THE PRODUCTION OF SUPEROXIDE ANION RADICALS IN THE REACTION OF REDUCED FLAVINS AND FLAVOPROTEINS WITH MOLECULAR OXYGEN.[1]

V. Massey, S. Strickland, S. G. Mayhew, L. G. Howell,[2] P. C. Engel,[3] R. G. Matthews,[4] M. Schuman,[5] and P. A. Sullivan.
Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48104

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The generation of $O_2^{\cdot-}$ in the reaction of reduced flavins and several flavoproteins has been shown. This has been demonstrated by the ability to catalyse the aerobic reduction of cytochrome c and the inhibition of this reduction by erythrocuprein. In contrast to the results with the intact enzyme, the reduction of cytochrome c by deflavoxanthine oxidase is not inhibited by erythrocuprein, indicating that the site of production of $O_2^{\cdot-}$ in the native enzyme is flavin, rather than non-heme iron.

In 1949, Horecker and Heppel (1) showed that the xanthine-cytochrome c reductase activity of milk xanthine oxidase was markedly increased by oxygen. Fridovich and Handler (2-5) proposed that this oxygen-dependent reduction of cytochrome c was due to reduction by $O_2^{\cdot-}$ produced by one-electron reduction of molecular oxygen by xanthine and xanthine oxidase. This proposal was based on the O_2-dependent reduction of cytochrome c, the initiation of a chain reaction oxidation of sulfite, and the burst of light emitted in the presence of luminol (the latter reaction was first shown by Totter et al. (6)). Similar results were also obtained with two other metalloflavoproteins, aldehyde oxidase and dihydro orotate dehydrogenase (7), but not with the simple flavoproteins, glucose oxidase, D-amino acid oxidase or L-amino acid oxidase (4). It was concluded that the non-heme iron rather than the flavin was responsible for the reduction of molecular oxygen to $O_2^{\cdot-}$. Similar conclusions were reached by Knowles et al. (8) who demonstrated by rapid-freezing EPR studies the produc-

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tion of $O_2^{\bullet-}$ during the reaction of xanthine oxidase with xanthine and O_2. Recently Komai et al. (9) succeeded in removing the FAD of milk xanthine oxidase and showed that reduced flavin rather than the non-heme iron was probably the site of reaction of this enzyme with O_2. The xanthine-cytochrome c reductase activity of the deflavo enzyme was not dependent on O_2 suggesting that the reaction of reduced flavin with O_2 was responsible for generation of $O_2^{\bullet-}$.

An important analytical tool for the investigation of the participation of $O_2^{\bullet-}$ in biological oxidations has recently become available through the discovery of the superoxide anion dismutase activity of erythrocuprein (10). McCord and Fridovich found that very low concentrations of erythrocuprein inhibited the O_2-dependent xanthine-cytochrome c reductase activity of xanthine oxidase and also inhibited the reduction of cytochrome c by $O_2^{\bullet-}$ generated electrolytically (11). Using erythrocuprein we have been able to demonstrate that $O_2^{\bullet-}$ is generated by the reaction of reduced flavin and several simple flavoproteins with O_2. The same technique gives a confirmation of our earlier suggestion that it is the reduced flavin of xanthine oxidase which is responsible for production of $O_2^{\bullet-}$ (9). The accompanying paper presents direct EPR evidence for the generation of $O_2^{\bullet-}$ in the reaction of reduced flavin with oxygen, and for erythrocuprein as a superoxide anion dismutase (12).

MATERIALS. The enzymes used were prepared as described previously; xanthine oxidase (13, 9); L- and D-amino acid oxidases (14, 15); lactate oxidase (16); glycolate oxidase (17); lipoyl dehydrogenase (18); glutathione reductase (19); flavodoxin (20); ferredoxin-TPN reductase (21); old yellow enzyme (22). Melilotate hydroxylase from an Arthrobacter sp. and p-hydroxybenzoate hydroxylase from Pseudomonas fluorescens were isolated by unpublished procedures. Erythrocuprein, from beef erythrocytes, (Reed et al. (23)) was the generous gift of Dr. D. E. Hultquist and Mr. P. Passon. Bovine catalase was from CalBiochem and horse heart cytochrome c (Type III) was from the Sigma Chemical Company.

