Observations on the Oxidation–Reduction Properties of Bovine Erythrocyte Superoxide Dismutase†

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ABSTRACT: The $E^{0\prime}$ value of the Cu ions of superoxide dismutase was found to be +0.42 V. Between pH 5 and 8.7 the reduction of each Cu(II) is accompanied by the uptake of a proton from the medium, being characterized by the half-reaction ECu(II) + e^- + H^+ ⇄ HECu(I). The proton is probably bound by a ligand which is released from Cu(I). The pK_a of this ligand must be greater than 9. Hydrogen peroxide reduces the Cu(II) ions with the concomitant formation of oxygen. The stoichiometry of this process is embodied in the reaction, 2Cu(II) + H_2O_2 → 2HCu(I) + O_2. In contrast to an earlier report (G. Rotilio, L. Morpurgo, L. Calabrese, and B. Mondovi, *Biochim. Biophys. Acta, 302*, 129 (1973)) there are no components of the enzyme system which undergo oxidation–reduction other than the Cu ions. On the basis of published kinetic data (D. Klug, J. Rabani, and I. Fridovich, *J. Biol. Chem., 247*, 4839 (1972)) calculations are made which indicate that free protons (hydronium ions) cannot be involved in the catalytic mechanism. Observations are presented which indicate that catalase effects a peroxide initiated catalytic reoxidation of reduced superoxide dismutase by molecular oxygen.

The superoxide dismutase from bovine erythrocytes contains 2 g-atoms each of Cu(II) and Zn(II) (Mann and Keilen, 1939; Carrico and Deutsch, 1970) presumably as two Cu–Zn pairs (Fee and Gaber, 1972; Fee, 1973b,c) each located on one of the two subunits of the molecule (Keele *et al.*, 1971; Weser *et al.*, 1971). The enzyme catalyzes the reaction

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad (1)$$

as was first shown by the work of McCord and Fridovich (1969) and confirmed with physical methods (Ballou *et al.*, 1969; Orme-Johnson and Beinert, 1969; Klug *et al.*, 1972; Rotilio *et al.*, 1972).

Reaction 1 proceeds spontaneously with a rate constant of 8.5×10^7 M^{-1} sec^{-1} at pH 4.8 and 1.2×10^5 M^{-1} sec^{-1} at pH 7.4 (Rabani and Nielsen, 1969). The catalyzed reaction has been studied by pulse radiolysis methods (Klug *et al.*, 1972; Rotilio *et al.*, 1972) and the catalytic rate constant has a value of $\sim 2 \times 10^9$ M^{-1} sec^{-1}, being essentially independent of pH in the range 4.8–9.7. The absence of a dependence on the hydronium ion concentration implies that the rate-limiting step does not involve proton transfer. This is unusual for the following reasons: (1) two protons must be obtained from the medium to complete the reaction, (2) the substrate becomes protonated at lower pH values, having a pK_a = 4.8 (Behar *et al.*, 1970), and (3) the hydronium ion concentration varies by five decades between pH 5 and 10 while the overall catalytic velocity is constant. Hence, any postulated mechanism must satisfactorily account for this result.

In this communication we present our results on the determination of the oxidation–reduction potential of the Cu(II) centers of superoxide dismutase in the pH range 5–9, and the pH independence of the catalytic reaction will be briefly discussed in terms of these results. Some results concerning the interaction between dismutase and H_2O_2 and O_2 will also be shown.

Experimental Section

Materials. Superoxide dismutase was prepared by the method of McCord and Fridovich (1969). Potassium hexacyanoferrate salts were obtained from J. T. Baker Chemical Co. The hexacyanoferrate(II) salt was recrystallized from ethanol–water before use and stored in the dark (Asperger, 1952). Cytochrome *c* (type III), L-ascorbic acid, catalase, and NADH were obtained from Sigma Chemical Co. Hydrogen peroxide was obtained from Fisher Scientific as a 30% aqueous solution. All other chemicals were of highest commercial quality and all solutions were prepared with glass-distilled water.

Instrumentation. Routine determination of pH and potential were made using a Heath Model EU-302A millivolt and pH meter with a Fisher combined glass|Ag–AgCl and a Metrohm combination Pt|Ag–AgCl electrode, respectively. The Ag–AgCl was referenced 0.182 V above the standard hydrogen couple. More critical experiments were made using a Radiometer Model 26 pH meter. Quantitative changes in oxygen concentration were determined using a Clark oxygen electrode (Yellow Springs Instruments Model 4004) and the circuitry described by Rikmenspoel (1969). The temperature of the solutions was maintained at 25 ± 0.1°.

Electron paramagnetic resonance (epr) measurements were made with a Varian E-9 spectrometer equipped with a nitrogen cooling system, and spectrophotometric measurements were made with a Cary Model 14 spectrophotometer.

Methods. General. Acid and base solutions were standardized against potassium acid phthalate. Dilute H_2O_2 solutions were standardized by iodometric titration (Jones and Sugget, 1968). A Gilmont microburet of all glass and plastic construction was used for titrations with H_2O_2.

