EVIDENCE FOR A COORDINATION POSITION AVAILABLE TO SOLUTE MOLECULES ON ONE OF THE METALS AT THE ACTIVE CENTER OF REDUCED BOVINE SUPEROXIDE DISMUTASE*

James A. Fee† and Raymond L. Ward

Biophysics Research Division and Department of Biological Chemistry,
The University of Michigan, Ann Arbor, MI 48109
and
Chemistry and Materials Science Department
The Lawrence Livermore Laboratory, Livermore, CA 94550

Received April 23,1976

SUMMARY: We have measured the contribution of the reduced form of bovine Zn/Cu superoxide dismutase to the relaxation of the ^{35}Cl nucleus of chloride ion. The reduced protein has a molar relaxivity approximately 2.5 greater than the metal free protein, and addition of a small excess of cyanide lowers the relaxivity of the reduced protein to that of the apo-protein. We have interpreted these observations in terms of an open coordination position on one of the two metal ions, and we have proposed a mechanism for the reduction of superoxide by reduced superoxide dismutase which requires that O_2^- binds to Cu^+ prior to electron transfer.

The reaction scheme whereby the copper and zinc containing protein termed superoxide dismutase catalyses the internal oxidation-reduction reaction of the superoxide ion has been shown to consist of alternate reduction and oxidation of the copper (1,2) as follows:

$$O_2^- + P\text{-}Cu^{2+} \rightarrow O_2 + P\text{-}Cu^+ \qquad (1)$$

$$O_2^- + 2H^+ + P\text{-}Cu^+ \rightarrow H_2O_2 + P\text{-}Cu^{2+} \qquad (2)$$

Reaction 1 is clearly the simplest of the two consisting only of a transfer of one electron from superoxide to a cupric ion. Fee and Gaber (3) speculated that this step may involve the entrance of the superoxide ion into the first coordination sphere of the cupric ion prior to electron transfer. While

*Supported in part by a grant from the U.S.P.H.S., GM-21519, and the U.S. Energy Research and Development Administration under contract No. W-7405-ENG-48.

†Address inquiries to this author at the University of Michigan.

this remains a reasonable possibility, supported by recent results (4), one electron reductions by superoxide appear to be extemely facile (5-7), and it is possible instead that rapid outer sphere electron transfer could occur. By contrast, Reaction 2 cannot be a simple electron transfer reaction since direct formation of the doubly charged O_2^{2-} ion is energetically unfeasible. Therefore, the charge density must be neutralized and this presumably involves juxtaposition of the superoxide with a positive charge prior to the actual electron transfer. The best evidence for this comes from the observation that the second order rate constant for the uncatalyzed dismutation reaction decreases 10-fold for each unit increase in pH above the pK_a of the superoxide ion. These data suggest that the transition state for the reaction is minimally composed of two molecules of superoxide and one proton (8).

Further evidence for the disparate natures of Reactions 1 and 2 comes from electrochemical studies of the reduction of dioxygen at the dropping mercury electrode (dme). Chevalet et al. (9) have examined the reduction of molecular oxygen at the dme in the absence and presence of a surfactant capable of forming a compact film on the surface of the electrode which excludes water molecules while still allowing electron transfer from the electrode to oxygen. In the absence of the surfactant dioxygen is reduced to hydrogen peroxide by two rapid electron transfer processes. The first of these appears to be simple electron transfer to form O_2^- while the second involves transfer of both an electron and a proton, the latter apparently from a water molecule adsorbed on the electrode surface. Under these conditions very few superoxide ions escape from the electrode before the second step occurs. When the electrode was coated with quinoline as a hydrophobic surfactant, Chevalet et al. (9) found that electrons could be easily transferred from the electrode through the film to dioxygen with formation of superoxide but that the second electron transfer step to form hydrogen peroxide did not occur at relevant potentials. Thus, the second step was blocked by the surfactant film and hydrogen peroxide accumulation in the

solution was shown to result from the dismutation of superoxide ions. These authors proposed that the reduction of superoxide at the dme explicitly involves an adsorbed water molecule which concomitant with electron transfer yields one of its proteins to form HO_2^-.

We propose that the reduction of superoxide at the enzyme surface is essentially analogous to the process occurring at the dme, and the purpose of this communication is to demonstrate that one of the metals of the cuprous form of bovine superoxide dismutase has an open coordination position. A mechanism requiring entrance of O_2^- into this coordination position for the second step of the catalytic dismutation is suggested.

