The Generation of Superoxide Radical during the Autoxidation of Ferredoxins*

HARA P. MISRA AND I. FRIDOVICH

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27706

SUMMARY

Clostridial and spinach ferredoxins, reduced enzymatically by the action of ferredoxin-TPN+ oxidoreductase, have been shown to carry out the univalent reduction of oxygen. The superoxide radicals, so generated, were detected by their ability to cause the oxidation of epinephrine to adrenochrome. Superoxide dismutase prevented the production of adrenochrome in these reaction mixtures, while having no effect on the rate of oxidation of TPNH. The ratio of the univalent reduction of oxygen to the sum of the univalent plus divalent reductions of oxygen was computed as a function of pH and of the concentrations of oxygen. This percentage of univalent flux increased with rising pH and with increasing concentrations of oxygen as has previously been observed in the case of milk xanthine oxidase. Under comparable conditions of pH and of oxygen concentration, milk xanthine oxidase generated O2- four times more rapidly than did clostridial ferredoxin.

Superoxide radicals have been shown to be produced during the aerobic action of milk xanthine oxidase (1-5). Both the iron-sulfur (6, 7) and the flavin (5) moieties of this enzyme have been proposed as the site responsible for the univalent reduction of oxygen. It is difficult to design experiments which would unequivocally settle this point. It is, however, possible to compare the behavior of xanthine oxidase with that of simpler iron-sulfur proteins in order to detect parallelisms which may exist in their modes of reduction of oxygen. That fraction of the total electron flux through xanthine oxidase which resulted in the univalent reduction of oxygen has been measured as a function of pH, oxygen tension, and concentration of xanthine (8). It appeared desirable to similarly investigate the reduction of oxygen by reduced ferredoxin, as a function of pH and of the concentration of oxygen. The present report describes such measurements which reveal considerable similarity in the reduction of oxygen by milk xanthine oxidase and by the ferredoxins from Clostridium pasteurianum and from spinach.

MATERIALS AND METHODS

Ferredoxins—Spinach ferredoxin was prepared by a modification of the method of Petering and Palmer (9). The concentrated solution of ferredoxin which they obtained by elution from DE-52 was treated with streptomycin to a final concentration of 5% and was then clarified by centrifugation at 9000 x g for 10 min. The supernatant solution (2 to 3 ml) was placed on a column (2.5 x 25 cm) of Sephadex G-75 and was eluted with 0.15 M Tris-HCl buffer at pH 7.8. Fractions for which the ratio of absorbance at 420 nm to that at 275 nm exceeded 0.45 were pooled and were concentrated by adsorption onto DE-32 and subsequent elution therefrom with 0.15 M Tris-HCl, 1.0 M NaCl, at pH 7.8 and at a slow flow rate. The resultant material gave a single band upon disc gel electrophoresis when stained with Amido black (10). Its absorption spectrum, recorded with a Cary 16, agreed with the published spectrum for this ferredoxin (11) and exhibited peaks at 462 nm, 420 nm, 320 nm, and 275 nm. This ferredoxin was also active in mediating the photoreduction of TPN+ by chloroplasts when tested by the method of San Pietro (12). The concentrations of solutions of spinach ferredoxin were determined on the basis of a molar extinction coefficient of 9400 M-1 cm-1 at 420 nm (13). Spinach ferredoxin was stored in the presence of 1.0 M NaCl and was diluted into reaction mixtures without prior desalting. Ferredoxin from C. pasteurianum was obtained from the Worthington Biochemical Corporation (Freehold, New Jersey). Concentrations were determined on the basis of a molar extinction coefficient of 26.8 x 103 M-1 cm-1 at 425 nm (14).

Other Materials—Superoxide dismutase was prepared from bovine erythrocytes and assayed as previously described (3). Ferredoxin-TPN+ oxidoreductase was prepared according to Shin, Tagawa, and Arnon (15). Xanthine oxidase, prepared from unpasteurized cream by a procedure which avoided treatment with proteolytic agents (16), was kindly provided by Dr. K. V. Rajagopalan. Catalase was obtained as a suspension of crystals from the Sigma Chemical Company. One unit of catalase was defined as that amount which decomposed 1 μmole of H2O2 per min when acting on 0.02 μM H2O2 in phosphate-buffered solutions at pH 7.8 and at 25°. TPN+ was obtained from Boehringer Mannheim and TPNH from P-L Biochemicals. All other materials were purchased at the highest available purities.