Oxidation–Reduction Potentials. The oxidation–reduction potential of the hexacyanoferrate(III)/(II) couple was determined, for each set of experimental conditions used in the

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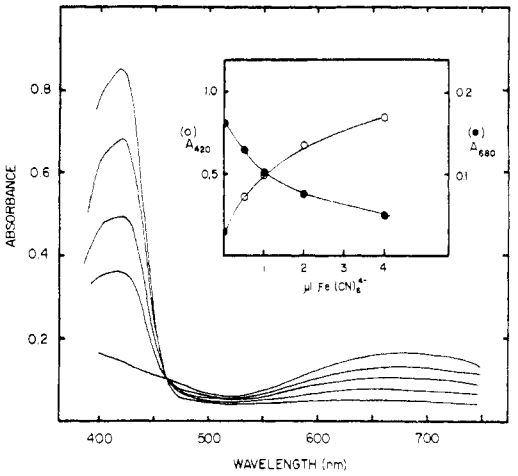


FIGURE 1: Optical titration of superoxide dismutase with hexacyanoferrate(II). The volume of the solution was 0.3 ml having $I = 0.1$ potassium phosphate (pH 6.56), [Cu(II)] = 1.08 mM. Titration was with 0.2 M $K_4Fe(CN)_6$; the total amount of this solution added was 0, 0.5, 1, 2, and 4 μl for the corresponding curves. The inset shows the decrease in A_{680} and the accompanying increase in A_{420}. The final pH was 6.65.

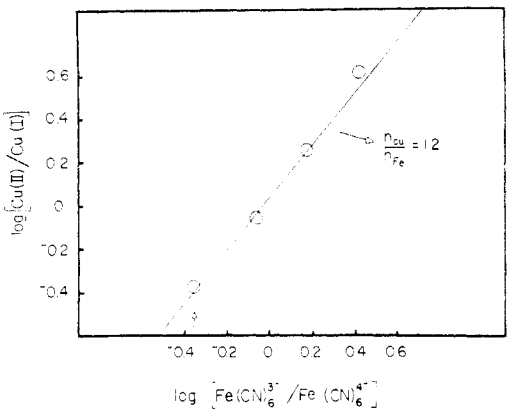


FIGURE 2: A Nernst plot of data taken from Figure 1. The concentrations of Cu(II) and Cu(I) were obtained from the A_{680} values, the concentration of $Fe(CN)_6^{3-}$ was set equal to the concentration of Cu(I), and the concentration of $Fe(CN)_6^{4-} = Fe_{tot} - Fe(CN)_6^{3-}$. The concentrations of the iron cyanide complexes can also be obtained from the A_{420} values and are similar. The arrow indicates the value of log $[Fe(CN)_6^{3-}/Fe(CN)_6^{4-}]$ obtained from the final potential of the solution and eq 3.

titration of superoxide dismutase (see below), from a plot of potential *vs.* log $[Fe(CN)_6^{3-}]/Fe(CN)_6^{4-}]$. For a given ionic strength the observed potentials agreed to within ±2% of those reported by Kolthoff and Tomsicek (1935).

The pH and ionic strength of all enzyme solutions were established by dialysis against multiple changes of appropriate buffer solution for 2 or more days at 4°. The protein solutions were then centrifuged to remove a small amount of precipitate and brought to the desired concentration with dialysate. The concentration of enzyme used varied from 0.7 to 1 mM.

Titrations with hexacyanoferrate(II) were carried out in open semimicro cuvettes equipped with a spacer which allowed an accurate absorption spectrum to be recorded on as little as 0.3 ml of solution. The absorbance at 680 nm was used to determine the concentration of enzyme, and the solution was then titrated incrementally with a 0.2 M solution of hexacyanoferrate(II). The titrant was added from a 10-μl Hamilton syringe, and the absorption spectrum was recorded between 750 and 380 nm after each addition of titrant. In most experiments the titration was carried to approximately 70% reduction (less above pH 7) of the enzyme after which the pH and the potential of the solution were determined.

Results

Studies with the Hexacyanoferrate(II)/(III) Couple. Addition of hexacyanoferrate(II) to solutions of superoxide dismutase results in a reduction of the Cu(II) to Cu(I) (Rotilio *et al.*, 1973). A titration of dismutase at pH 6.65 with $K_4Fe(CN)_6$ is shown in Figure 1. Each addition of titrant results in a decrease in absorbance at 680 nm and a related increase in absorbance at 420 nm due to the formation of hexacyanoferrate(III) ions. The spectra show an isosbestic point near 467 nm suggesting the presence of only two species absorbing in this spectral region. Using the following extinction coefficients and assuming that the two Cu ions of the dismutase molecule are identical and independent, it is possible to calculate the concentrations of oxidized and reduced Cu ions and oxidized and reduced iron cyanide ions: $\epsilon_{680} = 150$ l./(g-atom of Cu(II) cm), $\epsilon_{420} = 130$ l./(g-atom of Cu(II) cm) for the oxidized enzyme while the reduced enzyme does not absorb in the visible region and $\epsilon_{420} = 1000$ l./(mol cm) for hexacyanoferrate(III) ion and its reduced counterpart does not absorb in the visible region of the spectrum (McCord and Fridovich, 1969; Cohen and Plane, 1957). In addition, the ratio $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ can be determined by potentiometric measurements (see below) provided one ion is not present in large excess. Thus, two independent measures of the potential of the system can be obtained.

