On the Quaternary Structure of Copper–Zinc Superoxide Dismutases. Reversible Dissociation into Protomers of the Isozyme I from Wheat Germ†

Adelio Rigo,‡ Franco Marmocchi,§ Dina Cocco,¶ Paolo Viglino,‡ and Giuseppe Rotilio*,§

ABSTRACT: One of two isozymes of copper–zinc superoxide dismutase of wheat germ is dissociated into monomers by $NaDodSO_4$ (sodium dodecyl sulfate) (Beauchamp, C. O., and Fridovich, I. (1973), *Biochim. Biophys. Acta 317*, 50–64). The monomer in $NaDodSO_4$ is 500 times less active than the native enzyme. The kinetics of inactivation, as measured by the activity loss in $NaDodSO_4$, are a first-order process with $k = 0.28$ h^{-1}. The activity is fully restored, after removal of $NaDodSO_4$, in a biphasic process consisting of two first-order steps, each one accounting approximately for half the total activity. The former step has $k = 0.2$ min^{-1} (25 °C), the latter has $k = 1.4 \times 10^{-4}$ min^{-1}. Both rates are independent of protein concentration. Substantial recovery of the native electron paramagnetic resonance (EPR) line shape occurs in the first phase, but further small changes are observed during the slower phase. The protein is found by $NaDodSO_4$ disc gel electrophoresis to be essentially monomeric during the faster step of activity recovery; reassociation of dimers occurs in the slower phase. Apparently the first step corresponds to refolding of monomers, and the refolded monomer has approximately half the specific activity of the dimer. It can be concluded from activity and EPR evidence that the copper site of Cu–Zn superoxide dismutases can have an active configuration in monomeric molecules, but the native geometry and catalytic efficiency are fully recovered only in a dimeric structure.

Copper–zinc superoxide dismutases (Fridovich, 1974) are dimeric enzymes very similar to each other. In the bovine enzyme the protomers are identical chains (Steinman et al., 1974), each one containing two metal prosthetic groups, the active Cu^{2+} and the structural Zn^{2+}, in apparently identical environments (Richardson et al., 1975). Nevertheless, studies on the mechanism with the bovine enzyme (Fielden et al., 1974) have suggested that only half of the two active sites function in catalysis. Furthermore, protomers obtained from an analogous enzyme are practically inactive under conditions which do not impair the functions of dimeric molecules (Marmocchi et al., 1974). It seems, therefore, very likely that the quaternary assembly of the enzyme controls the catalytic activity. The aim of the present research was to follow, by activity and EPR[1] measurements, the change at the active site in a superoxide dismutase which is easily dissociated into protomers by sodium dodecyl sulfate.

Materials and Methods

Superoxide dismutases from wheat germ (isozymes I and II) were prepared according to Beauchamp and Fridovich (1973).

Protein was determined by a biuret method (Goa, 1953). The concentration of the two wheat germ isozymes was calculated routinely, in purified preparations, from their copper content, since the homogeneous enzymes were found to have 1.8–2 g-atoms of copper per mol of protein.

Copper was determined either by double integration of EPR spectra for concentrated samples, or by copper determination with an atomic absorption instrument (Hilger and Watts Atomspek, Model H 1170) for diluted solutions.

$NaDodSO_4$ disc gel electrophoresis, essentially according to Weber and Osborn (1969), and densitometric analysis of the electropherograms were carried out as described by Marmocchi et al. (1975).

Assays of the superoxide dismutase activity were performed with the polarographic method of kinetic currents (Rigo et al., 1975).

The proton spin-lattice relaxation time (T_1) of the water protons was measured by the 90 °C–τ–90 °C pulse sequence on a Polaron pulsed NMR apparatus working at 16 MHz. This relaxation time is typically lowered in solutions of copper–zinc superoxide dismutases (Fee and Gaber, 1972; Terenzi et al., 1974) due to the presence of at least one water molecule bound to the enzyme Cu^{2+} and in conditions of fast exchange with the bulk molecules of the solvent. T_1 is more conveniently expressed as a relaxation rate referred to a molar solution of the protein-bound Cu^{2+}, that is relaxivity, R:

$$R = \left(\frac{1}{T_1} - \frac{1}{T_{1(o)}}\right) \frac{1}{[Cu^{2+}]}$$

where $1/T_1$ and $1/T_{1(o)}$ are the measured proton spin-lattice relaxation rates of the paramagnetic oxidized protein solution and of the diamagnetic protein solution reduced by dithionite, respectively, and $[Cu^{2+}]$ is the concentration of the protein-bound copper. Relaxivity is thus expressed in M^{-1} s^{-1}.

