DIRECT DEMONSTRATION OF SUPEROXIDE ANION PRODUCTION DURING THE OXIDATION OF REDUCED FLAVIN AND OF ITS CATALYTIC DECOMPOSITION BY ERYTHROCUPREIN[1]

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SUMMARY The oxidation of reduced flavins by molecular oxygen at neutral to alkaline pH produces substantial yields of the superoxide anion, $O_2^{\cdot-}$. This species is rapidly destroyed by catalytic quantities of the copper protein, erythrocuprein, and by stoichiometric quantities of ferricytochrome c.

The involvement of superoxide anion in the aerobic reduction of cytochrome c catalyzed by xanthine oxidase and other metalloflavoproteins has been strongly suggested by the work of Fridovich and his colleagues (1,2,3). More recently, Knowles et al. (4) have demonstrated directly, by means of the rapid-freezing EPR (electron paramagnetic resonance) technique (5), that $O_2^{\cdot-}$ is indeed a product of the oxidation of substrate-reduced xanthine oxidase. McCord and Fridovich (6) have recently provided strong evidence that erythrocuprein is a potent superoxide dismutase and that it inhibits the xanthine oxidase catalyzed aerobic reduction of cytochrome c. In both the work of Knowles et al. (4) and the earlier work of Fridovich et al. it was suggested that the iron-sulfur component, rather than the flavin moiety, of these enzymes is responsible for the observed one electron reduction of oxygen. However, the observation that many metal free flavoproteins can catalyze an oxygen-dependent substrate-linked reduction of cytochrome c (7), which is inhibited by erythrocuprein, has led to the suggestion that reduced flavin can reduce oxygen to superoxide anion.

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In this paper we wish to report the direct demonstration that superoxide anion is produced in substantial yields during the oxidation of reduced flavins by molecular oxygen. Furthermore, the superoxide anion so produced has been shown to react rapidly with cytochrome c and to be decomposed extremely rapidly by catalytic quantities of erythrocuprein.

METHODS AND MATERIALS

Erythrocuprein was isolated from beef erythrocytes by the methods of Reed et al. (8), and was the generous gift of Dr. D. E. Hultquist and Mr. P. Passon. Its concentration was estimated from the molar extinction coefficient of 350 at 665 mμ (9). Cytochrome c, type III, was obtained from the Sigma Chemical Company, and tetra-acetyl riboflavin (TARF) was synthesized by the method of Hemmerich (10).

Reduced flavin solutions were prepared (and stored) under nitrogen in glass tonometers by irradiation with visible light in the presence of 1.33 x 10^{-2}M EDTA (11). Buffers were oxygenated by equilibration with oxygen (1 atm) at room temperature and stored in tonometers.

Rapid spectrophotometric studies were performed with a Gibson-Milnes stopped-flow spectrophotometer (12). Flavin oxidation was monitored at 500 nm.

The rapid-freezing technique of Bray (5) with modification by Ballou and Palmer (13) was employed to isolate the superoxide radical. Reaction of the superoxide radical with either erythrocuprein or cytochrome c was followed by employing a three syringe system (14) with subsequent rapid freezing at desired times. The plastic connecting hoses (0.022" and 0.046" bore, Bel Art Plastics) carrying the reduced flavin were maintained anaerobic by encasing them with thin-walled stainless steel sleeving. Repeated evacuation followed by flushing with oxygen-free helium in a desicator was carried out to minimize oxygen contamination.

EPR measurements were carried out with a Varian V4500-10A spectrometer equipped with a cryogenic device similar to that described by Hansen et al. (15).

