molecular weight standards in denaturing conditions". In fact, later on Malinowski & Fridovich (1979a) demonstrated by sedimentation equilibrium analysis that bovine CuZnSOD retained its native molecular weight in 8 M urea. Nevertheless, some destabilization of the quaternary structure of protein should actually be occurring in those conditions, as Marmocchi et al. (1978) showed that small but significant amounts of hybrid dimers formed when a mixture of Cu, Zn enzymes extracted from two different sources was incubated in 8 M urea and the urea was then dialyzed out, while they were not detectable in control incubations in buffer or when urea was not removed. Therefore, it is in principle possible that other agents could dissociate the dimer more extensively, without affecting the tertiary structure of the monomer itself. In other words, a condition may be found that stabilizes the transient intermediate observed during renaturation of the wheat germ isoenzyme (Rigo et al., 1978). In the present work succinylation of the accessible groups of the bovine protein was explored with a view to possible destabilizing effects on quaternary structure mediated by extensive changes of the surface charge.

Materials and Methods

Bovine erythrocyte superoxide dismutase (BESOD) was purified by the method of McCord & Fridovich (1969). The following proteins were obtained by the indicated sources: horse heart cytochrome *c* (type III), horse muscle myoglobin

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[1] Abbreviations: SOD, superoxide dismutase; BESOD, bovine erythrocyte superoxide dismutase; s-BESOD, succinylated bovine erythrocyte superoxide dismutase; $NaDodSO_4$, sodium dodecyl sulfate; EDC, *N*-ethyl-*N*-[3-(dimethylamino)propyl]carbodiimide hydrochloride; $BESOD_{ins}$, bovine erythrocyte superoxide dismutase coupled to a Sepharose gel; EDTA, ethylenediaminetetraacetic acid.

0006-2960/82/0421-2853$01.25/0

(type I), and bovine serum albumin from Sigma Chemical Co.; bovine erythrocyte carbonic anhydrase from the same preparation method as superoxide dismutase.

Ultrapure sodium dodecyl sulfate ($NaDodSO_4$) was purchased from Serva Feinbiochemia and urea from Merck. Succinic anhydride was obtained from K & K Laboratories and *N*-ethyl-*N*-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) from Fluka. Ultrogel AcA54 was purchased from LKB and CH-Sepharose-4B from Pharmacia.

Superoxide dismutase activity was assayed polarographically according to Rigo et al. (1975). Protein concentration was measured by the method of Lowry (1951). Metal analysis was made by atomic absorption (Hilger and Watts Atomspek Model H 1170). Copper concentration was also evaluated by double integration of the EPR signal against a copper–EDTA standard solution. EPR spectra were recorded at X band and liquid nitrogen temperature with a Varian E-9 spectrometer. Polyacrylamide gel electrophoresis was carried out according to Davis (1964) and $NaDodSO_4$–polyacrylamide gel electrophoresis according to Weber & Osborn (1969). Gel filtration chromatography was carried out on a column of Ultrogel AcA54 (1.6 cm × 83 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4, at an elution rate of 12 mL/h (2-mL fraction volume). Three milligrams of each protein was applied to the column. Succinylation was performed essentially according to Klotz (1967). Typically, proteins were dissolved in H_2O in the presence or absence of 8 M urea to give a final protein concentration of 5 mg/mL. Solid succinic anhydride (2 mg/mg of protein) was added in several small portions over 60 min at 25 °C with continuous stirring. During the course of the reaction the pH of the mixture was maintained between 8 and 9 by the addition of 0.2 N NaOH. Reagents were removed by dialysis against H_2O. The extent of modification of lysine side chains was evaluated by treating samples of the succinylated proteins with nitrous acid (Van Slyke, 1929), followed by extensive dialysis and 24-h hydrolysis at 105 °C with 6 N HCl. The number of lysine residues succinylated and hence protected against reaction with nitrous acid was then identified by amino acid analysis of the hydrolysates.

Coupling of BESOD to CH-Sepharose-4B was obtained by the following procedure. A 0.26-mL aliquot of a 0.7×10^{-3} M BESOD solution was mixed with 3 mL of swollen CH-Sepharose-4B suspended in 6 mL of H_2O in an end-over-end mixer. After 30 min the concentration of SOD in the aqueous solution as monitored by activity was 1.2×10^{-5} M. This figure indicates that practically no substantial amount of BESOD was retained by the gel by any kind of noncovalent interaction. At this stage EDC at a concentration of 1 mg/mL was dissolved in the coupling mixture, and the decrease of the enzyme activity in the supernatant was monitored with time. After 1 h from EDC addition the concentration of BESOD in the aqueous phase was 0.3×10^{-5} M. The gel was repeatedly washed with 0.5 M NaCl and then with water until the concentration of SOD in the washing solution decreases by orders of magnitude. The EPR spectrum of the coupled BESOD was identical with that of the soluble enzyme. No effect of EDC on the enzyme activity of BESOD was observed in parallel control measurements, including 5×10^{-4} M sodium acetate as the counterpart of CH-Sepharose, to allow amide formation as in the coupled enzyme. The concentration of BESOD coupled to the gel was 0.7×10^{-4} M from EPR spectra of gels and 0.5×10^{-4} M from activity measurements. The latter value was obtained as the difference between the initial amount of enzyme added to the gel and that measured in the washing solution after the coupling reaction. Both values

