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The Role of Superoxide Radical in the Autoxidation of Cytochrome *c*†

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ABSTRACT: The net rate of autoxidation of ferrocytochrome *c* was decreased by ferricytochrome *c*. Superoxide dismutase accelerated this autoxidation to a limit and overcame the inhibitory effect of ferricytochrome *c*. This was the case whether the autoxidation was observed in the presence or in the absence of denaturants, such as alcohols or urea, and whether the superoxide dismutase used was the Cu^{2+}-Zn^{2+} enzyme from bovine erythrocytes or the Mn^{3+}-enzyme from *Escherichia coli*. It can be deduced that the autoxidation of ferrocytochrome *c*, under a variety of conditions, generates O_2^- which can then dismute to $H_2O_2 + O_2$ or can reduce ferricytochrome *c* back to ferrocytochrome *c*. Superoxide dismutase, by accelerating the dismutation of O_2^-, prevents the back reaction and thus exposes the true rate of reaction of ferrocytochrome *c* with molecular oxygen.

Alcohols have been shown to facilitate the autoxidation of ferrocytochromes *c* and c_1 (Kaminsky et al., 1971; Yu et al., 1974). Higher alcohols were more effective in this regard than were lower homologs, and unbranched alcohols were more effective than the corresponding branched isomers. All of this is not surprising in view of the structure of ferrocytochrome *c* (Takano et al., 1973), in which the heme is enclosed in a hydrophobic crevice, and on the assumption that the situation in cytochrome c_1 is not very different. Thus alcohols could open the heme crevice both by partitioning into it and by changing the solvent properties of the bathing medium. The more hydrophobic the alcohol, the more effectively should it thus expose the heme and speed its reaction with dissolved oxygen. But what is the reduction product of oxygen in this autoxidation? If there is no aggregation of the ferrocytochrome prior to oxidation, and if the only oxidative change is the univalent conversion of ferroheme to the corresponding ferriheme, then the oxygen must be reduced to the superoxide radical (O_2^-).[1] If this were the case, it would create an interesting situation because O_2^- is a potent reductant of ferricytochrome *c* (McCord and Fridovich, 1968, 1969; Ballou et al., 1969; Land and Swallow, 1971). The autoxidation of ferrocytochrome *c* might therefore become self-limiting, since as ferricytochrome *c* accumulated, it would intercept an increasing fraction of the O_2^- generated. Ferricytochrome *c* would therefore have the apparent effect of inhibiting the autoxidation of ferrocytochrome *c*. In contrast, superoxide dismutase would, by catalytically scavenging O_2^- (McCord and Fridovich, 1969), prevent the reduction of the ferricytochrome *c*, and would thus expose the true rate of autoxidation. The following report demonstrates these effects and thus establishes that the autoxidation of ferrocytochrome *c* generates O_2^-.

Experimental Section

Materials. Horse heart cytochrome *c*, type III and type VI, was obtained from the Sigma Chemical Company, St. Louis, Mo. Bovine erythrocyte superoxide dismutase (SOD) was purchased from Truett Laboratories, Dallas, Texas, and was freed of carbonic anhydrase by passage through the affinity column described by Whitney (1974). Manganisuperoxide dismutase from *Escherichia coli B*, prepared by the method of Keele et al. (1970), was kindly provided by Dr. F. J. Yost, Jr. SOD was assayed by the method of McCord and Fridovich (1969). Reagent grade 1-butanol was purchased from Mallinckrodt Chemical Works, St. Louis, Mo., and 1-propanol from J. T. Baker Chemical Company, Phillipsburg, N.J. Deionized water was used throughout.

Methods. Cytochrome *c* concentrations were determined spectrophotometrically as described by Massey (1959). The concentration of oxygen was initially close to 0.2 m*M*. Since this exceeds the concentration of ferrocytochrome *c* by a factor of 5 there could not have been significant depletion of oxygen during the reactions observed.

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[1] Abbreviations used are: O_2^-, superoxide anion; SOD, superoxide dismutase.