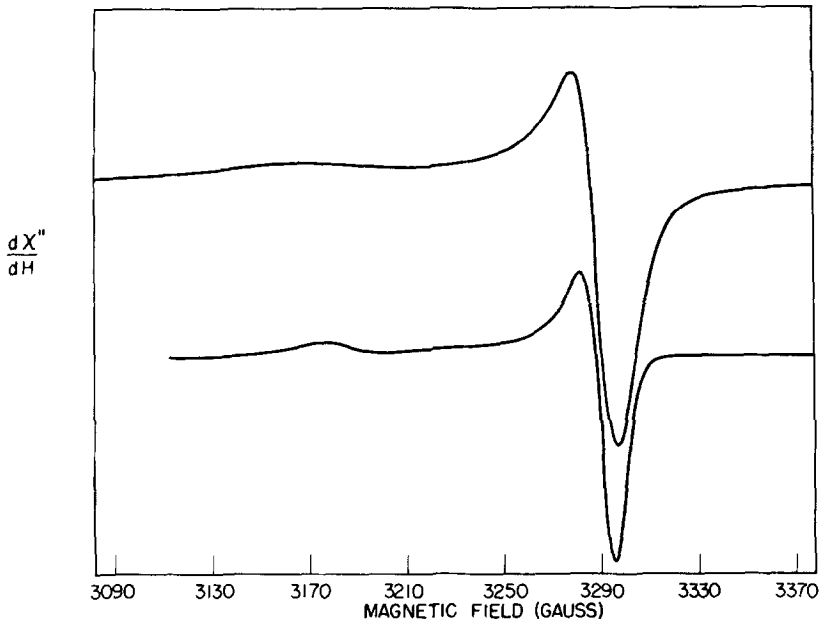

Figure 1 EPR spectra of $O_2^{\cdot-}$. (Top) Radical produced by the reaction of 0.075M KIO_4 with 0.15M H_2O_2 in 0.1 M glycine buffer pH 9.9 at 20° for 545 msec. EPR spectrum recorded at 87°K. (Bottom) Radical produced by reaction of 1.25 x 10^{-4}M $TARFH_2$ with 6.25 x 10^{-4}M oxygen in 0.095 M glycine pH 10.6 at 20° for 444 msec. EPR spectrum recorded at 92°K. Microwave power, 3m watts; modulation amplitude, 3 gauss at 100 kHz. Microwave frequency 9.235 GHz.

RESULTS

Figure 1 shows EPR spectra of the oxygen radical trapped by rapid freezing during both the reaction of reduced tetra-acetyl riboflavin ($TARFH_2$) and O_2, and the reaction of KIO_4 and H_2O_2. A similar spectrum was obtained by the reaction of dithionite with oxygen (4). The g-values and overall shape of the spectra are in close agreement with those obtained by Knowles *et al.* (4) in the KIO_4 vs H_2O_2 system and in the xanthine oxidase reaction. They also agree well with those obtained by Ichikawa *et al.* (16) and Bennett *et al.* (17) who report values of $g_{\parallel} = 2.088$ and $g_{\perp} = 2.008$.

Similar spectra were also obtained for the reaction of reduced FMN with O_2, but flavin impurities in the sample made kinetic analysis less straightforward than with $TARFH_2$ which was therefore used in the studies reported here.

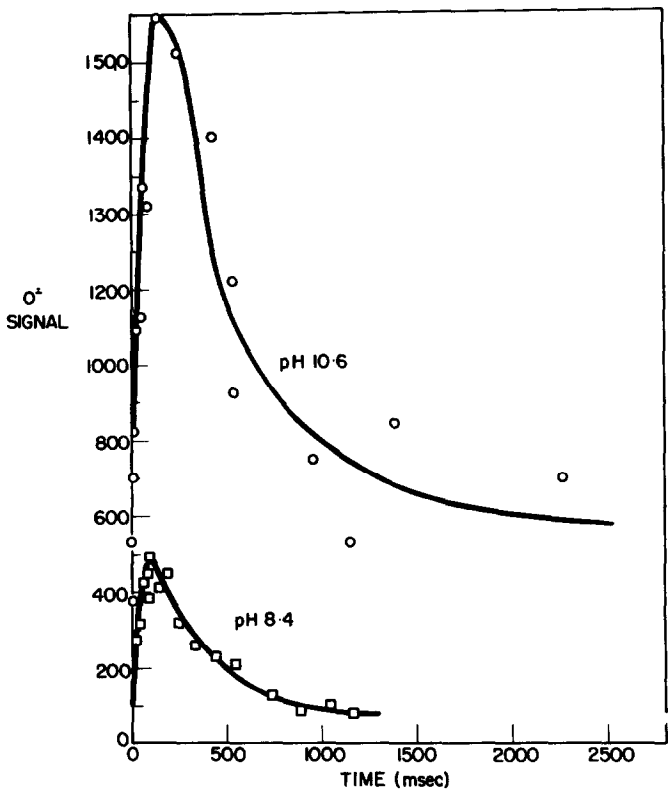

Figure 2 Time course of the appearance and decay of $O_2^{\cdot-}$ produced by reaction of 1.25 x 10^{-4}M $TARFH_2$ and 6.5 x 10^{-4}M O_2 at 20°. Top curve, in 0.1M glycine pH 10.6. Bottom curve, in 0.1M glycylglycine pH 8.4. All times include the 5 msec. quenching time. Maximum intensity at pH 10.6 corresponds to 1.19 x 10^{-4}M oxygen radical. Modulation amplitude 10 gauss at 100 kHz. Microwave power = 12 m watts. Microwave frequency = 9.2 GHz. Temperature = 84°K.