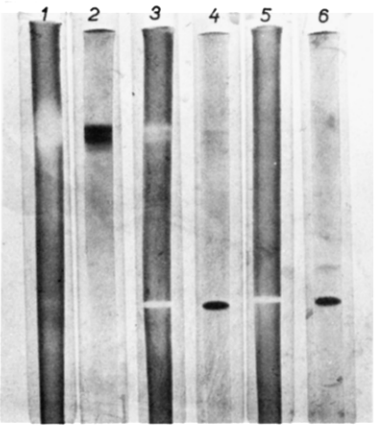

FIGURE 1: Polyacrylamide gel electrophoresis of BESOD: (1 and 2) native protein; (5 and 6) the protein treated with succinic anhydride as described in the text; (3 and 4) the protein treated with half the amount of succinic anhydride as used in gel 5. Gels 1, 3, and 5 were stained for enzyme activity (5 μg of protein was applied to the gels); gels 2, 4, and 6 were stained for protein (25 μg of protein was applied to the gels).

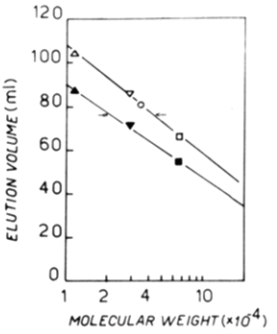

FIGURE 2: Gel filtration chromatography of BESOD and s-BESOD. The column was calibrated with native (open symbols) and succinylated (solid symbols) protein standards: cytochrome *c* (△, ▲); carbonic anhydrase (▽, ▼); serum albumin (□, ■). (○) BESOD; (→) points to the elution volume of s-BESOD on both calibration curves.

are within the error limits of the theoretical value of 0.6×10^{-4} M expected on the basis of the initial amount of enzyme added and subsequent dilution in the gel. For succinylation experiments on gel, succinic anhydride or sodium succinate was added in small aliquots up to a 0.1 M concentration to a stirred suspension (0.5 mL) of coupled gel with 1 mL of water. The pH of the suspension was kept in the range pH 6.5–9 by careful addition of 0.5 M NaOH. After complete dissolution of the anhydride or the salt, the solution was allowed to react for 1 h. Bovine carbonic anhydrase was coupled to the gels and subsequently treated in identical conditions.

Results

Succinylation of BESOD. Figure 1 shows polyacrylamide gel electrophoresis of BESOD after treatment with succinic anhydride. The gel of the native protein shows the three-band pattern typical of samples of pure BESOD (Malinowski & Fridovich, 1979a). The gels of protein treated with two increasing amounts of succinic anhydride show only one fast band in the sample treated with the higher reagent concentration, indicating extensive succinylation. In this case, amino acid analysis demonstrated complete modification of the lysine residues, irrespective of the presence of 8 M urea during the succinylation treatment.

Gel Exclusion Chromatography of Succinylated BESOD (s-BESOD). If the exclusion volume of s-BESOD in Ultrogel AcA54 is compared to that of succinylated proteins of known

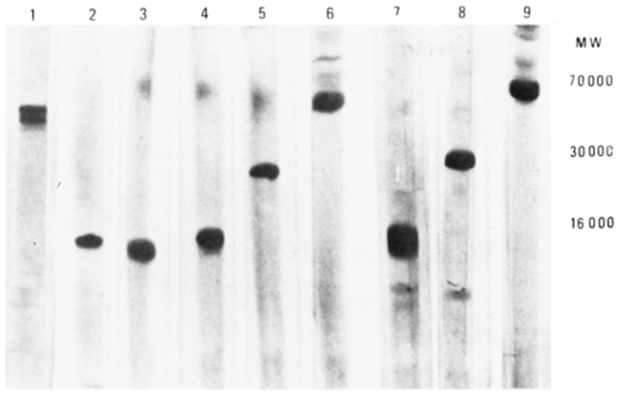

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of BESOD and monomeric proteins of known molecular weight: (1) BESOD; (2) denatured BESOD; (3) s-BESOD; (4) succinylated myoglobin; (5) succinylated carbonic anhydrase; (6) succinylated bovine serum albumin; (7, 8, and 9) same as gels 4, 5, and 6 but not succinylated. 50 μg of protein was applied to each gel.