Figure 2 shows a Nernst plot of the data taken from the experiment described in Figure 1. The midpoint potential was obtained from the expression

$$E_m^{Cu} = E_m^{Fe} + \frac{RT}{nF} \ln [(Fe(CN)_6^{3-})/(Fe(CN)_6^{4-})] - \frac{RT}{nF} \ln [(ECu(II))/(ECu(I))] = 0.439 \text{ V} \quad (2)$$

where $E_m^{Fe} = 0.441$ V under the conditions of the experiment and within the experimental error n is clearly unity. The ratios of concentrations shown in Figure 2 were obtained from the spectral data of Figure 1. The ratio $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ was also obtained independently at the end of the titration from the expression

$$E_{expt} = E_m^{Fe} + \frac{RT}{nF} \ln [(Fe(CN)_6^{3-})/(Fe(CN)_6^{4-})] = 0.421 \text{ V} \quad (3)$$

which when combined with log (ECu(II)/ECu(I)) = −0.372 in eq 2 gives a value of $E_m^{Cu} = 0.442$ V. Thus, the two values are in good agreement. In general, values of E_m^{Cu} obtained in this way differed by less than ±0.005 V.

The titration shown in Figure 1 was carried out in the presence of air. There is no evidence that the reduced enzyme is reoxidized by oxygen during this time period; thus, the spectrum of a partially reduced solution of dismutase is unchanged after 0.5 hr at room temperature (however, see below). This is consistent with the original observations of Mann and Keilen (1939) on the inability of the reduced form of this protein to be reoxidized by oxygen.

TABLE I: Proton Uptake of Superoxide Dismutase Upon Reduction by Hexacyanoferrate(II).[a]

pH	μmol of Cu(I)	μmol of H^+	H^+/e^-
5.96	0.581	0.748	1.27
6.53	0.557	0.667	1.18
6.79	0.531	0.587	1.10
7.15	0.471	0.506	1.06
7.35	0.437	0.506	1.14
7.54	0.353	0.390	1.08
7.71	0.366	0.414	1.13

[a] Each experiment was carried out as follows: 125 μl of a stock solution of dismutase, 0.483 μmol of Cu(II) (pH 7), 0.1 M $NaClO_4$, and 10^{-4} M in potassium phosphate salts, was diluted to 3 ml with the same buffer and the pH adjusted to the desired value with 1 M NaOH or 1 M HCl. The solution was enclosed in a vessel thermostated at 25 ± 0.1°, stirred, and scrubbed N_2 was passed over its surface. When a constant pH value was obtained 25 or 30 μl of a 0.2 M solution of potassium hexacyanoferrate(II) was added. This resulted in an increase in pH, and standardized HCl, ~0.01 M, was used to titrate the solution back to its original pH. When the pH reading had stabilized, the combination glass electrode was quickly exchanged for a combination Pt|Ag–AgCl electrode and the potential of the system recorded. Using the observed potential, the known E_m for Fe, and the E_m for Cu taken from Figure 3 the number of moles of Cu(I) formed were calculated using eq 2 and 3. The number of moles of H^+ required to obtain the original pH were calculated from the HCl concentration and the required volume. Corrections for the hexacyanoferrate(II) alone were less than 1% in the pH range used.

It is important to know if the iron cyanide ions are binding to the protein in such a manner as to affect the redox potential determinations. We have carried out two experiments which bear on this. At concentrations as high as 0.1 M, $Fe(CN)_6^{3-}$ does not alter the low-temperature epr spectrum while $Fe(CN)_6^{4-}$ leads to simple disappearance of the characteristic Cu(II) signal. In another control experiment, we first reduced the enzyme with a small excess of hexacyanoferrate(II) and then after 0.5 hr added an excess of hexacyanoferrate(III). This solution was then passed over a 25 × 1 cm Sephadex G-25 column. The protein appeared as a single band separate from the iron cyanide ions, and fractions containing protein were combined and concentrated overnight in a collodion bag. The material thus obtained had spectral properties identical with starting material and the yield was 75%. In a parallel experiment not involving reduction of the enzyme the yield was 70%. Attempts to remove the iron cyanide salts by dialysis against 0.1 M pH 5 acetate buffer always led to the formation of a small amount of brown colored material, apparently protein bound iron.[1] These experiments suggest that hexacyanoferrate ions probably have a rather low affinity for the dismutase molecule if they bind at all.

With this knowledge we titrated the protein with hexacyanoferrate(II) ions in separate experiments over the pH range 4–8.7 and calculated the midpoint potential in each case. The results are shown in Figure 3. At pH 4 the E_m for the Cu centers is 0.53 V while at pH 8.7 it is 0.33 V. Between pH 6 and 8.7 E_m is a linear function of pH, decreasing ~0.059 V for each unit of pH. Between pH 4 and 6 the change of E_m with pH is less, indicating a change in behavior between pH 5 and 6.