EPR spectra were recorded with a Varian V-4502 spectrometer at approximately 9.15 GHz and −160 °C.

Results

Effect of the $NaDodSO_4$ Treatment on the Superoxide Dismutase Activity of Wheat Germ Isozymes. Samples of the

† From the Institute of Biological Chemistry, University of Camerino, Camerino, Italy, the Institute of Physical Chemistry, University of Venice, Venice, Italy, and the CNR Center for Molecular Biology, Rome, Italy. *Received January 31, 1977; revised manuscript received June 27, 1977.*

‡ Present address: Institute of Physical Chemistry, University of Venice, Venice, Italy.

§ Present address: Institute of Biological Chemistry, University of Camerino, Camerino, Italy.

¶ Present address: Institute of Applied Biochemistry, University of Rome, Rome, Italy.

[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; EPR, electron paramagnetic resonance.

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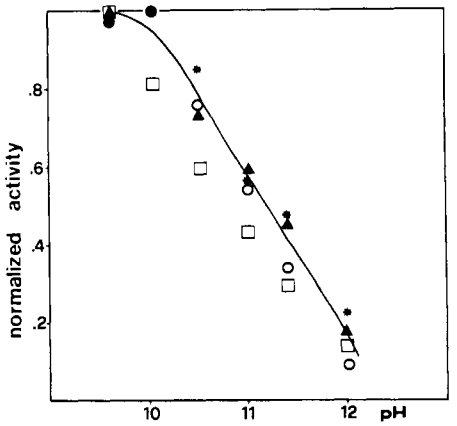

FIGURE 1: pH dependence of enzyme activity of wheat germ isozymes. The measured activities are normalized with respect to the activity at neutral pH; 0.1 M Tris, adjusted with NaOH to the desired pH value: (▲) isozyme I; (*) isozyme II; (○) isozyme I, after incubation in $NaDodSO_4$; (□) isozyme II, after incubation in $NaDodSO_4$.

TABLE I: Enzyme Activity of Wheat Germ Isozyme I after 48-h Incubation in $NaDodSO_4$.

[Protein] (M)	(Final act./initial act.) × 100
0.46×10^{-6}	0.21
1.86×10^{-6}	0.22
8.45×10^{-6}	0.19
15.3×10^{-6}	0.21

two isozymes and of bovine superoxide dismutase were incubated in 0.02 M sodium borate (pH 8.0) containing 4% $NaDodSO_4$, at 37 °C for 36–48 h. $NaDodSO_4$ disc gel electrophoresis shows (Beauchamp and Fridovich, 1973) that this treatment leads to complete dissociation into protomers of isozyme I, while it does not produce any form with the mobility of monomers in the other two enzymes. The final activities, referred to the native proteins, were: isozyme I, 0.2%; isozyme II, 60%; and bovine superoxide dismutase, 60%. The activity loss of the isozyme II and the bovine enzyme, which maintain the dimeric structure after this treatment, is likely to be due to partial denaturation of dimers as $NaDodSO_4$ itself does not affect the activity measurements (Rigo et al., 1975). The activity of the $NaDodSO_4$-treated proteins has the same pH dependence in the range pH 10–12 as the native enzymes (Figure 1). The final activity of isozyme I after incubation in $NaDodSO_4$ was found to be independent of the protein concentration as no changes in activity were observed over a 30-fold change in relative protein concentrations (Table I). Thus, the low residual activity of isozyme I appears to be the actual activity of dissociated protomers in $NaDodSO_4$, since if it were due to a small amount of dimer in equilibrium with the protomer molecules, it would be dependent on protein concentration.

