The Univalent Reduction of Oxygen by Reduced Flavins and Quinones*

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SUMMARY

FMN and flavodoxin, when reduced by the action of ferredoxin-TPN+ oxidoreductase, have been shown to cause both univalent and divalent reductions of oxygen. Superoxide radicals, so generated, were detected by their abilities to oxidize epinephrine to adrenochrome and to reduce cytochrome c. Superoxide dismutase inhibited these actions of the superoxide radical. The ratio of the univalent reduction of oxygen, to the sum of the univalent plus divalent reductions of oxygen, was determined as a function of pH and of oxygen concentration. These data were compared with the results of similar measurements made previously, with ferredoxins and xanthine oxidase. FMN and flavodoxin carry out the univalent reduction of oxygen much less effectively than either xanthine oxidase or the ferredoxins. Furthermore, the univalent reduction of oxygen by xanthine oxidase and by the ferredoxins showed similar responses to changes in pH and oxygen concentration; whereas the behavior of the flavodoxin and FMN mediated systems was distinctly different. These results suggest that the reduction of oxygen by native milk xanthine oxidase may well be a function of its non-heme iron centers.

The fully reduced form of menadione, generated by the action of Diaphorase, caused a predominantly divalent reduction of oxygen; whereas the semiquinone forms of this carrier, generated by the action of the ferredoxin-TPN+ oxidoreductase, caused primarily a univalent reduction of oxygen.

Milk xanthine oxidase causes both univalent and divalent reductions of oxygen (1). Both the iron-sulfur (1, 2) and the flavin (3) prosthetic groups of this enzyme have been proposed as the site of oxygen reduction. Experiments which would unequivocally settle this point have not yet been devised. It is possible however, to approach this problem by comparing the behavior of xanthine oxidase with that of simpler systems containing either non-heme iron or flavin. Thus, the univalent reduction of oxygen, mediated by ferredoxins, has been investigated as a function of pH and of oxygen concentration and has been found to mimic the comparable behavior of xanthine oxidase (4). Although these results are consistent with the position that oxygen reduction by milk xanthine oxidase is a function of its non-heme iron centers; it is obviously desirable to similarly explore the reduction of oxygen by flavins. Flavodoxin, which is elaborated in place of ferredoxin by a number of bacteria, when grown in iron-deficient media, and which can substitute for ferredoxin in a number of reactions (5-10) was selected as a suitable FMN-containing and iron-free analogue of ferredoxin. The effects of pH and of oxygen concentration on the univalent reduction of oxygen, mediated by FMN and by flavodoxin, are described in this report.

It has been reported that, when FMN or menadione mediated the reduction of oxygen by the ferredoxin-TPN+ oxidoreductase, univalent reduction of oxygen predominates over divalent reduction by a factor of 3 to 4 (11). This demonstration, that the reduced forms of these carriers could cause the univalent reduction of oxygen, gave no indication of the relative importance of the fully reduced as compared to the semiquinone forms. A means of investigating this point was provided by Iyanagi and Yamazaki (12) who showed that lipoyl dehydrogenase (Diaphorase) causes the divalent reduction of p-henoquinone, whereas ferredoxin-TPN+ oxidoreductase (FTR) causes the univalent reduction of this carrier. Measurements of the relationship of univalent to divalent reduction of oxygen by electron carriers, reduced by these enzymes, should be informative with respect to the mechanism of oxygen reduction by fully reduced and by semiquinone forms of electron carriers. The results of such measurements are also reported below.

MATERIALS AND METHODS

Superoxide dismutase was prepared from bovine erythrocytes and was assayed as previously described (13). FTR was prepared according to Shin et al. (14). Xanthine oxidase, prepared from unpasteurized cream by a procedure which avoided exposure to proteolytic agents (15), was kindly provided by Dr. K. V. Rajagopalan of the Duke University Medical Center. Flavodoxin, isolated from Clostridium pasteurianum, strain W-S (original ATCC 6013), was a generous gift of Dr. J. L. Fox of the University of Texas at Austin. Diaphorase type III (lipoyl dehydrogenase) from pig heart was obtained from Sigma as was cytochrome c type III. Solutions of flavodoxin were quantitated on the basis of Em = 10,400 M⁻¹ cm⁻¹ at 443 nm (16) and solutions of lipoyl dehydrogenase on the basis of Em = 11,300 M⁻¹ cm⁻¹ at 455 nm (16, 17). All other compounds were obtained from commercial sources at the highest obtainable purity.