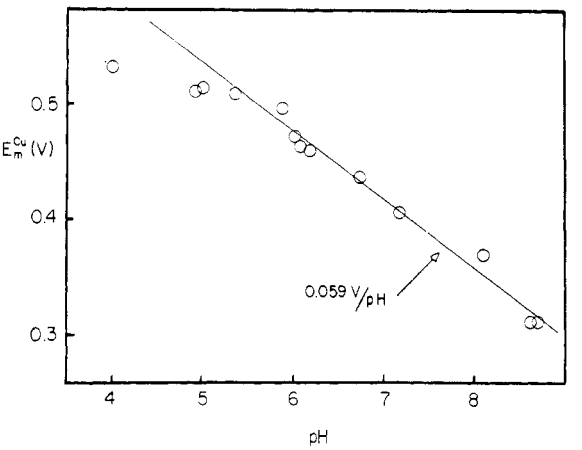


FIGURE 3: The variation of the midpoint potential of dismutase Cu with pH. The values were obtained as described in the text. The ionic strength was 0.1 with the exception of the three values just above pH 6 which were obtained at ionic strengths of 0.01, 0.1, and 0.2. There is no obvious variation of E_m with ionic strength or specific buffer ions. The solid line is not derived from the data points.

These data suggest that the half-reaction

$$ECu^{2+} + H^+ + e^- \rightleftharpoons H^+ECu^+ \quad (4)$$

which predicts E_m will decrease 0.059 V for each unit increment in pH, describes the reaction of the enzyme with the added electron donor.

To test this further, we have reduced the enzyme with hexacyanoferrate(II) in dilute buffer solutions, determined how much acid must be added to restore the original pH of the buffer, and finally calculated the extent of the reduction from the measured potential of the solution and the E_m of the Cu centers taken from Figure 3 at the final pH of the experiment. The results of these experiments are summarized in Table I. In the pH region where the experiments are feasible[2] one proton was taken up by the protein for each electron accepted by the Cu(II) ions. These results confirm the validity of eq 4.

Studies with Hydrogen Peroxide. It has been reported that H_2O_2 reduces the Cu(II) of dismutase and that 1[3] mol of H_2O_2 reduces only 1 mol of Cu(II) (Rotilio *et al.*, 1973). This is an unusual stoichiometry as the immediate product of Cu(II) reduction by H_2O_2 would be O_2^- which would dismute to H_2O_2 and oxygen. Rotilio *et al.* (1973) rationalize this stoichiometry by concluding that there must be additional redox components in the protein. Because of the possible significance of this observation, we have studied this further with the idea in mind of determining the products of the reaction and reinvestigating the stoichiometry.

It was first necessary to determine whether or not oxygen was released when H_2O_2 was added to solutions of dismutase. However, before such an experiment can be done in a meaningful way it must first be established that the solutions of dismutase do not catalytically decompose H_2O_2 to oxygen and

[1] Unpublished results from this laboratory indicate that Fe(III) binds to apoprotein to give an intensely brown material.

[2] The fourth ionization constant of hexacyanoferrate(II) ion lies between 10^{-3} and 10^{-4} M (Jordan and Ewing, 1962). Thus, protonation of this ion interferes in such experiments below pH ~5.

[3] A value of 1.6 H_2O_2/Cu is obtained by plotting the reported epr intensities *vs.* added peroxide (Rotilio *et al.*, 1973).

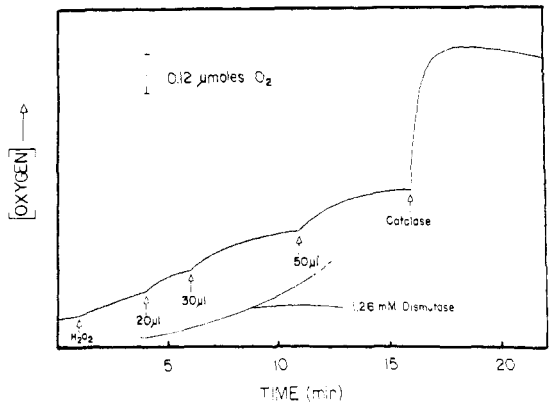


FIGURE 4: Test for possible catalatic activity of superoxide dismutase. The solution was 3 ml of 0.01 M potassium phosphate (pH 7.2)–0.1 mM EDTA, thermostated at 25°. An oxygen electrode was used to sense the concentration of oxygen. Peroxide, 12 μl of 0.081 M, was added at the beginning of the experiment and superoxide dismutase and catalase were added as indicated.

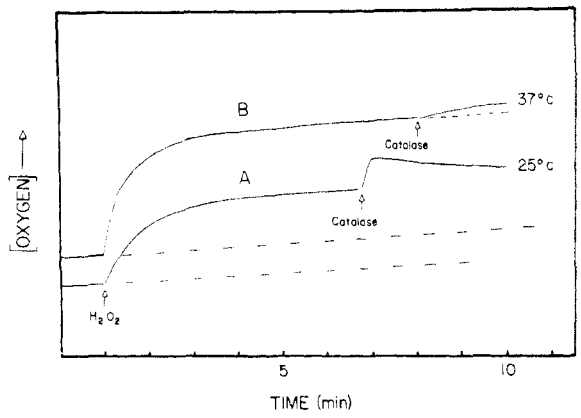


FIGURE 5: Formation of molecular oxygen upon adding H_2O_2 to solutions of superoxide dismutase. Trace A: 25°, 2.7 ml of 0.01 M potassium phosphate buffer–0.1 mM EDTA–0.3 ml of 1.26 mM dismutase; reaction initiated by addition of 5 μl of 0.081 M H_2O_2 and 0.3 μl of 0.22 mM catalase at point indicated. Trace B: 37°, 2.75 ml of above buffer solution, 0.25 ml of 1.26 mM dismutase, and amounts of peroxide and catalase same as above.

water. The results of this control experiment are shown in Figure 4 in which increasing concentrations of dismutase were added to a 0.27 mM H_2O_2 solution in an oxygen electrode chamber. Even at the highest concentration of dismutase obtained no catalytic decomposition of H_2O_2 to oxygen occurred, while the larger additions of dismutase showed a short burst of oxygen formation which was not sustained. These increments of oxygen production were invesgitated further and the results are shown in Figure 5.