The loss of activity by isozyme I was a first-order process, with a rate constant of 0.28 h^{-1} in the conditions reported above.

Reactivation of the $NaDodSO_4$-Treated Enzyme. A fast recovery of the activity with time was observed during the polarographic activity measurements of the $NaDodSO_4$-treated enzymes in borate buffer. On the contrary the activities in Tris buffer were constant with time and identical with those measured in 4% $NaDodSO_4$–borate buffer.

The activity recovery during the activity measurements is

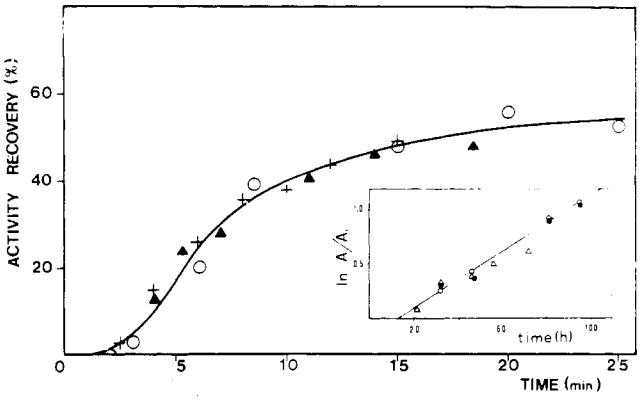

FIGURE 2: Activity recovery of $NaDodSO_4$-treated isozyme I after removal of $NaDodSO_4$. $NaDodSO_4$ was precipitated by addition of concentrated KCl (3 molar excesses) and the precipitate discarded after high-speed centrifugation for 3 min at 4 °C. Zero time is when the supernatant has been brought back to room temperature. Activity is expressed with respect to the activity of the native enzyme: (+) 1.7×10^{-8} M enzyme; (▲) 3.4×10^{-8} M enzyme; (○) 1.65×10^{-7} M enzyme. Insert: Slow phase of activity recovery of $NaDodSO_4$-treated isozyme I after removal of $NaDodSO_4$, recorded at three different enzyme concentrations: (△) 7.3×10^{-6} M; (●) 9×10^{-7} M; (○) 2×10^{-7} M.

apparently due to dilution of $NaDodSO_4$ in the assay mixture. In fact, the removal of $NaDodSO_4$ by precipitation as potassium salt leads to recovery of the enzyme activity. After a few days at room temperature, the activity recovery was total for the bovine enzyme and for both wheat germ isozymes. The kinetics of activity recovery after $NaDodSO_4$ removal were studied in greater detail with isozyme I and showed (Figure 2) an autocatalytic rise of activity lasting a few minutes (step I) followed by a relatively fast increase (step II) up to approximately 50% of the native activity value after 30 min at 25 °C. This kinetic behavior can be easily explained in terms of two consecutive first-order processes having $k = 0.3$ min^{-1} and $k = 0.2$ min^{-1}, respectively, the former giving still inactive species, from which active molecules are then formed. Both processes are independent of protein concentration in the range 1.7×10^{-8}–1×10^{-6} M. After steps I and II, there is a further slow first-order increase of activity (step III) up to native value ($k = 1.45 \times 10^{-4}$ min^{-1} at 25 °C), which is independent of protein concentration (Figure 2, insert).

Magnetic Resonance Measurements on $NaDodSO_4$-Treated Isozymes. The proton spin-lattice relaxation time (T_1) of the water protons was substantially unmodified in $NaDodSO_4$-treated samples of the isozymes. In fact, the relaxivity of enzyme solutions was 4.5×10^3 M^{-1} s^{-1} for the native enzymes and 4.1×10^3 M^{-1} s^{-1} after $NaDodSO_4$ treatment either in borate or in Tris solutions. The latter value demonstrates that practically all the copper is still bound to the protein after $NaDodSO_4$ treatments, as the relaxivity of a Cu^{2+}–Tris solution was 1.1×10^3 M^{-1} s^{-1}.