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1 The abbreviation used is: FTR, ferredoxin-TPN+ oxidoreductase.
Assays—All assays were performed at 25° in a Gilford model 2000 absorbance indicator. Reactions under controlled atmospheres were executed in cuvettes which allowed purging the reaction volume with the desired gas mixture. These cuvettes are similar in design to those described by Lazarow and Cooperstein (18) and were obtained from Pyrocell.

The transfer of electrons from TPNH, through the enzyme to oxygen, was mediated by FMN, flavodoxin, or menadione. The total flux of electrons, from TPNH to oxygen, was measured in terms of the oxidation of TPNH, followed at 340 nm. The oxidation of TPNH, by the FTR, was completely dependent upon electron carriers. Since oxygen was the only electron acceptor present in stoichiometric amounts, the net oxidation of TPNH is presumed to have been dependent upon the oxidation of the reduced carriers by oxygen. The extent of univalent reduction of oxygen was measured by using epinephrine. It has been presumed to have been dependent upon the oxidation of the reduced carriers by oxygen.

Results

FMN as Mediator of TPNH Oxidation. As shown in Fig. 1, the oxidation of TPNH by FTR was dependent upon FMN. The data approximates a rectangular hyperbola and half of the maximal rate was observed at 5 X 10⁻⁴ M FMN. The data represented by the triangular points on this figure demonstrate that superoxide dismutase was without effect on the ability of FMN to mediate the oxidation of TPNH by FTR. These results are in accord with a sequence of electron transfers going from TPNH to FTR to FMN to O₂. If O₂⁻ was generated by the reoxidation of reduced FMN; its rate of dismutation would have no influence on the rate of TPNH oxidation. The inactivity of superoxide dismutase in this assay, is therefore the expected result. It does however establish that superoxide dismutase does not directly inhibit FTR; which is in accord with previous observations (4).

Epinephrine as Detector of O₂⁻—The action of FTR on TPNH in the presence of FMN, epinephrine, and oxygen, resulted in the production of adrenochrome. As shown in Fig. 2 the rate of accumulation of adrenochrome was a function of the concentration of FMN. When the maximal rate was achieved at 2.5 X 10⁻⁵ M FMN. The apparent difference between this value and that arrived at by measurement of TPNH oxidation (Fig. 1) is not considered to be significant. The effect of varying the concentration of epinephrine was also investigated and these results are shown in Fig. 3. Increasing the concentration of epinephrine should result in the interception of an ever larger fraction of the O₂⁻ generated. Saturation with epinephrine would occur when essentially all of the O₂⁻ generated, reacted with epinephrine, and none of the O₂⁻ was lost to the dismutation reaction. The rate of adrenochrome formation was half saturated at 2 X 10⁻⁸ M epinephrine. Elimination of oxygen from these reaction mixtures completely prevented the formation of adrenochrome.

Superoxide dismutase was used to verify the production of O₂⁻ in this reaction mixture. Thus, O₂⁻, generated by the oxidation of reduced FMN or flavodoxin, could either dismute to H₂O₂ + O₂ or could react with epinephrine or with ferricytochrome c. Superoxide dismutase by catalyzing the dismutation reaction should decrease the availability of O₂⁻ for the oxidation of epinephrine or for the reduction of cytochrome c. Fig. 4 demonstrates that this was the case. Superoxide dismutase inhibited the production of adrenochrome when either FMN or flavodoxin was used as the electron carrier. The results of Fig. 4 are similar to those obtained previously (4) and were obtained from Pyrocell.