When 0.405 μmol of H_2O_2 was added to a solution at 25° containing 0.756 μmol of Cu(II) bound to dismutase oxygen evolution occurred as is shown in Figure 5, tracing A. After several minutes, oxygen production appears to cease; however, upon addition of catalase there was an additional increase in oxygen concentration followed by a slow consumption (see below). In this experiment 94% of the peroxide was accounted for if the first burst of oxygen was considered to be formed in a reaction such as

$$2Cu(II) + H_2O_2 \longrightarrow 2Cu(I) + 2H^+ + O_2 \quad (5)$$

and the oxygen released upon addition of catalase to be due to the catalatic reaction

$$2H_2O_2 \longrightarrow O_2 + 2H_2O \quad (6)$$

In addition to the fact that evolution of oxygen results from the reduction of dismutase by H_2O_2, it is important that not all the peroxide was consumed even after 5 min, apparently because the reaction is sluggish at this temperature and the lower concentration of H_2O_2. The reaction appears to go to completion at higher temperature, however (Figure 5, tracing B). In this experiment 0.405 μmol of H_2O_2 was added to 0.63 μmol of dismutase Cu(II) at 37°. Most of the peroxide disappeared after 6 min, and, assuming the above stoichiometry, 83% of the peroxide was accounted for with less than 10% being acted on by catalase. The results of these experiments are consistent with the stoichiometry of eq 5.

To test the stoichiometry further, we have titrated the protein with H_2O_2 at 25° under reduced oxygen tensions using the intensity of the 680-nm absorption band to determine Cu(II) reduction. The results of this experiment are shown in Figure 6A. The data can be extrapolated to a value of ~0.75 mol of H_2O_2 added per mol of Cu(II) reduced. Since the titration was carried out at 25° not all the H_2O_2 was consumed after each addition, as demonstrated in Figure 5, and each point could be corrected for this, which would result in a value lower than 0.75. Thus, fewer than 0.75 molar equiv of H_2O_2 reduce 1 equiv of Cu(II). An attempt was made to titrate the protein at 37°; however, this experiment was done in the presence of air where a significant reoxidation during incubation at the higher temperature interfered with the titration. Nevertheless, a single measurement was obtained which indicated that 0.52 equiv of Cu(II) was reduced by 0.25 molar equiv of added H_2O_2.

While the experiments described above can be rationalized in terms of the stoichiometry of eq 5 the question of whether other redox centers are involved can be tested further. This was approached by first reducing the dismutase to a given level with H_2O_2 followed by reoxidation with hexacyanoferrate(III) ion. The results shown in Figure 6B are calculated from the reappearance of absorbance at 680 nm and clearly show that only 1 equiv of this oxidizing agent is required to oxidize one Cu(I) to Cu(II). The absorbance at 420 nm was also monitored during the titration, and a plot of A_{420} *vs.* equivalents of oxidizing agent showed a break of 1 equiv per Cu(I) present and, upon complete reoxidation of the Cu(I), rises with a slope corresponding to ϵ_{420} of 1000 M^{-1} cm^{-1}, which is the molar extinction coefficient of the oxidizing agent (Cohen and Plane, 1957). These results demonstrate that there are no redox centers in the superoxide dismutase molecule operating in the potential region involved in these experiments other than the two copper ions.[4]

Action of Various Reducing Agents on Superoxide Dismutase. With the exception of dithionite, classical reducing agents such as ascorbate and quinol were found to react rather slowly with superoxide dismutase and NADH did not reduce the Cu(II) at all. However, at pH 5 and 40° reduction of both Cu(II) ions was achieved with 1–2 mol of ascorbate in *ca.* 15 min (Weser *et al.*, 1971). Similarly, reduced cytochrome *c* reduced the enzyme but only slowly and at elevated temperatures.

The rate of electron exchange between the copper ions of superoxide dismutase and the hexacyanoferrate(III)/(II)

[4] It is noteworthy that dismutase reduced with H_2O_2, dismutase reduced with H_2O_2 and reoxidized with $Fe(CN)_6^{3-}$, and native protein all have the same enzymatic activity in the xanthine oxidase–xanthine–cytochrome *c* assay (McCord and Fridovich, 1969). Unpublished results of R. Briggs and J. A. Fee.