On the other hand the EPR spectra were significantly modified by the $NaDodSO_4$ treatment, as shown in Figure 3. Incubation in $NaDodSO_4$ gives rise to a new species with more axial line shape which is far more evident in the isozyme I samples (see, for comparison, curves b and f). This species is not due to removal of some copper from the enzyme, as the Cu^{2+}–$NaDodSO_4$ complex has a different EPR spectrum, under the same conditions.

The native rhombic line shape is fully restored after removal of $NaDodSO_4$. It is also evident that this restoration is mostly accomplished in the fast phase of the activity recovery.

Recovery of the Dimeric Structure by Isozyme I after Removal of $NaDodSO_4$. In order to evaluate the relative con-

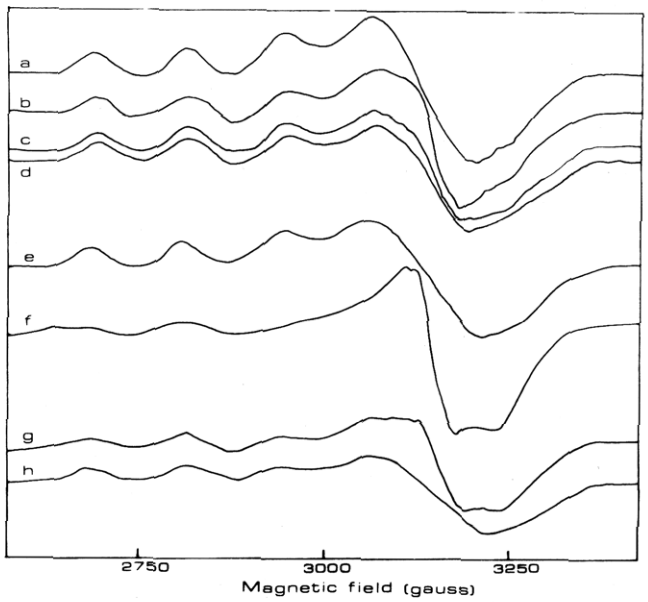

FIGURE 3: EPR spectra of the wheat germ isozymes; 0.1 M sodium phosphate buffer, pH 7.4: (a) 3×10^{-4} M isozyme II; (b) after 22-h incubation in $NaDodSO_4$ as described in the text; (c) 1 min at room temperature after removal of $NaDodSO_4$ as described in Figure 2; (d) 20 min at room temperature after removal of $NaDodSO_4$; (e) 2.3×10^{-4} M isozyme I; (f) same as b; (g) same as d; (h) 24 h at room temperature after removal of $NaDodSO_4$.

centrations of dimer and protomer molecules at the various times of the recovery process, the mobility in $NaDodSO_4$ disc gel electrophoresis was analyzed after readdition of 1.5% $NaDodSO_4$ at room temperature, which was found, by activity measurements, to stop the recovery process keeping the activity constant at the value reached before the readdition of $NaDodSO_4$.

Figure 4 shows that the great majority of the enzyme molecules still have the mobility of the protomeric form when the first phase of the activity recovery was completed and the EPR spectrum was already substantially of the native type (Figure 3g). On the other hand, dimeric molecules are mostly present during the slow phase of activity recovery, where further smaller changes of the EPR spectrum toward the native line shape were also observed (Figure 3h).

Discussion

The incubation of wheat germ isozyme I in $NaDodSO_4$ leads to dissociation of the enzyme into protomers (Beauchamp and Fridovich, 1973; Marmocchi et al., 1975) and most likely to the unfolding of protomers. The process should occur by consecutive steps and the disappearance of the dismutase activity, in the $NaDodSO_4$-treated enzyme, is probably related to both dissociation and unfolding. At variance with the reactivation process where more than one step was observed, the decrease of activity follows first-order kinetics. On account of the principle of microscopic reversibility it appears that inactivation of the active sites occurs in more steps, the first of which is the rate-determining step.