molecular weights (Figure 2), its apparent radius of gyration is between that of cytochrome *c* and that of carbonic anhydrase. This would indicate that its molecular weight approaches that of the BESOD subunit. If, however, s-BESOD is compared to native proteins, including native BESOD, its radius of gyration lies in the molecular weight range of 40 000–50 000. These results demonstrate that succinylation increases the radius of the proteins examined and that the method can be applied to the evaluation of molecular weights only in the case that the extent of succinylation be comparable for all proteins examined and the radius expansion be proportional to the succinylation extent according to an identical dependence law. Therefore gel exclusion experiments cannot give conclusive evidence on the quaternary state of s-BESOD, although they do not rule out that the molecule with a larger radius than that of the native protein might be the succinylated subunit, as suggested by empirical correlation with other succinylated monomeric molecules.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis of s-BESOD. Figure 3 shows the results of a polyacrylamide gel electrophoresis in 0.1% $NaDodSO_4$. In these conditions the calibration proteins moved identically, either succinylated or not, showing that succinylation did not affect $NaDodSO_4$ binding to protein and consequently the evaluation of molecular weight by this method. In the same conditions, while BESOD moved as an apparent tetramer according to previous observations (Weser et al., 1971; Malinowski & Fridovich, 1979a), s-BESOD had a mobility comparable to that of denatured BESOD and myoglobin and much higher than that of succinylated carbonic anhydrase ($M_r \simeq 30\,000$). This suggests that the mobility of s-BESOD in the presence of 0.1% $NaDodSO_4$ may represent dissociation into subunits and not simply an increase in negative charge, conferred by succinylation, of the undissociated dimer.

This behavior did not require either prior incubation in $NaDodSO_4$ or heating. On the other hand the mobility of BESOD was not affected by long incubation in the presence of much higher $NaDodSO_4$ concentrations (Rigo et al., 1978) and compared with that of proteins of molecular weights in the range of SOD subunits only if incubated in 1% $NaDodSO_4$ with concurrent heating at 60 °C or treatment with β-mercaptoethanol (Figure 3, gel 2, and Malinowski & Fridovich, 1979b). Therefore the very mild conditions that lead to dissociation of subunits of s-BESOD are a valid indication of at least a very much weakened interaction between subunits after succinylation.

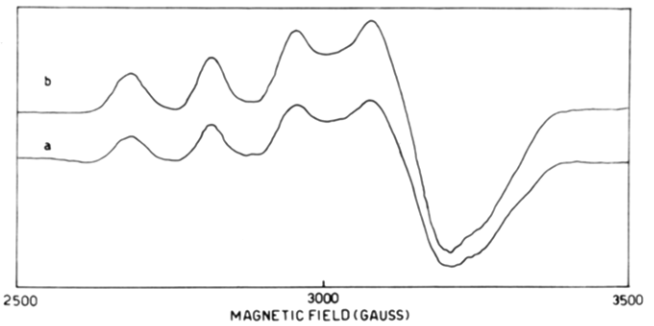

FIGURE 4: EPR spectra of BESOD: (a) Native protein (1.2×10^{-4} M copper) in 0.05 M phosphate buffer, pH 8.0; (b) succinylated protein (3.6×10^{-4} M copper) in the same buffer. EPR conditions: frequency 9.14 GHz; modulation amplitude 10 G; microwave power 10 mW; temperature −170 °C. Amplification factor of spectrum a = ×2.

EPR Spectra and Enzyme Activity of s-BESOD. EPR spectra (Figure 4) show that the copper coordination typical of native BESOD is not affected by succinylation. On the contrary, the activity of s-BESOD was approximately 10% of that of the native enzyme [$k = (1.64 \pm 0.01) \times 10^8$ M^{-1} s^{-1} from three separate succinylation experiments, as referenced to the copper content of the sample]. Incubation of s-BESOD in $NaDodSO_4$ under the conditions of gel electrophoresis had no effect on either the EPR spectrum or the activity of the protein.

Succinylation of Sepharose-Coupled BESOD. Treatment of BESOD coupled to a Sepharose gel ($BESOD_{ins}$) with succinic anhydride under the conditions used for the soluble protein led to a 50% decrease of the EPR copper signal of the enzyme bound to the gel. This value is that expected if complete dissociation of $BESOD_{ins}$ had occurred in the case that all of the BESOD dimers were coupled to gel particles through only one subunit. A confirmatory result was given by measurements carried out on the supernatant obtained after treating the gel with succinic anhydride. The soluble protein material recovered after such a treatment was found to be completely succinylated. The same amount of protein was detected in the supernatant either by amino acid analysis or by taking the kinetic constant of 1.64×10^8 M^{-1} s^{-1} for soluble s-BESOD. On the basis of this value approximately 60% of $BESOD_{ins}$ was detected in solution after succinylation. No activity was found in the supernatant solution after repeating the treatment with succinic anhydride.