Assays—All spectrophotometric assays were performed at 25° in a Gilford model 2000 recording spectrophotometer equipped with a thermostated cell compartment. Reactions under controlled atmospheres were performed in cuvettes which allowed purging the reaction volume with the desired gas mixture. These cuvettes are similar to those described by Lazarev and Cooperstein (17) and were obtained from Pyrocell. Oxygen consumption was measured with a Gilson Medical Electronics (Middleton, Wisconsin) oxygraph equipped with a Clark oxygen electrode.

* The work reported herein was supported in full by Grant GM-10287 from the National Institutes of Health, Bethesda, Maryland.
**RESULTS**

**Ferredoxins as Mediators of TPNH Oxidation**—As shown in Fig. 1, the rate of aerobic oxidation of TPNH by ferredoxin-TPN+ reductase was a linear function of the concentration of ferredoxin, in the range 0 to $2 \times 10^{-6}$ M. It is also clear that superoxide dismutase had no effect on the activity of clostridial ferredoxin to mediate the oxidation of TPNH. This was also shown to be the case with spinach ferredoxin. These results follow directly from the ability of FT-reductase1 to transfer electrons from TPNH to ferredoxins and from the autooxidizability of reduced ferredoxins. The linear relationship between the concentration of ferredoxin and the rate of TPNH oxidation indicates that the dissociation constant of the FT-reductase-ferredoxin complex was greater than $2 \times 10^{-8}$ M under these conditions. This is in accord with the results of Foust, Mayhew, and Massey (19). If superoxide anions are generated during the oxidation of reduced ferredoxin, their rate of dismutation should have no influence on the rate of TPNH oxidation. The inability of superoxide dismutase to inhibit this assay was therefore anticipated. It does, however, establish that superoxide dismutase does not interact directly with and thus influence the activity of FT-reductase. The single measurement made with the spinach ferredoxin under these conditions demonstrated that it was approximately half as active as an equimolar amount of the clostridial ferredoxin.

**Epinephrine as Detector of O$_2$—**The action of FT-reductase upon TPNH and ferredoxin in the presence of 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 ferredoxin, and clostridial ferredoxin was a more effective mediator of adrenochrome formation than was spinach ferredoxin. The effect of varying the concentration of epinephrine was also investigated and these results are shown in Fig. 3. Elimination of oxygen from these reaction mixtures completely prevented the formation of adrenochrome.

Superoxide dismutase was used to verify the role of O$_2^-$ in the oxidation of epinephrine to adrenochrome. Thus, O$_2^-$, generated by the oxidation of reduced ferredoxin, could either dismute to H$_2$O and O$_2$ or could react with epinephrine. Superoxide dismutase, by catalyzing the former reaction, should inhibit the formation of adrenochrome. Fig. 4 demonstrates that this was the case. Superoxide dismutase inhibited the production of adrenochrome when either spinach ferredoxin or clostridial ferredoxin was used as the electron carrier. It follows that both of these ferredoxins are capable of the univalent reduction of oxygen. Since superoxide dismutase is in competition with epinephrine for the available O$_2^-$, the sensitivity of this reaction to inhibition by superoxide dismutase could be enhanced or diminished at will by lowering or raising the concentration of epinephrine.

Superoxide dismutase must also be considered to be in competition with the spontaneous dismutation reaction (1). Therefore, the greater the rate of generation of O$_2^-$ in any system, the greater the amount of superoxide dismutase which will be required to effect a given reduction in the steady state concentration of O$_2^-$. This reasoning provides an explanation for the apparently greater sensitivity toward superoxide dismutase exhibited in Fig. 4.