MATERIALS AND METHODS

Superoxide dismutase was prepared from bovine erythrocytes by the procedure of McCord and Fridovich (10), apoprotein, and the 2 Zn derivatives were prepared as previously described (11). The reduced protein was prepared by treatment with an excess of dithionite in a well-buffered solution at pH 7.4 followed by extensive dialysis against 5 mM potassium phosphate buffer and at pH 7.4 and being 1 M in NaCl. The copper was not lost during this procedure as would be predicted from earlier studies which demonstrated a very tight binding of Cu (12), and low temperature electron paramagnetic resonance examination of the samples indicated no oxidation had occurred prior to the NMR experiments described below. All protein samples were transported from Ann Arbor to Livermore at dry ice temperature.

The magnetic resonance measurements of the ^{35}Cl nucleus were made at 5.88 MHz as described previously (13). The line width of the resonance was measured as the full width at half-maximum amplitude. All spectra were obtained at the probe temperature of 29°, and the sample was purged with nitrogen gas prior to the measurement and the probe was flushed with nitrogen during the experiments.

THEORY

The line width of the ^{35}Cl resonance, as measured by the full width at half-maximum amplitude, is related to the relaxation time of the nucleus by the expression $\pi\Delta\nu = 1/T_2$. When a moderately fast chemical exchange of the chloride ion occurs between a free solvated environment and another environment (in this case bound to the protein) in which the nucleus has an intrinsically different relaxation time, the observed relaxation time will be the weighted average of the relaxation time characteristic of the two environments (14). Thus, if $1/T_{2(0)}$ represents the relaxation rate of the free chloride

ion and $1/T_2$ the observed relaxation rate then the relaxation due to the bound chloride ion is given by:

$$1/T_{2p} = 1/T_2 - 1/T_{2(0)}. \qquad (3)$$

When no chemical shift results from the different environment experienced by the chloride ion on binding (14)

$$1/T_{2p} = \underline{f}/(T_{2M} + \tau_M), \qquad (4)$$

where T_{2M} is the transverse relaxation time of the protein bound chloride ion, τ_M is the exchange lifetime, and $\underline{f}$ is the fraction of the total chloride ion which is bound to the protein. A detailed analysis of the relaxation process yields a more complete expression, but similar in functional form, for $1/T_{2p}$.

The relaxation mechanism for the ^{35}Cl nucleus in the absence of paramagnetic species is normally the interaction between the nuclear quadrupole moment of the nucleus (eQ) and the fluctuating electrostatic field gradient (eq) determined by the environment and sensed at the nucleus. The full NMR line width for a particular environment is given by (16)

$$\Delta\nu_i = \frac{2}{5}\pi \cdot (e^2 q_i Q)^2 g(\tau_c) \qquad (5)$$

$$\text{where } g(\tau_c) = 0.6\,\tau_c + \frac{\tau_c}{1+\omega^2\tau_c^2} + \frac{0.4\,\tau_c}{1+4\omega^2\tau_c^2}$$

where τ_c is the characteristic correlation time of the nucleus in that environment and ω is the nuclear precessional frequency.

Because the solvation shell of the free chloride ion produces only a small field gradient at the nucleus, the experimental line width is quite small, on the order of 10 to 12 Hz. On entering the coordination sphere of a divalent ion such as Zn^{2+} or Hg^{2+} or any environment having a different eq the ^{35}Cl nucleus experiences a relatively larger electric field gradient and nuclear relaxation occurs at a correspondingly higher rate.

It is important to distinguish between exchange limited and rapid exchange cases, and this can usually be done by examining the temperature dependence of the line width (see for example Ref. 17). For a nucleus which relaxes by a

quadrupole related process the rate is proportional to the correlation time for molecular tumbing (Eq. 5) which decreases with increasing temperature, thus the line width would be expected to decrease with increasing temperature for the rapid exchange situation. By contrast, τ_m invariably decreases with increasing temperature and from equation 4 it is clear that a smaller line width will result with increasing temperature. In the first exchange situation Equation 4 reduces to:

$$\frac{1}{T_{2p}} = \frac{f}{T_{2m}} \tag{6}$$

while in the exchange limited case the equation reduces to:

$$\frac{1}{T_{2p}} = \frac{f}{\tau_m} \tag{7}$$

RESULTS

The data shown in Fig. 1 constitute the evidence for the presence of a

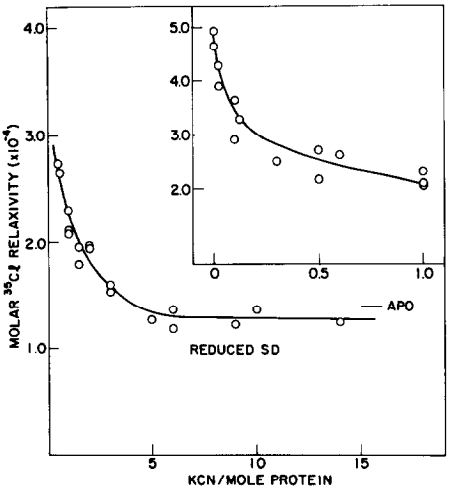


Figure 1 Effect of KCN on the chloride relaxivity of reduced bovine superoxide dismutase. The intial concentration of protein was 1.40 mM, the volume was 0.35 ml, the sample was titrated with freshly prepared 0.1 M KCN. The concentration of NaCl was maintained at 1.0 M. Relaxivity is expressed in terms of protein concentration, and appropriate corrections for dilution were made. In some experiments EDTA was included at 2.8 mM; this is the case for the data shown in the inset. The temperature within the probe was 31°C.

binding site for chloride ion on the reduced protein which is associated with a metal ion. It can be seen that there is a relatively weak molar relaxivity associated with the reduced protein, compared to the oxidized protein, Fig. 2, which is lowered to that of the apoprotein by titration with a small excess of potassium cyanide. It is this sensitivity to cyanide which constitutes the evidence for the metal nature of the chloride binding site. The inset of Fig. 1 shows the behavior of the relaxivity during the very early stages of the titration. It can be seen that there is considerable relaxivity which is effectively removed by a very small amount of cyanide, less than 0.2 moles KCN/mole of protein. EPR studies of these samples after the NMR experiments were completed showed the presence of small amounts of non-native cupric ion. Double integration of these signals indicated that 7 - 16% of the total copper was cupric ion.

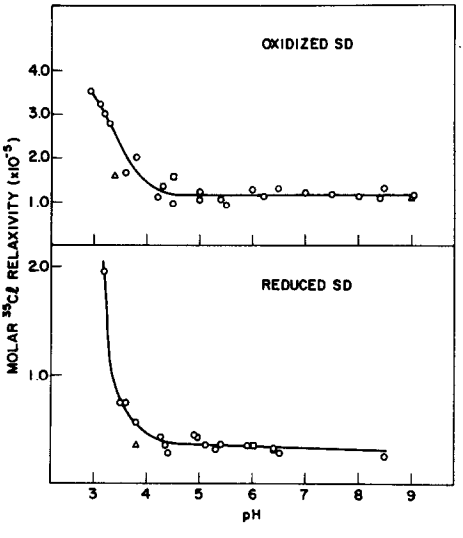


Figure 2 Effect of pH on the chloride relaxivity of oxidized (upper) and reduced (lower) bovine superoxide dismutase. The concentration of oxidized protein was 0.123 mM and that of the reduced protein 1.40 mM. In the latter experiments 0.5 Meq KCN was added to inhibit a small amount of strongly relaxing material present in these solutions. Conditions were generally as described in Fig. 1. The points indicated by triangles indicate the presence of EDTA and the points indicated by squares derive from samples which had been adjusted to extreme low pH (3.5 in the case of the reduced protein).

It is presumably this Cu^{2+} which accounts for the cyanide sensitive relaxivity greater than 3 x 10^4 M^{-1} sec^{-1} in Fig. 1 since this region showed great variation from one sample to another. In contrast, the relaxivity measured in the presence of $\bar{>}$0.3 KCN per mole of reduced protein was within error identical with the several samples used. The contribution of the extraneous Cu^{2+} to the cloride relaxivity of reduced protein was avoided by including 0.3 molar equivalents KCN in the remainder of the experiments described.

We have also measured the relaxivity of the 2 Zn^{2+} - copper free, protein (11) (data not shown) and have found its relaxivity to be identical to apoprotein and insensitive to cyanide. These observations suggest that the metal ion exposed to the solvent is Cu.

The molar relaxivities of the reduced and oxidized protein are shown as a

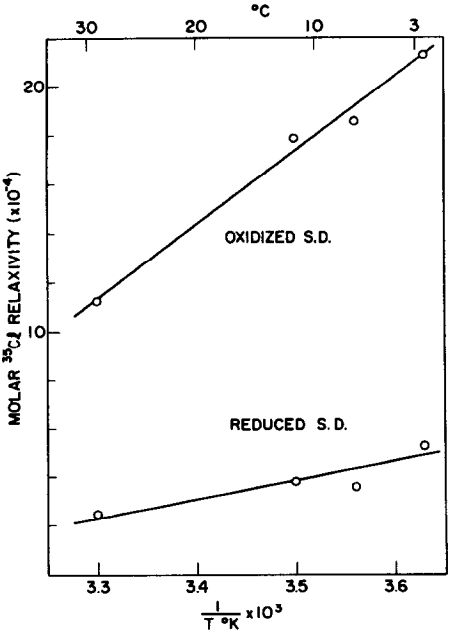


Figure 3 Temperature dependence of the molar relaxivity of oxidized and reduced bovine superoxide dismutase. Except for the temperature differences the conditions were generally as described in the legend to Fig. 2.