It was found that the kinetics and the yield of the $O_2^{\cdot-}$ radical were markedly affected by pH. Figure 2 shows the kinetic progress curves for identical reactions of $TARFH_2$ with oxygenated buffer at pH 10.6 and pH 8.4. The most striking feature is the difference in the yields of the radical species produced at the two pH values. At pH 10.6 the maximum yield (obtained by double integration using a copper sulfate - EDTA standard) was 96% of the initial reduced flavin concentration whereas at pH 8.4 the maximum yield was only 14%. Although the decay of the radical species is slower at the higher pH, this factor alone cannot account for the difference

in yields. This result suggests that the superoxide anion reacts only slowly with reduced flavin species at the higher pH whereas it reacts much more readily at the lower pH. That $O_2^{\cdot-}$ is involved in the autocatalytic reoxidation of reduced flavin was shown by stopped-flow studies; in the presence of 10^{-7}M erythrocuprein the reoxidation of $TARFH_2$ by O_2 was slowed down four-fold. These results will be published in full later.

Having obtained direct evidence that oxidation of reduced flavins can produce substantial amounts of the superoxide anion, it became desirable to confirm directly the proposal that erythrocuprein is a superoxide dismutase (1). Rapid-freezing studies were performed employing a three syringe system which produced the superoxide radical by the reaction at pH 8.4 of $TARFH_2$ and O_2 contained in the first two syringes respectively, and, after a delay of 181 msec., introduced erythrocuprein via the third syringe. By varying the dead volume from the second mixing chamber to the exit nozzles, a kinetic progress curve of the reaction catalyzed by erythrocuprein could be obtained. Stopped-flow studies showed that more than 98% of the flavin had been oxidized at the time at which erythrocuprein was introduced. It can be seen from Figure 2 that at 181 msec. nearly 80% of the maximum yield of superoxide anion (or about 2×10^{-5}M) was available for reaction with erythrocuprein. At erythrocuprein concentrations greater than 6.2×10^{-7}M, no superoxide radical could be detected at times longer than 12 msec. after introduction of erythrocuprein. (This includes the 5 msec. necessary for quenching (13).)

Limited kinetic studies of the decomposition of superoxide anion by this direct method are shown in Figure 3. The decay of the superoxide signal followed second order kinetics implying that at this concentration of $O_2^{\cdot-}$ the erythrocuprein is not saturated with substrate. Under these experimental conditions (i.e. with limiting $O_2^{\cdot-}$ concentrations), the turnover number for erythrocuprein as a superoxide anion dismutase can be estimated to be at least 3 million (moles $O_2^{\cdot-}$ disappearing per minute per mole erythrocuprein).

When in this three syringe experiment the erythrocuprein was replaced

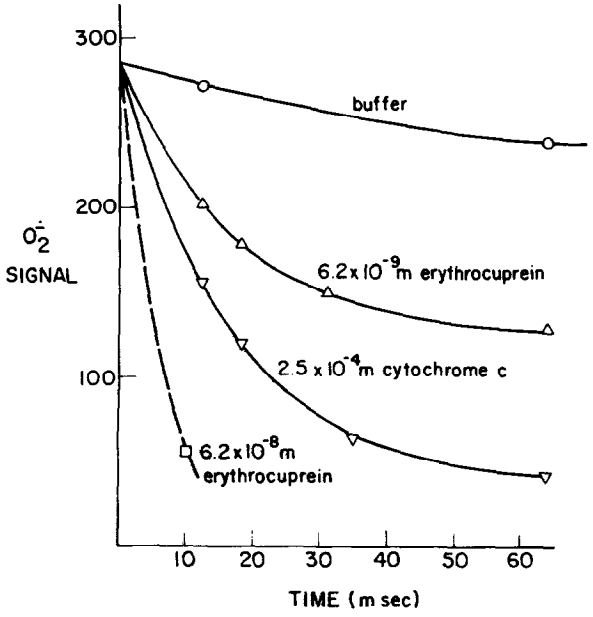

Figure 3 Reaction of O_2^- with erythrocuprein and cytochrome c. O_2^- was produced by the reaction of 1.25×10^{-4}M $TARFH_2$ with 6.5×10^{-4}M O_2 in 0.1 M glycylglycine pH 8.4 at 20° for 181 msec., before reaction from a third syringe with erythrocuprein, cytochrome c, or buffer as shown. The reaction times shown are those from 181 msec. and include the 5 msec. quenching time. The signal intensities in this experiment are 67% of those reported in Figure 2 (bottom) because of the dilution produced by the third syringe. Modulation amplitude 10 gauss at 100 kHz. Microwave power = 12m watts. Microwave frequency 9.2 GHz. Temperature = 84°K.

with cytochrome c, rapid disappearance of O_2^- was again observed (Figure 3). The decay of the O_2^- signal follows first order kinetics with a pseudo first order rate constant of 40 sec^{-1} which is equivalent to a second order rate constant of $1.6 \times 10^5 M^{-1}$ sec^{-1}.

In summary these observations provide direct experimental proof that O_2^- is a significant product in the reaction of oxygen with reduced flavins, and that it can be rapidly decomposed by erythrocuprein. By the above techniques it should be possible to investigate the role of O_2^- in a wide range of biological oxidation-reduction reactions.

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