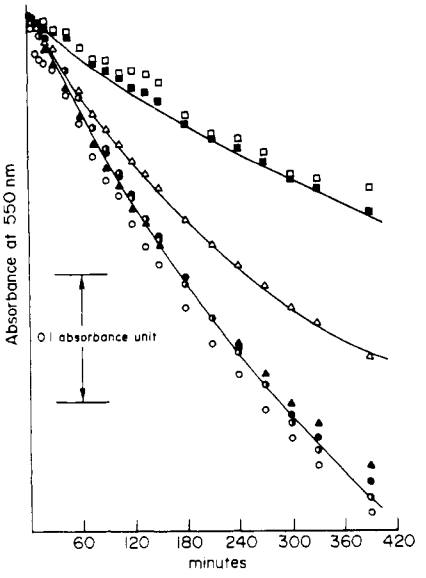

FIGURE 1: Effect of superoxide dismutase on the rate of autoxidation of ferrocytochrome *c* in 1-butanol. Reaction mixtures contained 1 mol % 1-butanol, 0.05 *M* potassium phosphate buffer (pH 7.8), 1×10^{-4} *M* EDTA, 4.0×10^{-5} *M* ferrocytochrome *c*, type III, and 4.0×10^{-6} *M* ferricytochrome *c*. Varying concentrations of bovine erythrocyte superoxide dismutase were present, as follows: (□) none; (■) 10.4 ng/ml; (△) 104 ng/ml; (▲) 1.04 μg/ml; (○) 10.4 μg/ml; (●) 104 μg/ml; (◐) 208 μg/ml. Reaction mixtures were incubated at 35° and absorbance at 550 nm was read at the indicated times.

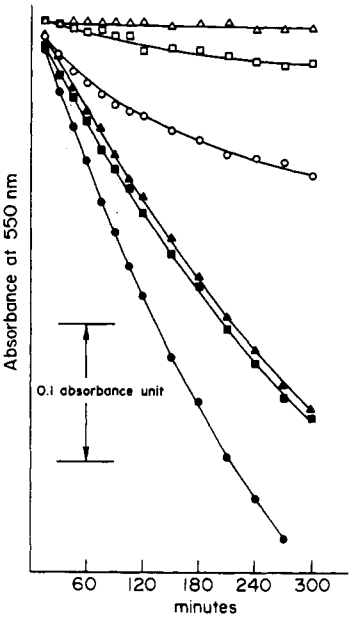

FIGURE 2: Effect of ferricytochrome *c* on the rate of autoxidation of ferrocytochrome *c*. The conditions were the same as those described in the legend to Figure 1, except that 3.99×10^{-5} *M* ferrocytochrome *c*, type VI, was used. Ferricytochrome *c* and bovine erythrocyte superoxide dismutase were varied, as follows: (○) none; (□) 4.75×10^{-6} *M* ferricytochrome *c*; (△) 9.79×10^{-6} *M* ferricytochrome *c*; (●) 10.4 μg/ml of SOD; (■) 4.75×10^{-6} *M* ferricytochrome *c* and 10.4 μg/ml of SOD; (▲) 9.79×10^{-6} *M* ferricytochrome *c* and 10.4 μg/ml of SOD.

Ferrocytochrome *c* was prepared by adding a slight excess of powdered sodium dithionite to a solution of cytochrome *c* in 0.05 *M* potassium phosphate buffer, pH 7.8, 1×10^{-4} *M* in EDTA. The sample was then either dialyzed under nitrogen against the potassium phosphate buffer or passed rapidly through a Sephadex G-25 column equilibrated with the same buffer. Completely reduced cytochrome *c* was obtained only by passage through the Sephadex column. Ferricytochrome *c* was prepared by adding a slight excess of potassium ferricyanide to a cytochrome *c* solution and then dialyzing against the phosphate buffer.

In certain experiments, a 10% (w/v) solution of trichloroacetic acid was added to an equal volume of cytochrome *c* solution. The precipitated cytochrome *c* was immediately collected by centrifugation and was redissolved in the potassium phosphate buffer and reduced and treated as previously described. Urea was dissolved in water and passed through Amberlite MB-3 to remove cyanates immediately before use.

The oxidation of ferrocytochrome *c* was observed at 35° by following the decrease in absorbance at 550 nm. In all cases the reaction mixtures were 0.05 *M* in potassium phosphate buffer at pH 7.8, and 1×10^{-4} *M* in EDTA. Reactions were started by the addition of ferrocytochrome *c* as the last component of the mixture.