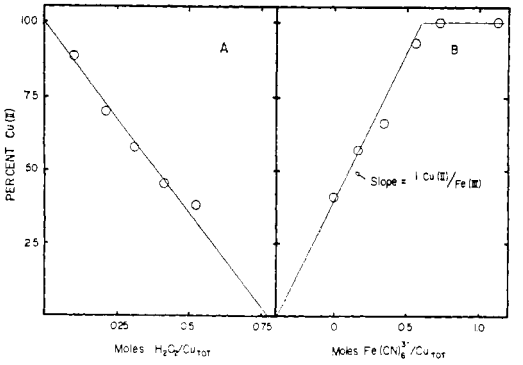


FIGURE 6: Spectrophotometric titration of superoxide dismutase with H_2O_2 (A), followed by titration with hexacyanoferrate(III) (B). Titrations were done at room temperature in a 3-ml cuvette equipped with an insert for a glass electrode, and wet nitrogen gas was passed through the system several minutes prior to the start of the experiment. The solution contained 1.0 ml of 1.26 mM dismutase in water plus 0.9 ml of 10^{-4} M potassium phosphate buffer, 0.067 M in $NaClO_4$ (pH 7.2). A. (Peroxide reduction): H_2O_2, 0.081 M, was delivered through plastic tubing from a Gilmont microburet. Approximately 3–4 min after each addition of peroxide the pH and the absorption spectrum were recorded. The final point in the titration corresponds to 15 μl of H_2O_2 solution. There was no change in pH. B. Reoxidation with hexacyanoferrate(III): the pH of the solution was adjusted from 7.15 to 9.0 with 1 M NaOH and the back-titration done with 0.1 M $K_3Fe(CN)_6$. After each addition of oxidizing agent the pH was adjusted back to 9 and the absorption spectrum recorded. The fifth point corresponds to the addition of 17 μl of oxidant solution. The solid line in B is the expected result if 1 mol of $Fe(CN)_6^{3-}$ oxidizes 1 mol of Cu(I). The second point of the titration was arbitrarily placed on the line. Per cent Cu(II) values were calculated from A_{680}.

couple is slow, equilibrium being effectively obtained only after 3–4 min. The rate of the reduction by H_2O_2 appears to be comparable to that with hexacyanoferrate(II) ion.

Studies on the Autoxidation of Reduced Superoxide Dismutase. In 1939, Mann and Keilen (1939) observed that the reduced protein was not readily reoxidized by molecular oxygen. It was slowly reoxidized, however, as typified by the data shown in Figure 7A. In this experiment, a sample of dismutase reduced to the 40% Cu(II) level by the addition of 0.5 mol of H_2O_2 per mol of Cu(II) present was reoxidized only to 50% over a period of 90 min at 22°. At that point, catalase was added to the solution with no change in A_{680} over a period of 3 min. However, upon adding H_2O_2 a faster rate of reoxidation ensued with ~95% reoxidation resulting after 176 min. The optical spectrum of this solution identifies it as native superoxide dismutase.

It would appear that both catalase and peroxide are necessary for this more rapid reoxidation, and an attempt to demonstrate this is shown in tracing 1, Figure 7B. In this experiment, 0.76 μmol of copper as dismutase was reduced to the 20% Cu(II) level with 0.5 molar equiv of ascorbic acid per Cu(II) and placed in the oxygen electrode chamber. It can be seen that adding catalase does not effect any consumption of oxygen. When peroxide was added (not shown) a short burst of oxygen evolution occurred but a significant oxygen consumption was not observed (see Discussion). In tracing 2 of Figure 7B an experiment is described which involved adding 1.7 μmol of H_2O_2 to 0.56 μmol of dismutase Cu(II) over a period of 24 min followed by addition of catalase. The latter resulted in a rapid oxygen evolution followed by a slower consumption of oxygen apparently due to a reoxidation of reduced dismutase Cu(II). Approximately 75% of the total H_2O_2 was accounted for in this experiment as was described above.