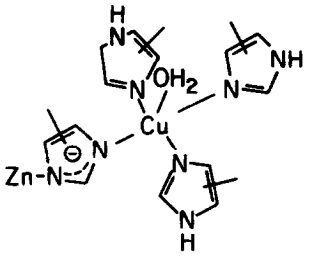
function of pH in Fig. 2. It is evident that between pH values of approximately 4 and 9 the values are constant suggesting that no ionization, which affects the Cl^- relaxivity, occurs in the region of the active site in either oxidized or reduced forms of the protein. Below pH 4.5 there is a significant increase in relaxivity shown by both the oxidized and reduced forms of the protein. In the first case this is presumably associated with ionization involving the active center as reported earlier (18) while the increase observed with the reduced protein is not completely reversible suggesting a general opening of the catalytic center at these lower pH values.

Fig. 3 shows the molar relaxivity of both the oxidized and reduced proteins at different temperatures. From the temperature dependence of the relaxivity it is evident that we are dealing with a rapidly exchanging chloride binding site and the residence time of the ion is small compared to T_{2m}.

It should be pointed out that the larger relaxivity of the oxidized protein is probably due in part to a paramagnetic contribution to the relaxation of the ^{35}Cl nucleus. The oxidized protein has been studied in some detail by measuring the molar relaxativity of the protein toward the protons of water molecules (19), and from that work it was concluded that the cupric ion binds at least one water molecule subject to rapid exchange.

DISUCSSION

The recent crystallographic results from the Richardsons' group (20) suggest that the metal binding region of bovine superoxide dismutase can be pictured as a cupric ion and a divalent zinc ion bridged by an imidazalato ion. As discussed in recent communications from one of our laboratories (12, 18), all of the presently known chemical facts are explicable in terms of reasonable chemical hypotheses involving this structural arrangement of the metal ions.

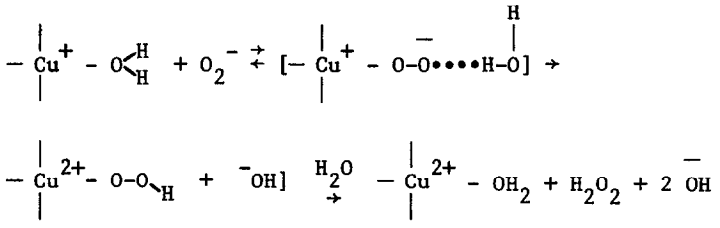


Much less is known about the structure of the metal binding sites when the cupric ion is reduced. The metals clearly remain tightly bound (12), and some sort of general conformational change occurs as evidenced by high resolution NMR spectra (18,21). Measurements of the midpoint potentials of the copper redox couple as a function of pH suggested the presence of a ligand associated with the cupric ion which would take up a proton concomitantly with reduction (22). This ligand has been postulated (12,18) to be the bridging imidazolato ion observed by the crystallographers.

The present results suggest that at least one coordination position on one of the metals remains open for coordinating solvent species in the cuprous form of the protein, and since chloride ion readily enters this position it is probably normally occupied by a water molecule. From the pH dependence of the relaxivity it is evident that ionization of the bound water molecule does not occur in the pH range 4 to 9 such as to impede entrance of the chloride ion to the coordination position. Unfortunately, the data do not reveal which metal ion, Cu^{+} or Zn^{2+}, is open to the solvent, but because apoprotein and 2 Zn^{2+}-protein have the same molar relaxivity we speculate that Cu^{+} is responsible for the chloride relaxation effects and is the site of ^{-}CN binding.

Based on the presently known facts, and following the mechanisms proposed by Chevalet et al. (9) for the reduction of superoxide ion at the mercury electrode, we suggest that the formation of hydrogen peroxide in the catalytic reaction of superoxide dismutase involves an open coordination position on the copper ion. A reasonable hypothesis may properly include formation of a cupric

peroxide complex as this undoubtedly forms in the reverse reaction, oxidation of hydrogen peroxide to molecular oxygen (22-24).



The lack of any pH dependence of the catalysis in the region 5 to 10 (25,26) suggests that protons are donated from water molecules in the region of the superoxide bound to the copper; and general acid catalysis by amino acid side chains is probably not occurring.

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