---

1 The abbreviation used is: FT-reductase, ferredoxin-TPN+ oxidoreductase.
Fig. 3. The epinephrine dependence of adrenochrome formation. Reaction mixtures contained 3.3 × 10⁻⁵ M TPNH, 1.8 × 10⁻⁴ M ferredoxin, 1.5 × 10⁻⁴ M ferredoxin-TPN⁺ oxidoreductase, 1.4 × 10⁻⁴ M EDTA, and the indicated concentrations of epinephrine in a final volume of 3.0 ml buffered at pH 7.8 by 0.05 M potassium phosphate and at 25°. The data obtained are here presented on linear and on reciprocal coordinates. Half-maximal adrenochrome formation was achieved at 1.25 × 10⁻⁴ M epinephrine.

Fig. 4. Inhibition of adrenochrome formation by superoxide dismutase. Reaction mixtures contained 3.3 × 10⁻⁴ M epinephrine, 3.3 × 10⁻⁵ M TPNH, 1.8 × 10⁻⁴ M ferredoxin-TPN⁺ oxidoreductase, and either 1.4 × 10⁻⁴ M spinach ferredoxin (●) or 3.83 × 10⁻⁵ M clostridial ferredoxin (▲) and the indicated concentrations of superoxide dismutase in a final volume of 3.0 ml buffered at pH 7.8 by 0.05 M potassium phosphate containing 1 × 10⁻⁴ M EDTA and at 25°.

Effects of Epinephrine and of Superoxide Dismutase on Oxygen Uptake—If O₂⁻ reacts with epinephrine, in a manner which leads to the formation of adrenochrome, then adding epinephrine to reaction mixtures which generate O₂⁻ should result in an enhancement of oxygen consumption due to the co-oxidation of epinephrine. Superoxide dismutase should prevent this co-oxidation and should thus inhibit the extra oxygen uptake. The results shown in Fig. 5 demonstrate that this was the case. Line 3 represents the oxygen consumption by 1 × 10⁻⁸ M xanthine oxidase acting on 1.07 × 10⁻⁴ M xanthine in 0.05 M potassium phosphate, 1 × 10⁻⁴ M EDTA at pH 7.8 and 25°. Line 1 demonstrates that adding 1.11 × 10⁻⁴ M epinephrine to this reaction mixture enhanced the rate and the extent of oxygen uptake by a factor of 1.6. Line 2 shows that 2.22 µg per ml of superoxide dismutase completely prevented the effect of epinephrine on oxygen utilization. In all cases, the addition of catalase after oxygen uptake had ceased resulted in an evolution of oxygen in an amount equal to half of that which had been consumed. This is the expected result when the final product of oxygen reduction is H₂O₂.

Proportion of Univalent Reduction of Oxygen by Ferredoxin—The oxidation of TPNH in these reaction mixtures was totally dependent upon the presence of the FT-reductase, oxygen, and ferredoxin. We assume that the only available pathway for electrons from TPNH to oxygen traversed both the oxidoreductase and the ferredoxin. The total flux of electrons from ferredoxin to oxygen was computed in terms of the oxidation of TPNH. Since the conversion of epinephrine to adrenochrome was totally inhibitable by superoxide dismutase, we conclude that O₂⁻ was the agent responsible for causing this oxidation. Increasing the concentration of epinephrine should result in the interception of an ever increasing proportion of the O₂⁻ generated and should therefore yield a saturation curve. This was shown to be the case in Fig. 3. It follows that saturating concentrations of epinephrine will trap virtually all of the O₂⁻ generated and that it should therefore be possible to quantitatively determine the rate of univalent transfer of electrons from ferredoxin to oxygen, in terms of the rate of conversion of epinephrine to adrenochrome. The saturation of the rate of adrenochrome formation by raising the concentration of epinephrine (Fig. 3) also demonstrates that chain mechanisms, involving epinephrine, were not quantitatively significant under these conditions.

If the stoichiometry of the reaction of epinephrine with O₂⁻ to yield adrenochrome were known, it would then be possible to calculate what fraction of the total electron flux, from ferredoxin to oxygen, was accomplished by univalent electron transfers. The
The univalent fraction of the total flux of electrons between xanthine oxidase and oxygen has been seen (8) to increase with increasing concentration of oxygen and with rising pH. The ferredoxins from spinach and from C. pasteurianum have now been shown to behave similarly. Xanthine oxidase and the ferredoxins share a number of properties among which are low oxidation-reduction potential (6, 14), \( G = 1.94 \), low temperature electron paramagnetic resonance spectrum when in the reduced state (20, 21), equimolar amounts of nonheme iron and acid-labile sulfide (14, 21), and a characteristic absorption spectrum in the visible region (14). This constellation of properties has been associated with an iron-sulfur chromophore which contains two iron atoms close enough to share one electron (22, 23) or to engage in antiferromagnetic coupling (24).