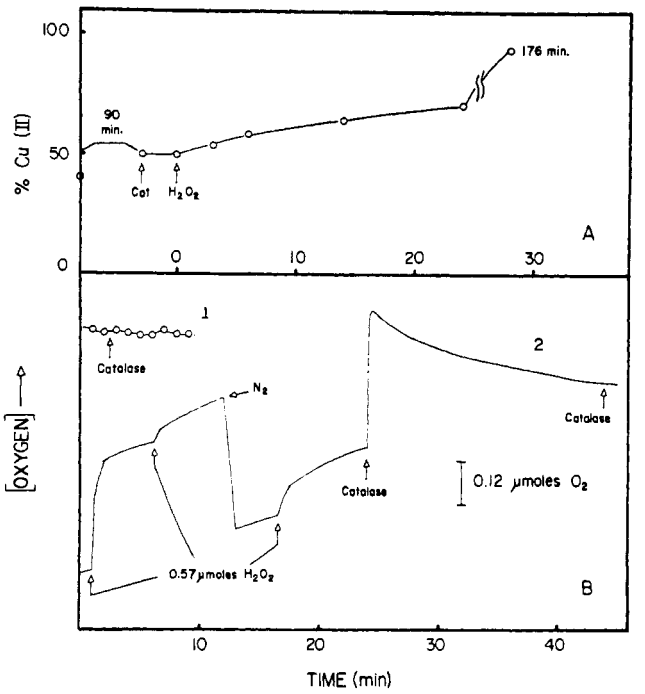


FIGURE 7: Experiments on the autoxidation of reduced superoxide dismutase. A. Spectrophotometric observations: 0.5 μmol of dismutase in 0.7 ml of dilute potassium phosphate buffer–0.05 mM EDTA (pH 7.2) were partially reduced with approximately 1 μmol of H_2O_2 followed by heating at 37° for 10 min to remove excess H_2O_2. A 60% reduction was achieved in this manner (see text). The solution was then allowed to stand at room temperature (~22°) for 90 min. Catalase, 0.2 μl of 0.22 mM, was then added and 3 min later 2 μl of 0.081 M H_2O_2. A_{680} was recorded at each of the times indicated. B. Observations using the oxygen electrode. Trace 1: 0.38 μmol of dismutase in 3 ml of 0.01 M potassium phosphate buffer–0.1 mM EDTA (pH 7.4). The dismutase was reduced to approximately 80% by 1 molar equiv of ascorbate prior to the beginning of the experiment; 1 μl of 0.22 mM catalase was added at the indicated point. Trace 2: 0.28 μmol of dismutase in 3 ml of above buffer, addition of peroxide as indicated. The first and second additions of catalase were 0.2 and 1.0 μl, respectively, of 0.22 mM enzyme (see text). Nitrogen was used to deoxygenate the chamber.

The rate of oxygen consumption represents a somewhat faster rate of reoxidation than was observed in the spectrophotometric experiment (Figure 6A), and if the reoxidation proceeds by

$$O_2 + 2Cu(I) + 2H^+ \longrightarrow 2Cu(II) + H_2O_2 \longrightarrow {}^1/_2O_2 + H_2O \quad (7)$$

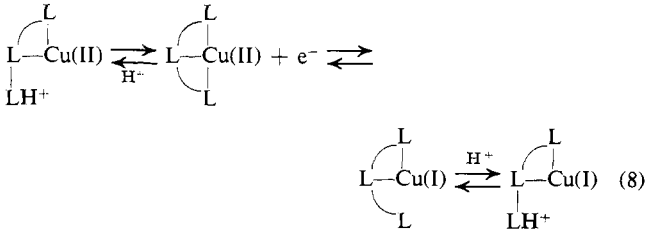
then (assuming full reduction of the Cu(II)) 75% reoxidation had occurred after 20 min.

Discussion

The value of the midpoint potential of the Cu(II) ions of dismutase at any pH is not unusual and lies in the same range as several cuproproteins containing so-called type 1 or "blue" Cu centers (Reinhammar, 1972). However, the dependence of E_m on pH is striking, and only in the case of one of these "blue" proteins has E_m been studied in this manner.[5] Thus, with plastocyanin E_m is constant at 0.37 V above pH 5.4 and increases 0.06 V with each unit of pH below this point (Katoh

[5] The oxidation–reduction potential of the type 1 Cu of fungal laccase varies only a few millivolts between pH 4 and 8 (unpublished results of J. A. Fee and B. G. Malmström).

et al., 1962). This behavior is related to that shown in Figure 3 for dismutase and has been explained for small complexes of Cu (Brill *et al.*, 1964) by the loss of a ligand from the low-valent form followed by its association with a proton. In general, such results can be understood in terms of (8). In the



case of dismutase, above pH 5, protonation of the Cu(II) form is negligible and up to pH 8.7 protonation of the reduced form appears to be essentially complete. The apparent pK of the dissociated ligand would thus seem to be well above 9. Below pH 5, there is an apparent deviation from eq 4 suggesting that some protonation of the Cu(II) form is occurring. While this type of analysis is adequate for simple Cu complexes, with proteins, perturbation of ionizable groups near but not liganding the Cu must be considered as a possible source of the pH variation of E_m. However, the rather close fit of the data on plastocyanin (Katoh *et al.*, 1962) and dismutase to eq 4 suggests that a ligand is lost from the first coordination sphere of the Cu(II) upon its reduction, and that the apparent pK of this ligand is above 9 in the case of dismutase. The nature of this ligand is not known; arginine and peptide nitrogen are possible but tyrosine is excluded (Fee, 1973a).

There have been several suggestions that the catalytic mechanism of superoxide dismutase involved a valence shuttle of the Cu ions (Fee and Gaber, 1971; Fielden *et al.*, 1973; Klug *et al.*, 1972,1973) as described by eq 9 and 10. There are,

$$ECu(II) + O_2^- \longrightarrow ECu(I) + O_2 \qquad (9)$$

$$ECu(I) + O_2^- + 2H^+ \longrightarrow ECu(II) + H_2O_2 \qquad (10)$$

however, conflicting observations regarding a reduction–oxidation cycle for the Cu(II) centers. Fielden *et al.* (1973) observed reduction of the Cu(II) subsequent to a 5 meV/1.5 μsec electron pulse which produced a solution 20 μM in O_2^-, but these authors did not demonstrate that this reduction was catalytically significant. On the other hand, Klug *et al.* (1972) report being unable to observe any decrease of the 680-nm absorption subsequent to an electron pulse but later (Klug *et al.*, 1973) reported significant reduction of dismutase Cu(II) which occurred within a time comparable to the catalytic turnover time of the enzyme. It will be assumed in the following discussion that the enzyme functions by a mechanism involving cyclic reduction and oxidation of the copper ions. Thus, the present results suggest that expressions 9 and 10 might be rewritten as

$$ECu(II) + O_2^- + H^+ \longrightarrow HECu(I) + O_2 \qquad (11)$$

$$HECu(I) + O_2^- + H^+ \longrightarrow ECu(II) + H_2O_2 \qquad (12)$$

where ECu(II) and HECu(II) are the equilibrium oxidation–reduction states.

