INITIATION OF SULFITE OXIDATION BY SPINACH FERREDOXIN-NADP REDUCTASE AND FERREDOXIN SYSTEM : A MODEL EXPERIMENT ON THE SUPEROXIDE ANION RADICAL PRODUCTION BY METALLOFLAVOPROTEINS*

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SUMMARY: By means of the radical-initiation of sulfite oxidation, spinach ferredoxin-NADP reductase was shown to produce O_2^- in the course of NADPH oxidation by molecular oxygen. With the reductase and NADPH as the electron donating system, ferredoxin was found to stimulate the sulfite oxidation even more effectively than the flavoprotein at high ionic strength. Under O_2^- producing conditions, the semiquinoid form of the flavoprotein was found to be involved in the steady state absorption spectrum which was measured by "stopped-flow" technique.

It has been well demonstrated by the studies of Fridovich and Handler (1,2,3) that milk xanthine oxidase produce superoxide anion radical (O_2^-) in the course of the substrate oxidation by molecular oxygen. According to them, the site for O_2^- generation should be the iron-sulfur moiety of the metallo-flavoprotein, which was based on the facts that only metal-containing flavo-proteins produce the radical while simple flavoproteins, such as glucose oxidase, D- and L-amino-acid oxidases, can not. By rapid-freezing EPR studies, Knowles et al. (4) demonstrated O_2^- signal, and considered that the iron-sulfur moiety of xanthine oxidase will be responsible for O_2^- generation, taking into account their earlier proposal that the reaction site of the enzyme with oxygen should be the iron moiety rather than the FAD and molybdenum moie-ties. On the other hand, Komai et al. (5) prepared "deflavo" xanthine oxidase and proposed that the site for oxygen binding might be the FAD moiety. Massey et al.(6) and Ballou et al.(7) showed by "superoxide dismutase" inhibition and EPR studies that a flavin derivative and some simple flavoproteins, e.g., old

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yellow enzyme or ferredoxin-NADP reductase, could produce a substantial amount of O_2^-, insisting that the site of O_2^- production is the FAD moiety of the enzyme rather than iron moiety. Orme-Johnson and Beinert (8) also supported this interpretation, since they could not observe O_2^- signal by EPR on re-oxidation of chemically reduced iron-sulfur proteins with an exception of Clostridium pasteurianum ferredoxin.

Xanthine oxidase, per se, is a rather complex enzyme because of its many functional groups, its instability with aging (9), and the accompanying inactive form. These facts seem to cause the conflicting conclusions. In this context, an artificial iron-flavin complex model, if any, would be preferred to elucidate the mechanism of O_2^- production. It has been confirmed by many workers (10, 11, 12) that spinach ferredoxin and ferredoxin-NADP reductase can form a tightly-bound 1:1 complex at low ionic strength, which is easily dissociated by increasing ionic strength. This system is an excellent model of a metallo-flavoprotein.

This paper describes some findings on O_2^- generation by this system by means of the initiation of sulfite oxidation and on the roles of the flavo-protein by "stopped-flow" method.

Materials and Methods. Ferredoxin and ferredoxin-NADP reductase (FNR) were purified from spinach by the methods of Tagawa and Arnon (13) and Shin et al. (14), respectively. Bovine adrenal iron-sulfur protein (adrenodoxin) was prepared as reported previously (15). Oxygen uptake was measured with a Gilson KM Oxygraph equipped with a Clark oxygen electrode. Stopped-flow experiment was with a Durrm-Gibson Stopped Flow apparatus with a 2-cm cuvette. All measurements were done in a 0.01 M Tris-HCl buffer, pH 7.4, at 25° unless otherwise specified.

Results and Discussion. A quantitative comparison of oxygen uptake and NADPH oxidation, which was measured spectrophotometrically at 340 mμ under the same conditions, is shown in Table I. FNR was found to have an NADPH oxidase activity, although the turnover number is very low (about 5.5 moles/mole of

TABLE I

Comparison of Oxygen Uptake and NADPH Oxidation by Ferredoxin-NADP Reductase (FNR) and Ferredoxin (Fd) in the presence and the Absence of Sodium Sulfite.

Addition	O_2 Uptake (μM/min)	NADPH Oxidation* (μM/min)
NADPH & FNR	12.3	14.7
NADPH, Sulfite & FNR	22.3	14.4
NADPH, Sulfite, Fd & FNR	19.7	14.6
NADPH, Sulfite, Fd & FNR in 0.2 M NaCl	36.4	15.4

* NADPH oxidation was measured with a Hitachi-Perkin Elmer spectrophotometer, Model 139, with a Beckman recorder. Concentrations: FNR, 1.6 μM; ferredoxin, 4.5 μM; NADPH, 0.13 mM; sodium sulfite, 5.5 mM.

enzyme/min) as compared with other activities (e.g., DCPIP-diaphorase activity is about 300). By the oxidase activity, one mole of NADPH was oxidized by consuming one mole of oxygen, suggesting that the main product is hydrogen peroxide rather than water. This was supported by the observation that the rate was reduced to half by addition of bovine liver catalase.