Experimental

The transfer of electrons from TPNH, through the enzyme to oxygen, was mediated by FMN, flavodoxin, or menadione. The total flux of electrons, from TPNH to oxygen, was measured in terms of the oxidation of TPNH, followed at 340 nm. The oxidation of TPNH, by the FTR, was completely dependent upon electron carriers. Since oxygen was the only electron acceptor present in stoichiometric amounts, the net oxidation of TPNH is presumed to have been dependent upon the oxidation of the reduced carriers by oxygen. The extent of univalent reduction of oxygen was measured by using epinephrine. It has been presumed to have been dependent upon the oxidation of the reduced carriers by oxygen. The extent of univalent reduction of oxygen was measured by using epinephrine. It has been presumed to have been dependent upon the oxidation of the reduced carriers by oxygen. The extent of univalent reduction of oxygen was measured by using epinephrine. It has been presumed to have been dependent upon the oxidation of the reduced carriers by oxygen. The extent of univalent reduction of oxygen was measured by using epinephrine. It has been presumed to have been dependent upon the oxidation of the reduced carriers by oxygen.
duce cytochrome c without the involvement of $O_2^-$. Under the conditions of these experiments approximately 20% of the reduction of cytochrome c occurred by an $O_2^-$-independent, superoxide dismutase-insensitive route. The formation of adrenochrome, in contrast, is an oxidative reaction, dependent upon the oxidizing properties of $O_2^-$. It cannot be caused directly by reduced forms of FMN and was therefore totally dependent upon the $O_2^-$-dependent and superoxide dismutase-sensitive route of electron transfer.

The Proportion of Univalent Reduction of Oxygen by FMN and Flavodoxin—Electrons from TPNH were ultimately transferred to oxygen singly or in pairs. In the former case $O_2^-$ was produced and it could be measured in terms of the accumulation of adrenochrome; as has previously been described (4). That fraction of the total reduction of oxygen by TPNH which occurred by single electron transfers could therefore be calculated and when multiplied by 100 could be expressed as the percentage of univalent flux. Such measurements were made as a function of oxygen concentration and at pH 6.8 and 7.8. Fig. 5 presents the results obtained when FMN was the mediator of electron transfer from FTR to oxygen; while Fig. 6 presents comparable data obtained with flavodoxin as the electron carrier.

Oxygen Reduction by Fully Reduced Menadione—When menadione was used to mediate the aerobic reduction of cytochrome c by TPNH, as catalyzed by FTR, approximately 70% of the electrons were found to be traversing the $O_2^-$ pathway (11). It was not possible at that time to decide whether the observed univalent reduction of oxygen was primarily due to fully reduced menadione or to its semiquinone. Inayagi and Yamazaki (12) have shown that FTR carries out the univalent reduction of quinones, whereas lipoyl dehydrogenase causes divalent reductions. These two enzymes were therefore used to catalyze the reductions of cytochrome c and of oxygen by reduced pyridine coenzymes as mediated by menadione, and the percentage of univalent reduction of oxygen was measured. When FTR was used, 64% of the oxygen reduction was univalent; whereas when lipoyl dehydrogenase was used, only 21%; of the electrons passing to oxygen did so in univalent steps. Thus, when $6.1 \times 10^{-4}$ M FTR acted upon aerobic reaction mixtures containing $6.7 \times 10^{-5}$ M TPNH and $8.3 \times 10^{-7}$ M menadione in 0.05 M Tris-HCl at pH 8.7 and at 25°, the rate of TPNH oxidation was $2.1 \times 10^{-5}$ M per min. When 1 $\times 10^{-5}$ M cytochrome c was added, it was reduced at a rate of $3.55 \times 10^{-6}$ M per min and this cytochrome c
The univalent and divalent reductions of oxygen by milk xanthine oxidase and by both spinach and chloroplast ferredoxins have previously been compared (1, 4) and been found to share several properties. Thus, raising the concentration of oxygen, in the range 0 to 1 \times 10^{-4} \text{ M}, caused a smooth increase in the proportion of univalent reductions and raising the pH also increased the proportion of univalent reduction. Furthermore, the rates of univalent reduction of oxygen by xanthine oxidase and by the ferredoxins were not grossly dissimilar. Thus, when operating at \( V_{\text{max}} \) at pH 7.0 in the presence of \( 2.4 \times 10^{-4} \text{ M} \) O2 and at 25\(^\circ\)C, xanthine oxidase generated approximately 242 molecules of O2 per min per molecule of enzyme. With chloroplast ferredoxin, under comparable conditions, the corresponding number was 60 (4).