The residual activity, which is about 500 times lower than the initial one, can be explained either in terms of the protomer specific activity or of the native enzyme in equilibrium with inactive protomers. The same pH dependence of activity in the $NaDodSO_4$-treated and native samples (Figure 1) could rather favor the second hypothesis, which is, however, in contrast with the independence of protein concentration of the residual activity (Table I). It appears, therefore, likely that $NaDodSO_4$ produces an almost quantitative yield of poorly active monomers as also indicated by $NaDodSO_4$ disc gel electrophoresis (Figure 4).

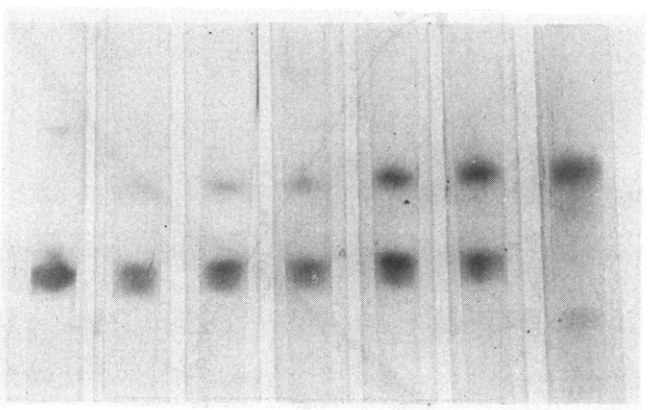

FIGURE 4: $NaDodSO_4$ disc gel electrophoresis of the isozyme I; 110 μg of protein was applied to each gel. From the left: the enzyme (3 mg/mL) incubated in 0.02 M borate buffer (pH 8.0) for 48 h at 37 °C, and 10 min, 30 min, 70 min, 2 h, 3 h, and 48 h at room temperature after removal of $NaDodSO_4$ as described in Figure 2. The respective percentages of dimer and protomer, as evaluated by densitometric analysis of gels, were 10 and 90% at 10 min, 22 and 78% at 30 min, 25 and 75% at 70 min, 37 and 63% at 2 h, and 47 and 53% at 3 h. For other details see text.

Some properties of the protomers in $NaDodSO_4$, such as T_1 and pH dependence of activity, support the hypothesis that the copper is still at its original binding site in the monomer, though not in the native configuration, as evidenced by the low catalytic efficiency and by the EPR spectrum. The copper binding site appears also to be more accessible to chelating agents, as the effect of Tris on the activity restoration can be attributed to occupation by Tris of some coordination position of the metal, which however remains still bound to the active site. This conclusion is supported by the fact that the relaxivity of the $NaDodSO_4$-treated enzyme, either in borate or in Tris buffer, is practically the same as that of the native enzyme and is four times higher than that of the Cu^{2+}-Tris complex in $NaDodSO_4$. The good reversibility of activity and EPR properties after removal of $NaDodSO_4$ is further evidence that the copper retains its ligands in the monomer in $NaDodSO_4$. In the process of reconstitution of native Cu^{2+} binding sites from $NaDodSO_4$-treated protomers, dimerization does not appear to be the rate-determining step because the rate of activity recovery is independent of protein concentration. The presence of a great deal of protomer (>50%) 30 min after the removal of the detergent indicates that the fast activity recovery is essentially determined by the refolding of protomers to a conformation similar to the native one. This process should occur in more than one step such as formation of the first intramolecular bonds inside the unfolded subunits followed by rearrangement of the Cu^{2+} active site, as monitored by the fast EPR changes. These consecutive steps could account both for the initial autocatalytic rise of activity and for its subsequent fast first-order increase.

This process, however, accounted for only 50% of the total activity recovery. In our view the association between the protomers should occur after step II and is followed by a much slower step III, during which the subunits attain the fully active conformation. The end of this step corresponds to the full recovery of activity.

The following kinetic scheme, which fully describes the time course of the activity recovery, can be tentatively proposed:

$$M \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} M' \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} M^* \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} M^*M^* \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} D$$

where M is the unfolded inactive monomer, M′ is the refolded inactive monomer, M* is the active monomer, M*M* is the dimer not yet in the native configuration, whose specific rate constant, i.e. the rate constant relative to each copper ion, is similar to that of M*, and D is the dimer in the final native configuration, where the specific rate constant is about twice that of M*. On the basis of our measurements we can estimate $k_1 = 0.3\ min^{-1}$, $k_2 = 0.2\ min^{-1}$, $k_3 \gg k_4$, $k_4 = 1.4 \times 10^{-4}\ min^{-1}$, $k_{-4} \ll k_{-3}$, k_{-2} . . . .