Addition of sulfite was found to increase the rate of oxygen uptake about 2-fold, but had no effect on NADPH oxidation. The results clearly indicate that the chain reaction of sulfite oxidation was initiated by O_2^-, which was produced by the reduced flavoprotein and molecular oxygen, and then the sulfite-radical reaction took up oxygen independently of NADPH oxidation. This finding is in agreement with Massey et al. (6).

Figure 1 shows the effect of ferredoxin on the oxygen uptake by the FNR-sulfite system in buffers of various ionic strength. Ferredoxin had no significant effect on total oxygen uptake at low ionic strength, however, it became stimulatory with increasing concentrations of NaCl: the rate of oxygen uptake was increased about 5 times by the addition of ferredoxin in the presence of 0.4 M NaCl. The increase in oxygen uptake by the iron-sulfur protein at high ionic strength can be attributed to that of sulfite-linked oxygen uptake,

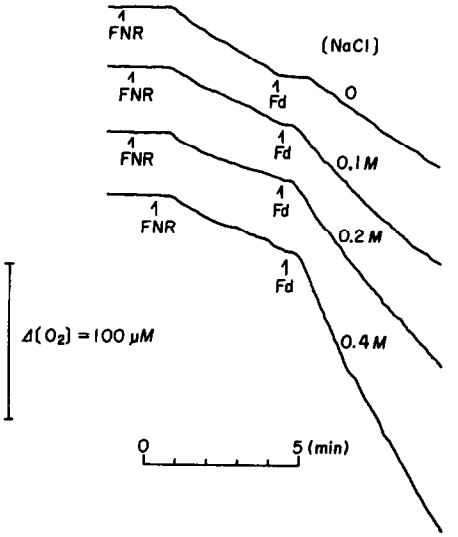

Figure 1. Effect of ferredoxin (Fd) on the oxygen uptake by the FNR-sulfite system at various ionic strength.
Concentrations: FNR, 1.6 μM; ferredoxin, 4.6 μM; NADPH, 0.23 mM; sodium sulfite, 5.5 mM. NaCl concentrations are as indicated in the figure. Rates of oxygen uptake after the addition of ferredoxin were: 14.2 μM/min (NaCl, 0); 23.0 (0.1 M); 24.8 (0.2 M); 47.2 (0.4 M).

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since NADPH oxidation was found to be essentially the same under the given conditions (Table I). A similar effect was observed when NaCl was replaced by KCl, indicating that this was not a specific ion effect but that of ionic strength. This phenomenon is quite in parallel with the dissociation of the FNR-ferredoxin complex (11,12), hence ferredoxin could stimulate the sulfite-linked oxygen uptake only in the dissociated state and not in the form of a complex with FNR. It seems reasonable to say that the iron-sulfur protein can produce O_2^- even more effectively than the flavoprotein itself under suitable conditions. In a high ionic strength medium, however, ferredoxin must still maintain a specific interaction with FNR so that it can accept electrons from NADPH-FNR system. As shown in Figure 2A, native adrenodoxin could not replace ferredoxin at either high or low ionic strength; but when the protein was denatured by heat, it was able to stimulate the oxygen uptake