This common structural feature coupled with similar changes in percentage of univalent flux, in response to changes in pH and oxygen tension, does not prove that the site of oxygen reduction in intact xanthine oxidase is at the iron-sulfur chromophore, but it does make that proposal increasingly attractive.

It is of interest to compute the rate at which ferredoxin can effect the univalent reduction of oxygen. This can conveniently be expressed in terms of the molecules of \( O_2^- \) generated per molecule of ferredoxin per min under conditions of limiting ferredoxin. In reaction mixtures containing \( 1.12 \times 10^{-4} \text{ M FT-reductase}, 6 \times 10^{-4} \text{ M clostridial ferredoxin}, 3.33 \times 10^{-4} \text{ M TPNH}, 3.33 \times 10^{-4} \text{ M epinephrine}, 2.4 \times 10^{-4} \text{ M oxygen}, 1 \times 10^{-2} \text{ M EDTA}, \) and 0.05 \( \text{ M potassium phosphate at pH 7.8 and at 25°, the rate of adrenochrome formation was 47 molecules of adrenochrome per min per molecule of ferredoxin. Since this formation of adrenochrome was inhibited 90\% by 1.39 \mu \text{ g per ml of superoxide dismutase and since each adrenochrome corresponded to 1.39 O_2^-} \) (Table I), we may calculate that the rate of generation of \( O_2^- \) was 65 \( O_2^- \) per min per ferredoxin. This rate of generation of \( O_2^- \) by clostridial ferredoxin may be compared with that found with milk xanthine oxidase. Thus, at pH 7.0 and at 25°, milk xanthine oxidase generated 242 molecules of \( O_2^- \) per min per molecule of enzyme, when operating at \( V_{\text{max}} \) in air. At pH 10.2 the corresponding number was 1060. Clostridial ferredoxin is thus one-fourth as effective a source of \( O_2^- \) as is xanthine oxidase under comparable conditions.

In contrast, the flavoenzymes (5) were less effective than xanthine oxidase by factors of 50 or more. This similarity between milk xanthine oxidase and the ferredoxins, in terms of the rate of univalent electron transfer to oxygen, coupled with the grossly dissimilar behavior of a variety of flavoenzymes, provides yet another reason for suspecting that univalent reduction of oxygen by milk xanthine oxidase occurs at its iron prosthetic groups.

Electron paramagnetic resonance spectrometry has been used to detect \( O_2^- \) generated during the oxidation of reduced clostridial

| \( O_2^- \) | pH 7.8 | | pH 6.8 | |
|---|---|---|---|---|
| Adrenochrome/ \( O_2^- \) | Percentage of univalent flux | Adrenochrome/ \( O_2^- \) | Percentage of univalent flux |
| % | | | | |
| 0 | 0.62 | 0 | 0.50 | 0 |
| 10 | 0.72 | 66.7 | 0.57 | 30.5 |
| 20 | 0.83 | 67.9 | 0.81 | 61.6 |
| 100 | 0.86 | 98.8 | 0.86 | 72.0 |

![Fig. 6. Percentage of univalent reduction of oxygen as a function of oxygen concentration. That percentage of the total electron flux through ferredoxin to oxygen which resulted in the univalent reduction of oxygen is here presented as a function of the percentage of oxygen in the gas phase. Cuvettes contained 3.3 \( \times 10^{-4} \text{ M epinephrine, 3.3 \( \times 10^{-5} \text{ M TPNH, 1.4 \( \times 10^{-4} \text{ M ferredoxin, and 1.8 \( \times 10^{-4} \text{ M ferredoxin-TPN+ oxidoreductase in a mixture of nitrogen and oxygen for 10 min at 25° prior to sealing the cuvettes and starting the reaction by tipping the enzyme in from a side arm.} \)\) } \text{ M}