FMN and flavodoxin have now been seen to reduce oxygen in a way which differs substantially from that of the non-heme iron containing proteins (1, 4). As shown in Fig. 5, the proportion of univalent reduction of oxygen, mediated by FMN, increased with increasing \( O_2 \), in the range 0 to \( 1.2 \times 10^{-4} \text{ M} \), but decreased with further increases in oxygen concentration. Furthermore, changing the pH from 6.8 to 7.8 was without effect on the proportion of univalent oxygen reduction. The effect of increasing the concentration of oxygen on the proportion of univalent electron transfers from flavodoxin to oxygen, was similar to that observed with FMN. Thus, as shown in Fig. 6, there was an increase followed by a decrease. Flavodoxin-mediated univalent reduction of oxygen did, however, respond to changing the pH from 6.8 to 7.8. This change caused maximum univalent flux to be achieved at \( 2.4 \times 10^{-4} \text{ M} \) O2 rather than at \( 6.0 \times 10^{-4} \text{ M} \) O2.

Massey, Palmer, and Ballou in an elegant study of the reaction of reduced flavins with oxygen, have demonstrated the reality of a reduced flavin-oxygen complex and of the role of \( O_2^- \) in the air oxidation of these compounds (20). Their work provides a basis for explaining the biphasic effect of oxygen concentration on the proportion of univalent reduction of oxygen (Figs. 5 and 6). Consider the following reactions, which only partially describe the processes involved:

\[ \text{FTRH}^+ + 2 F \leftrightarrow \text{FTR} + 2 \text{FH} \]  
\[ 2 \text{FH} \leftrightarrow \text{FH}_2 + \text{F} \]  
\[ \text{FH}^- + \text{O}_2 \leftrightarrow \text{F} + \text{H}^+ + \text{O}_2^- \]  
\[ \text{FH}_2 + \text{O}_2 \leftrightarrow \text{FH}_2\text{O}_2 \]  
\[ \text{FH}_2\text{O}_2 \leftrightarrow \text{F} + \text{H}_2\text{O}_2 \]

At very low concentrations of \( O_2 \) the rate of reoxidation of reduced forms will be rate-limiting and the FMN will accumulate in the fully reduced form (Reactions a + b). Most of the oxygen will therefore react with FH2 (Reaction d) and the proportion of univalent reduction will be small. In this region of low oxygen, raising the concentration of oxygen would increase the relative abundance of oxidized flavin and therefore of the semiquinone form (Reaction b). Thus raising \( O_2 \) would increase the percentage of univalent reduction of oxygen. However, once the oxygen concentration became high enough to convert most of the available FH2 to the oxygen complex (Reaction d), there would be very little FH2 available for Reaction b and the availability of FH1 would decrease as would the percentage of univalent reduction of oxygen. This reasoning would be valid only if the fully reduced flavin reduced oxygen primarily to \( \text{H}_2\text{O}_2 \); whereas the semiquinone reduced it primarily to \( \text{O}_2^- \). This was shown to be the case for menadione and is probably also the case for FMN.
min. Doubling the concentration of FMN in these reaction mixtures, had no effect on this molecular activity. The activity of flavodoxin was measured under identical conditions with the exception that $1.7 \times 10^{-7}$ M flavodoxin was used as the electron carrier. Adrenochrome was accumulated at a rate of 1.75 per flavodoxin per min. This corresponds with a rate of generation of $O_2^-$ of 2.4 per flavodoxin per min.

FMN and the FMN-containing flavodoxin are thus much less effective reductants of oxygen than are the non-heme iron containing ferredoxins and xanthine oxidase. This statement applies to the rates of both univalent and divalent reduction of oxygen by these electron carriers. Although this does not prove the point, it does suggest that the reduction of oxygen by native milk xanthine oxidase is a function of its non-heme iron center.

REFERENCES