The velocity of either of these reactions as written is proportional to the concentration of free hydronium ions, and at high pH each reaction would necessarily proceed more slowly. This is not observed. Indeed, the rate-limiting step in catalysis cannot involve an expression of the type

$$V = k[X][H^+] \qquad (13)$$

where X is any form of the enzyme dependent on obtaining a free proton from the solution before the next step in the catalysis can be effected. This statement can easily be proven using the following data from Figure 1b of Klug *et al.* (1972): initial velocity $\cong$ 0.02 M sec^{-1}, $[E]_0 = 0.35$ μM, and $[O_2^-]_0 = 20$ μM, and pH = 9.5 (taken as 10 for convenience). The highest concentration that X can achieve is $[E]_0$, and

$$k = 0.02 \text{ M sec}^{-1}/(0.35 \times 10^{-6} \text{ M})(10^{-10} \text{ M}) =$$

$$5.7 \times 10^{14} \text{ M}^{-1} \text{ sec}^{-1}$$

a value which is nominally four orders of magnitude above the diffusion limit (Alberty and Hammes, 1958; Koenig and Brown, 1972) and approximately five orders of magnitude above the observed rate constant. This calculation leads to the conclusion that hydronium ions cannot be involved in the catalytic process as indicated by either (9), (10), or (11), (12) because the observed velocity is simply too great to include free diffusion of hydronium ions. Clearly, the mechanism whereby the oxidizing O_2^- obtains two protons in the redox process of Klug *et al.* (1973) is not understood. It must be emphasized that there is no evidence which shows that during turnover the enzyme itself accepts protons from the medium and donates them to substrate as might be suggested by (8).

The reduction of the Cu(II) ions of dismutase by H_2O_2 is clearly occurring by reaction 5, and if the Cu centers are truly independent the superoxide anion will be formed during this process

$$Cu(II) + H_2O_2 \longrightarrow Cu(I) + 2H^+ + O_2^- \qquad (14)$$

However, it will rapidly dismute to oxygen and peroxide and the latter will serve to reduce another Cu(II). Reaction 1 is thermodynamically favorable ($\Delta G^{\circ\prime} \cong -18$ kcal/mol of O_2^-) while reaction 13 is unfavorable ($\Delta G^{\circ\prime} \cong 13$ kcal/mol) (George, 1965) which may account for the relative slowness of the reaction which overall is favorable ($\Delta G^{\circ\prime} \cong -6.9$ kcal/mol of H_2O_2).[6] While the reduction of dismutase Cu(II) by peroxide may be of no mechanistic or physiological significance, it is important to assert that the results demonstrate the absence of redox centers other than the two Cu ions. Recently, Symonyan and Nalbandyan (1972) have also concluded that (5) describes the reaction between peroxide and dismutase.

The extremely slow autoxidation of reduced dismutase which was first observed by Mann and Keilen (1939) can be understood in terms of the oxidation–reduction potential of the Cu ions. Thus, reaction 15 is very unfavorable (George,

$$HECu(I) + O_2 \rightleftarrows ECu(II) + O_2^- + H^+ \qquad (15)$$

1965) having $\Delta G^{\circ\prime} \cong +23$ kcal/mol, corresponding to the minimum free energy of activation of the reaction at 25°. This

[6] Potentials for the oxygen system used in these arguments are taken from the article of George (1965). The reduction potential of the $O_2 + e^- \rightleftarrows O_2$ half-reaction is taken to be -0.59 (*cf.* Discussion comment of N. Sutin to George (1965)). The recent report by Rao and Hayon (1973) that the reduction potential of this couple is -0.15 V is clearly incorrect.

barrier is undoubtedly the root of the phenomenon which seems to be shared by other cuproproteins containing isolated Cu ions (Nakamura and Ogura, 1967; Fee *et al.*, 1969).[7]

The catalytic reoxidation of the enzyme by catalase is dependent on the prior presence or addition of H_2O_2 (apparently to initiate the reaction) and this is consistent with the suggestion of Fielden *et al.* (1973) that intermediates in the catalase reaction with peroxide are responsible for the reoxidation. However, the system is clearly more complicated as all H_2O_2 is surely decomposed within seconds after addition of catalase, making regeneration of the intermediate catalase H_2O_2 complexes impossible (Chance *et al.*, 1952).

The effect of catalase, then, might be partially explained by coupling reactions 15, 1, and 6. The overall reaction would then be thermodynamically favorable, as opposed to the reverse of reaction 5

$$2Cu(I) + 2H^+ + O_2 \rightleftharpoons 2Cu(II) + H_2O_2$$

which is unfavorable by ~7 kcal as written. However, if this were the case, catalase should catalyze the reoxidation of ascorbate reduced enzyme which it does not (Figure 7). Perhaps one form of the ascorbate is binding to the enzyme in a manner similar to that described by Rapp *et al.* (1973) for the reaction between dismutase and catechols and preventing this type of reoxidation. The relationship between catalase and superoxide dismutase is presently under study in this laboratory.

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[7] While the reverse of reaction 5 is thermodynamically unfavorable (+~7 kcal/mol H_2O_2) the equilibrium does not appear to be established, apparently because the initial oxidation of Cu(I) is very slow. Nevertheless, only a partial reoxidation could be obtained, and reduction of O_2 to H_2O must occur to account for complete reoxidation shown in Figure 7A.