Results and Discussion

Effect of Superoxide Dismutase. The rate of autoxidation of ferrocytochrome *c*, as accelerated by either 1-butanol or 1-propanol, was augmented by superoxide dismutase. This is illustrated in Figure 1. If SOD was increasing this autoxidation by scavenging O_2^- and thus preventing the reduction of ferricytochrome *c*, then its effect should reach a limit at that concentration of SOD which could successfully compete with the ferricytochrome *c* for the entire flux of O_2^-. Figure 1 demonstrates that this limit was reached at 1 μg/ml of SOD and that further increases in its concentration, up to 208 μg/ml, had no further effect. This result precludes the possibility that SOD directly catalyzed the autoxidation of ferrocytochrome *c*. Although the data in Figure 1 were obtained in the presence of 1-butanol, essentially similar results were seen in the presence of 7.5 or 10.0 mol % 1-propanol. The bovine erythrocyte SOD, which contains Cu^{2+} and Zn^{2+}, could be replaced by the manganese-containing enzyme from *Escherichia coli B* without in any way affecting these results.

Bovine erythrocyte SOD retains its activity in the presence of high concentrations of urea (Forman and Fridovich, 1973) and urea affects the absorption spectrum of cytochrome *c* much as do alcohols (Kaminsky and Davison, 1969). It could therefore by anticipated that urea would accelerate the autoxidation of ferrocytochrome *c* and that SOD would give the appearance of enhancing this autoxidation. Urea at 4.0 *M* did augment the autoxidation of ferrocytochrome *c* and SOD at 104 μg/ml increased the initial rate of this autoxidation by 250%. SOD did, in fact, also stimulate the slow autoxidation of ferrocytochrome *c* which is observed in the absence of denaturants.

Effects of Ferricytochrome c. If the autoxidation of ferrocytochrome *c* can be described by

$$\text{cyt. } c^{2+} + O_2 \rightleftarrows \text{cyt. } c^{3+} + O_2^- \quad (1)$$

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

then this autoxidation should be inhibited by ferricytochrome *c*. Thus as the concentration of ferricytochrome *c* rises, the likelihood the O_2^- will react with ferricytochrome *c* (reaction 1), rather than be removed from the field of action by dismutation (reaction 2), increases. Furthermore, SOD should, by accelerating reaction 2, oppose this effect of ferricytochrome *c*. Figure 2 demonstrates these effects. It is clear that ferricytochrome *c* did strongly inhibit the aut-

oxidation of ferrocytochrome *c* and that SOD minimized this inhibition. These results are fully in accord with reactions 1 and 2.

The often encountered difficulty of preparing solutions of fully reduced cytochrome *c* and the apparently great stability of partially reduced cytochrome *c* are now easily explained in the terms presented above.

Effects of Trichloroacetic Acid Precipitation on the Autoxidation of Ferrocytochrome c. Purification procedures for cytochrome *c* frequently employ precipitation of the protein with trichloroacetic or other acids (Keilin and Hartree, 1945; Margoliash, 1954a; Margoliash and Schejter, 1966). Treatment with this reagent produces a variable proportion of a new species of cytochrome *c* (Margoliash, 1954b) and renders the cytochrome *c* more readily autoxidizable (Okunuki, 1961). We were interested in determining whether this treatment changed the oxygen product of the autoxidation.

In these experiments cytochrome *c* (type VI) which had been prepared without exposure to trichloroacetic acid was used. This was once precipitated with 5% trichloroacetic acid and further treated as described in the Experimental Section. Ferrocytochrome *c*, type VI, without trichloroacetic acid treatment, was used as a control, and both samples were allowed to autoxidize at 35°. Ferricytochrome *c* was added to the untreated sample to compensate for incomplete reduction of the treated sample. The trichloroacetic acid treated ferrocytochrome *c* exhibited a biphasic autoxidation profile. The initial rate of oxidation was much faster than that of the control but after about 90 min this rate decreased to an approximation of that of the control. SOD sharply stimulated the rates of all phases of the autoxidation for both the treated and the control samples. It can be concluded that the modification of cytochrome *c* by treatment with trichloroacetic acid, while augmenting its reactivity toward molecular oxygen, does not change the valency of this oxidation.

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