It is clear that these results cannot be used to generalize in terms of the influence of the quaternary structure on the activity of all Cu-Zn superoxide dismutase. Nevertheless, they represent further evidence that interaction between subunits is relevant to the regulation of the catalytic mechanism in this class of enzymes, as already suggested in the case of the bovine enzyme (Fielden et al., 1974), though the observed facts do not offer a direct explanation for the half-site mechanism proposed by these authors. It should, however, be kept in mind that the situation of isolated monomers investigated in the present report is clearly different from that of dimers where intersubunit interactions prevent one active site from functioning while the other site is catalytically working.

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Isolation and Partial Purification of Catfish Pancreatic Islet Messenger RNA†

M. Alan Permutt,*,‡ Irving Boime, Rose Chyn, and Marc Goldford

ABSTRACT: Poly(a)-rich mRNA has been isolated from catfish pancreatic islet total nucleic acid. Cell-free translation of the mRNA by wheat germ extracts yielded a protein of 11 000-12 000 molecular weight, estimated by sodium dodecyl sulfate-urea polyacrylamide gel electrophoresis. This peptide is larger than catfish proinsulin, but contains tryptic peptides of proinsulin. Its synthesis comprises up to 23% of the cell-free product, depending on the conditions of cell-free synthesis. Synthesis is inhibited by 7-methylguanosine 5′-monophosphate suggesting the presence of a 7-methylguanosine cap on the 5′ end of catfish proinsulin mRNA. Sucrose gradient centrifugation of the islet poly(A)-rich mRNA yielded 8S and 12S peaks. These fractions were translated with wheat germ extracts and it was determined that over 60% of the islet mRNA-dependent protein from the 8S fraction was preproinsulin. The 8S mRNA fraction was electrophoresed on 3% agarose-6 M urea gels and demonstrated to be several bands, ranging from 100 000-200 000 molecular weight.

Proinsulin is the most abundant protein synthesized by pancreatic islet cells, comprising as much as 25% of the newly synthesized protein under optimal conditions (Morris and Korner, 1970; Steiner et al., 1972; Permutt and Kipnis, 1972a). Proinsulin mRNA[1] should constitute a major fraction of the total islet mRNA. Unfortunately, mammalian islet tissue is embedded within the exocrine pancreas and comprises only about 1% of total pancreas (Falkmer and Patent, 1972). In contrast, islet tissue in teleost fish exists as a single relatively large gland, up to 100 mg (Bencosme et al., 1965), with less than 25% exocrine tissue in many species (Moule, 1972). Total poly(A)-rich mRNA has been extracted from catfish (Permutt et al., 1976), carp (Rapoport et al., 1976), angler fish and sea raven (Shields and Blobel, 1977), and translated in wheat germ and ascites cell-free protein synthesizing systems. Cell-free products included proteins of approximately 12 000-14 000 mol wt, but nothing as small as proinsulin (9000 mol wt). Anglerfish and sea raven cell-free products (Shields and Blobel, 1977) contain 23-25 extra amino acids on the amino terminus of proinsulin. The structure of the amino-terminal extension of these fish preproinsulins was shown to be very similar to that

† From the Department of Medicine, Metabolism Division, Washington University School of Medicine, St. Louis, Missouri 63110. *Received August 25, 1977.* This work was supported by National Institutes of Health Grant No. AM-16746 and by the St. Louis Diabetic Children's Welfare Association.

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[1] Abbreviations: mRNA and rRNA, messenger and ribosomal ribonucleic acids, respectively: mol wt, molecular weight; poly(A), poly(adenylic acid); dT, deoxythymidine; $NaDodSO_4$, sodium dodecyl sulfate; m^7pG, 7-methylguanosine 5′-monophosphate; m^7G, 7-methylguanosine; PPO, 2,5-diphenyloxazole.

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