RESULTS

Effect of Erythrocuprein on Xanthine-cytochrome c Reductase Activity of Xanthine Oxidase. In agreement with McCord and Fridovich (10) we find that erythrocuprein is a very potent inhibitor of the aerobic reduction of cytochrome c catalysed by xanthine oxidase and xanthine. Marked inhibition was obtained at concentrations of erythrocuprein as low as 10^{-9} M. In contrast, 10^{-6} M erythrocuprein had no effect on the anaerobic cytochrome c reduction catalysed by native enzyme, or on the O_2-independent cytochrome c reduction catalysed by deflavo xanthine oxidase (Table I).

TABLE I

Effect of Erythrocuprein on Xanthine-cytochrome c Reductase Activity of Milk Xanthine Oxidase

The reduction of cytochrome c was measured at 550 mμ using a Gilford recording spectrophotometer. Conditions; 0.1 M pyrophosphate, pH 8.5, 10^{-4} M xanthine, 1.67×10^{-5} M cytochrome c, xanthine oxidase 6×10^{-9} M to 3×10^{-7} M, temperature 25°.

Conditions	Molecular Activity*
A. Native xanthine oxidase (aerobic)	336
plus 1.23×10^{-9} M erythrocuprein	218
" 2.47×10^{-9} M "	165
" 3.7×10^{-9} M "	127
" 6.2×10^{-9} M "	94
" 1.23×10^{-8} M "	50
" 6.2×10^{-7} M "	4
B. Native xanthine oxidase (anaerobic)	3.5
plus 6.2×10^{-7} M erythrocuprein	3.5
C. Deflavo xanthine oxidase (aerobic)	150
plus 1.24×10^{-6} M erythrocuprein	143

* molecules of cytochrome c reduced per min. per atom of enzyme-bound molybdenum.

Reduction of Cytochrome c by Reduced Flavin and Oxygen. The results with xanthine oxidase strongly suggested that reduced flavin could generate $O_2^{\bullet-}$. A simple model system to demonstrate this is shown in Fig. 1. EDTA reduces free flavins readily on exposure to visible light (24); use was made of this

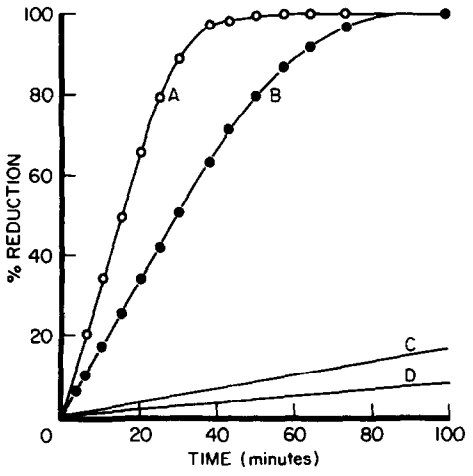

Figure 1. Reduced flavin-O_2 induced reduction of cytochrome c. Quartz spectrophotometer cells containing in a total volume of 3 ml., 0.1 M pyrophosphate, pH 8.5, 3.33×10^{-5} M cytochrome c, 0.01 M EDTA, 10 µg catalase and the following additions were exposed to room light for the indicated times: A. 3.33×10^{-6} M tetraacetyl riboflavin (TARF); B. 1.67×10^{-6} M TARF; C. 3.33×10^{-6} M TARF plus 6.2×10^{-7} M erythrocuprein; D. 1.67×10^{-6} M TARF plus 6.2×10^{-7} M erythrocuprein.

reaction to provide a continuous low concentration of reduced flavin for reaction with cytochrome c under aerobic conditions. Rapid reduction of cytochrome c occurs under these conditions (Fig. 1), such reduction is inhibited approximately 95% by 6.2×10^{-7} M erythrocuprein. The reduction did not proceed in the dark, nor in the absence of flavin. Similar reduction was observed with other flavin analogues tested, e.g. lumiflavin and lumiflavin acetic acid. Flavin photo-catalysed reduction of cytochrome c was in fact observed by Vernon (25) in 1959 and initiation of chain reaction oxidation of sulfite by Fridovich and Handler in 1960 (26). However, these results were interpreted as being due to flavin radical; the present demonstration of erythrocuprein inhibition clearly implies that $O_2^{\bullet -}$ rather than flavin radicals are responsible. The above results also indicate that the reaction of reduced flavin with O_2 to produce $O_2^{\bullet -}$ is a more efficient process than that of reduced flavin with cytochrome c. Stopped flow studies show that reduced flavin reacts rapidly anaerobically with

cytochrome c; the fact that erythrocuprein is such an effective inhibitor of the aerobic reaction shows that the generation of $O_2^{\bullet-}$ with reduced flavin and O_2 and the subsequent reduction of cytochrome c must be even more rapid.