\( \text{TPNH, 1.4 \times 10^{-4} M \text{ ferredoxin, and 1.8 \times 10^{-4} M \text{ ferredoxin-TPN+ oxidoreductase in a total volume of 3.0 mL buffered at pH 7.8 or at pH 6.8 by 0.05 M potassium phosphate containing 1 \times 10^{-4} M \text{ EDTA. The reaction mixtures were bubbled with mixtures of nitrogen and oxygen for 10 min at 25° prior to sealing the cuvettes and starting the reaction by tipping the enzyme in from a side arm.} \)\) \text{ M}

\( \text{EDTA, and 0.05 M potassium phosphate at pH 7.8 and at 25°, the rate of adrenochrome formation was 47 molecules of adrenochrome per min per molecule of ferredoxin. Since this formation of adrenochrome was inhibited 90\% by 1.39 \mu \text{ g per ml of superoxide dismutase and since each adrenochrome corresponded to 1.39 O_2^-} \) (Table I), we may calculate that the rate of generation of \( O_2^- \) was 65 \( O_2^- \) per min per ferredoxin. This rate of generation of \( O_2^- \) by clostridial ferredoxin may be compared with that found with milk xanthine oxidase. Thus, at pH 7.0 and at 25°, milk xanthine oxidase generated 242 molecules of \( O_2^- \) per min per molecule of enzyme, when operating at \( V_{\text{max}} \) in air. At pH 10.2 the corresponding number was 1060. Clostridial ferredoxin is thus one-fourth as effective a source of \( O_2^- \) as is xanthine oxidase under comparable conditions. In contrast, the flavoenzymes (5) were less effective than xanthine oxidase by factors of 50 or more. This similarity between milk xanthine oxidase and the ferredoxins, in terms of the rate of univalent electron transfer to oxygen, coupled with the grossly dissimilar behavior of a variety of flavoenzymes, provides yet another reason for suspecting that univalent reduction of oxygen by milk xanthine oxidase occurs at its iron prosthetic groups. Electron paramagnetic resonance spectrometry has been used to detect \( O_2^- \) generated during the oxidation of reduced clostridial
ferredoxin but failed to detect \( \text{O}_2^- \) when similarly applied to the spinach ferredoxin (25, 26). Chemical and enzymatic methods have now allowed the demonstration that spinach and clostridial ferredoxins are both capable of the univalent reduction of oxygen. This result is not surprising in view of the reported similarities between these ferredoxins (14). Quick freeze electron paramagnetic resonance methods can detect the steady state level of \( \text{O}_2^- \) present in a reaction mixture at the instant of freezing, whereas chemical trapping methods can detect all of the \( \text{O}_2^- \) generated in a reaction mixture over a period of minutes. The electron paramagnetic resonance methods of detecting \( \text{O}_2^- \) are thus inherently less sensitive and more difficult to apply than the chemical methods. In view of the difficulties and limitations of the methods, it appears likely that the reported (25, 26) inability to demonstrate \( \text{O}_2^- \) would bring about any divalent reduction of oxygen. One would expect it to be capable of only univalent reductions of oxygen. Since spinach ferredoxin has been found capable of transferring only 1 electron per molecule (14, 27, 28) we must rather consider how it can bring about any divalent reduction of oxygen.

Since spinach ferredoxin is known to associate with FT-reductase (19, 29) it is possible that part of the reduction of oxygen was accomplished by the FT-reductase-ferredoxin complex. In such a situation, electrons could flow in sequential univalent steps from FT-reductase to ferredoxin to oxygen. Univalent reduction of oxygen would then occur when oxygen separated from the FT-reductase-ferredoxin complex after the transfer of a single electron, whereas divalent reduction would depend upon oxygen remaining associated with the complex until the second electron had been transferred. Another possibility is that association with ferredoxin so modifies FT-reductase that it becomes capable of directly transferring electrons to oxygen by a route not traversing ferredoxin. In this case ferredoxin-dependent, divalent reduction of oxygen by FT-reductase plus TPNH could be observed. The actual mechanism of the divalent reduction of oxygen by the FT-reductase plus ferredoxin system remains to be ascertained.