If $BESOD_{ins}$ was extensively washed with buffer (or sodium succinate), 8 M urea, or 4% $NaDodSO_4$, activity and protein were not practically lost into solution and a decrease in the EPR signal of $BESOD_{ins}$ was not observed. If a monomeric enzyme of comparable isoelectric point and size, such as carbonic anhydrase, was coupled to the gel, no protein was found in the solution, as measured by amino acid analysis, after treatment with either succinic anhydride or sodium succinate.

Discussion

Succinylation of BESOD led to full derivatization of the lysine residues of the protein independently of the presence of 8 M urea during the treatment. This result indicates that all lysines are accessible in the native protein. Treatment with succinic anhydride produces a derivative (s-BESOD) with a much increased negative charge (see Figure 1). This modification is reflected by a 10-fold decrease of enzyme activity, although the active site, as probed by EPR, is not affected by the treatment (Figure 4). The extent of inactivation is the same as the limit inactivation value obtained by Malinowski & Fridovich (1979c) by neutralization of a critical positive

charge located near the copper active site and provided by Arg-141 in the linear sequence of the protein. This result confirms the suggestion (Malinowski & Fridovich, 1979c) that positive charges near the copper are necessary for optimal encounter between O_2^- and copper. However, in the experimental conditions used by these authors the ligand field of the copper was apparently distorted as monitored by changes of the optical spectrum and by the decreased tightness of copper binding. In the experimental conditions of the present work no such alterations were observed, and therefore the results with s-BESOD definitively demonstrate that the integrity of the copper coordination is not the only factor that determines the catalytic efficiency of the enzyme. The present suggestion that derivatization of lysine residues with succinic anhydride alters activity by increasing the negative charge of the protein is in apparent conflict with the relatively constant activity of the enzyme between pH 5 and pH 10. However, the original activity data of Klug et al. (1972) do actually show a 2-fold decrease of the catalytic constant above pH 7.0, and this result has been confirmed by recent measurements (E. Argese, B. De Carli, E. Orsega, A. Rigo, and G. Rotilio, unpublished results).

Furthermore, succinylation led to solubilization of 50% of the BESOD molecules coupled to Sepharose ($BESOD_{ins}$) while no such effect was observed with carbonic anhydrase, which is monomeric. This result can only be explained if dissociation of noncoupled subunits of dimers attached to the gel through just one subunit had occurred. In fact neither urea nor Na-DodSO_4 nor succinate brought about even minimal solubilization on exhaustive gel washing. The lack of effect by succinate excludes binding of superoxide dismutase and carbonic anhydrase by charge interaction, while lack of effect by the anhydride on the bound carbonic anhydrase rules out that the reagent might just reverse the covalent attachment of the enzyme to the support, by transamidation, for example.

The same conclusion that succinylation strongly destabilizes the dimeric structure of BESOD is suggested by the results obtained in NaDodSO_4 electrophoresis experiments (Figure 3), where under the relatively mild conditions used (short exposure to 0.1% detergent without preheating) s-BESOD had a mobility comparable to that of succinylated cytochrome *c* or myoglobin. However, for this case, it is difficult to discriminate between an actual dissociation and a very weak association of the subunits, which then dissociate with Na-DodSO_4. Anyway, it should be kept in mind that 0.1% Na-DodSO_4 affected neither the EPR spectrum nor the activity of s-BESOD, while all treatments reported up to date that lead to dissociation of subunits, even in the presence of concentrated NaDodSO_4, such as 6 M guanidinium chloride, β-mercaptoethanol, or heating at 60 °C, are known to denature the protein as well (Malinowski & Fridovich, 1979b).

In conclusion, all evidence supports the contention that succinylation of BESOD is the first reported condition where stable and active monomers of BESOD, with an apparently unaltered copper site, can be studied in aqueous solution. Although it should be kept in mind that results of gel filtration chromatography indicate that partial unfolding may occur on succinylation, it is clearly not relevant to the integrity of the active site. Therefore, the results presented here are in line with previous evidence that a native subunit of BESOD exhibits the same activity, whether paired with another native subunit, as in the natural dimeric enzyme, or with catalytically inactive subunits, as obtained by either chemical modification of the protein moiety (Malinowski & Fridovich, 1979a) or inert metal substitution (Cocco et al., 1981).

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