Evidence for $O_2^{\bullet-}$ Production with Simple Flavoproteins. We recently suggested that the initial step in the oxygen reaction of reduced flavin is the formation of a covalent linkage of O_2 with the reduced flavin (17). It was proposed that the fate of the oxygenated flavin determined whether a particular flavoprotein was a typical oxidase, dehydrogenase or hydroxylase, and that the oxygen compound of the flavoprotein dehydrogenases probably breaks down preferentially into flavin radical and $O_2^{\bullet-}$, while other routes are probably taken by the flavoprotein oxidases and hydroxylases. It was therefore with considerable interest that we looked for erythrocuprein-inhibited cytochrome c reduction with various flavoproteins reacting with their specific substrates in the presence of O_2. It is seen that in general the flavoprotein oxidases and hydroxylases do not seem to produce significant amounts of $O_2^{\bullet-}$, whereas the flavoprotein dehydrogenases do (Table II). It should also be noted that several enzymes tested exhibit much smaller cytochrome c reductase activity anaerobically than aerobically.

While a positive effect of erythrocuprein in inhibiting flavoprotein catalysed cytochrome c reduction is clearly a strong indication of $O_2^{\bullet-}$ production, a negative result is not capable of such clear-cut interpretation. The possibility exists in the case of the flavoprotein hydroxylases and oxidases that $O_2^{\bullet-}$ might indeed be produced in the reaction of the reduced flavoproteins with O_2, but that the radicals are sufficiently tightly bound to the enzyme that both cytochrome c and erythrocuprein would be ineffective scavengers of the $O_2^{\bullet-}$. As the reaction of cytochrome c with $O_2^{\bullet-}$ is presumably a bimolecular reaction whereas the catalytic reaction of erythrocuprein would require each molecule of erythrocuprein to react with two molecules of $O_2^{\bullet-}$, it is conceivable that in such cases (where the $O_2^{\bullet-}$ does not succeed in leaving easily the solvent cage of the flavoprotein) cytochrome c reduction could be

TABLE II

Effect of Erythrocuprein on Cytochrome c Reductase Activity of Flavoproteins

The cytochrome c reductase activity was measured in air-equilibrated solutions containing 0.1 M pyrophosphate, pH 8.5, in the presence of 3.33×10^{-5} M cytochrome c and 10 µg bovine catalase. When erythrocuprein was added, it was present at a concentration of 6.2×10^{-7} M. Temperature, 25°.

Enzyme	Reductant	Molecular Activity* In absence of erythrocuprein	In presence of erythrocuprein	Under anaerobic conditions
Glucose oxidase	0.1M glucose	0.038	0.034	
D-amino oxidase	1.86×10^{-2} M D-alanine	0.054	0.060	
L-amino acid oxidase	1.25×10^{-3} M L-leucine	0.127	0.126	
Glycollate oxidase	6.7×10^{-4} M glycollate	0.68	0.50	
Lactate oxidase	3.33×10^{-4} M L-lactate	<0.010	<0.010	
P-hydroxy benzoate hydroxylase**	1.5×10^{-4} M TPNH + 3.3×10^{-4} M p-hydroxybenzoate	213	213	
Melilotate hydroxylase**	2×10^{-4} M DPNH + 1.5×10^{-4} M melilotate	72	60	
Old yellow enzyme	2×10^{-4} M TPNH	5.3	0.37	1.27
Ferredoxin TPN reductase	2×10^{-4} M TPNH	4.6	1.25	2.3
Flavodoxin***	2×10^{-4} M TPNH + 3.2×10^{-8} M ferredoxin TPN reductase	0.102	0.047	
Lipoyl dehydrogenase	2×10^{-4} M DPNH	2.7	0.26	0.21
Glutathione reductase	2×10^{-4} M TPNH	0.9	0.072	0.10

* Molecules of cytochrome c reduced per min. per molecule of enzyme-bound flavin.

** The quoted values are due in part (and possibly completely) to the non-enzymic reduction of cytochrome c by the hydroxylated products of these enzymes.

***A coupled system containing 5×10^{-5} M flavodoxin (27). Results are corrected for the blank rate due to the ferredoxin TPN reductase alone.

observed without inhibition by erythrocuprein. We plan in future studies to use the rapid freezing EPR technique described in the accompanying paper to provide a direct test for the possible participation of $O_2^{\cdot -}$ in reactions with the flavoprotein oxidases and hydroxylases.

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