REFERENCES

1. McCord, J. M., and Fridovich, I., J. Biol. Chem., 243, 5753 (1968).
2. McCord, J. M., and Fridovich, I., Fed. Proc., 28, 346 (1969).
3. McCord, J. M., and Fridovich, I., J. Biol. Chem., 244, 6049 (1969).
4. Knowles, P. F., Gibson, J. F., Pick, F. M., and Bray, R. C., Biochem. J., 111, 53 (1969).
5. Massey, V., Strickland, S., Mathew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., and Solomon, P. A., Biochem. Biophys. Res. Commun., 36, 891 (1969).
6. Rajagopalan, K. V., and Handler, P., in T. P. Singer (Editor), Biological oxidations, Interscience Publishers, Inc., New York, 1968, p. 301.
7. McCord, J. M., and Fridovich, I., J. Biol. Chem., 244, 6056 (1969).
8. Fridovich, I., J. Biol. Chem., 245, 4033 (1970).
9. Petering, D. H., and Palmer, G., Arch. Biochem. Biophys., 141, 456 (1970).
10. Davis, B. J., Ann. N. Y. Acad. Sci., 121, 404 (1964).
11. Petering, D., Fee, J. A., and Palmer, G., J. Biol. Chem., 246, 643 (1971).
12. San Pietro, A., in S. P. Colowick and N. O. Kaplan (Editors), Methods in enzymology, Vol. 6, Academic Press, New York, 1963, p. 439.
13. Moss, T. H., Petering, D., and Palmer, G., J. Biol. Chem., 244, 2275 (1969).
14. Tagawa, K., and Arnon, D. I., Biochim. Biophys. Acta, 153, 602 (1968).
15. Shin, M., Tagawa, K., and Arnon, D. I., Biochem. Z., 338, 84 (1963).
16. Brady, F. O., Ph.D. Thesis, Duke University, 1969.
17. Lazzaroni, S. J., and Cooperstein, N. J., Science, 120, 674 (1954).
18. Green, S., Mazur, A., and Szoor, E., J. Biol. Chem., 220, 237 (1956).
19. Foust, G. P., Mayhew, S. G., and Massey, V., J. Biol. Chem., 244, 964 (1969).
20. Brinckert, H., in A. San Pietro (Editor), Nonheme iron proteins: role in energy conversion, The Antioch Press, Yellow Springs, Ohio, 1965, p. 23.
21. Britton, R. H., Palmer, G., and Sando, R. H., Biochemistry, 55, 307 (1966).
22. Tsebris, J. C. M., Tsai, R. L., Gunsalus, I. C., Orme-Johnson, W. H., Hansen, R. E., and Brinckert, H., Proc. Nat. Acad. Sci. U. S. A., 69, 5099 (1968).
23. Orme-Johnson, W. H., Hansen, R. E., Brinckert, H., Tsebris, J. C. M., Bartholomous, K. C., and Gunsalus, I. C., Proc. Nat. Acad. Sci. U. S. A., 60, 398 (1968).
24. Johnson, C. E., Commack, R., Rao, K. K., and Hall, D. O., Biochem. Biophys. Res. Commun., 43, 564 (1971).
25. Orme-Johnson, W. H., and Brinckert, H., Biochem. Biophys. Res. Commun., 36, 905 (1969).
26. Nilsson, R., Pick, F. M., and Bray, R. C., Biochim. Biophys. Acta, 132, 145 (1968).
27. Mayhew, S. G., Petering, D., Palmer, G., and Foust, G. P., J. Biol. Chem., 244, 2830 (1969).
28. Evans, M. C. W., Hall, D. O., Botha, H., and Whatley, F. R., Biochim. J., 110, 465 (1969).
29. Nelson, N., Neumann, J., Biochim. Biophys. Res. Commun., 30, 142 (1968); J. Biol. Chem., 244, 1926, 1932 (1969).
The Generation of Superoxide Radical during the Autoxidation of Ferredoxins
Hara P. Misra and I. Fridovich

*J. Biol. Chem.* 1971, 246:6886-6890.

Access the most updated version of this article at [http://www.jbc.org/content/246/22/6886](http://www.jbc.org/content/246/22/6886)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/246/22/6886.full.html#ref-list-1](http://www.jbc.org/content/246/22/6886.full.html#ref-list-1)