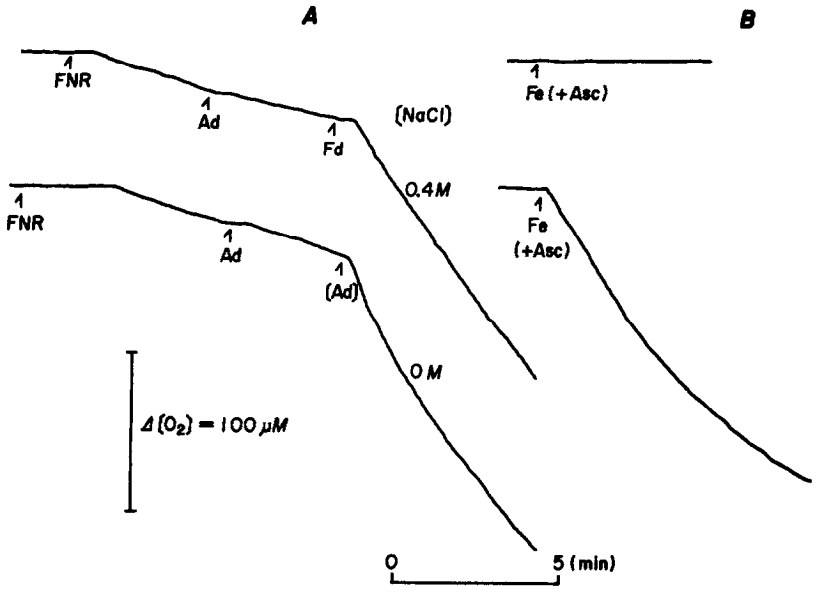

Figure 2. (A) Effect of native and heat-denatured adrenodoxin on the oxygen uptake by the FNR-sulfite system.
Concentrations: FNR, 0.65 μM; NADPH, 0.12 mM; native adrenodoxin (Ad), 23 μM; denatured adrenodoxin (Ad), 23 μM; ferredoxin, 4.5 μM; sodium sulfite, 5.5 mM.
(B) Oxygen uptake by ascorbate (Asc)-ferric iron system in the absence (upper) and the presence (lower) of sulfite.
Concentrations: ascorbate, 3.5 mM; ferric nitrate, 35 μM; sodium sulfite, 5.5 mM.

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even in a low ionic strength medium. It is interesting that iron-sulfur proteins could be replaced by ferric nitrate. Furthermore, ascorbate-ferric ion system could also initiate the sulfite oxidation (Figure 2B). These observations lead to the conclusion that the iron atom, whether it is bound with a protein or not, can produce O_2^- provided that a suitable electron donor system is present, and that the effective species for O_2^- production is ferrous iron, which is known to form perferryl ion (Fe^{2+}-O_2) as the intermediate of oxidation (16).

The steady state absorption spectrum of FNR was measured by stopped-flow technique in the presence of NADPH, sulfite, and ferric iron. The results shown in Figure 3 implicate the semiquinoid form of the FAD moiety as well as

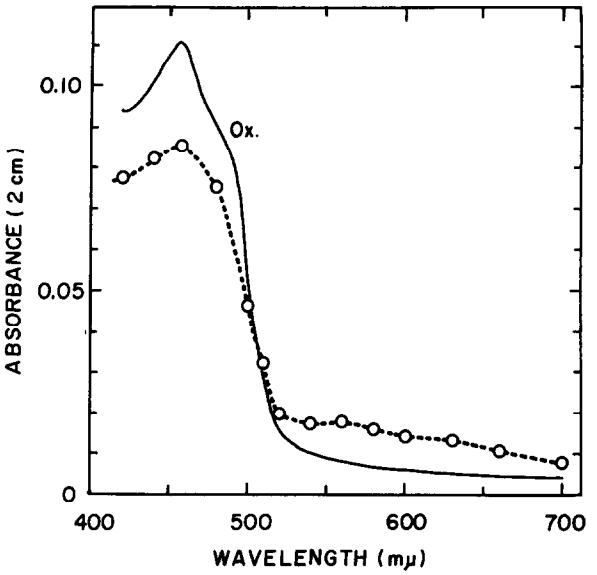

Figure 3. Steady state absorption spectrum of FNR under O_2^- producing conditions measured by "stopped-flow" method.
Steady state (-o--o-) was attained about 4 seconds after mixing.
Concentrations: FNR, 5.0 μM; NADPH, 54 μM; ferric nitrate, 25 μM; sodium sulfite, 2.5 mM.

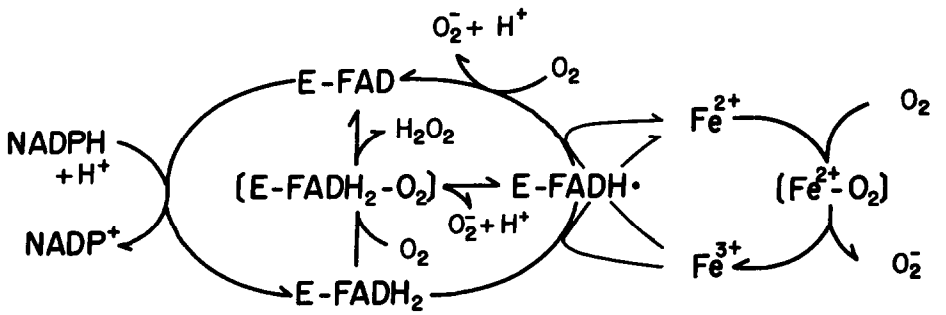

Figure 4. A schematic representation of O_2^- production by the flavin-iron system.

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the oxidized form in the turnover of the enzyme. The fully reduced form could not be identified in the spectrum because of its less characteristic absorption in the visible region, but it might also be involved in the catalytic cycle, since the product of NADPH oxidation was hydrogen peroxide as stated before. A relevant scheme for O_2^- production by the flavin-iron system is shown in

Figure 4. In this scheme, reduced FAD-O_2 complex (17) and perferryl ion complex are assumed as intermediates.

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