A TWO-STEP MECHANISM FOR THE PHOTOSYNTHETIC REDUCTION OF OXYGEN BY FERREDOXIN

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SUMMARY It is proposed that autoxidation of plant-type ferredoxin involves the reduction of oxygen to superoxide and of superoxide to peroxide. This mechanism accounts for an inhibition by superoxide dismutase of ferredoxin-mediated, photosynthetic oxygen uptake by isolated chloroplasts, and for an insensitivity of this reaction to ascorbate. Preliminary evidence for an inhibition by superoxide dismutase of autoxidation of chemically-reduced ferredoxin is presented, and some implications of the two-step mechanism are discussed.

Photosynthetic oxygen uptake is a result of autoxidation of a low-potential electron acceptor. Where an artificial electron acceptor such as methyl viologen is present an initially univalent reduction of oxygen may be followed by either dismutation or reduction of the superoxide so formed (1,2). Univalent reduction of oxygen by methyl viologen followed by superoxide dismutation may be written as follows;

$$2MV^{+} + 2O_2 \longrightarrow 2MV^{2+} + 2O_2^{-} \qquad \text{(i)}$$

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \qquad \text{(ii)}$$

overall: $2MV^{+} + O_2 + 2H^{+} \longrightarrow 2MV^{2+} + H_2O_2$ (iii)

The replacement of superoxide dismutation (equation (ii)) by a reduction of superoxide accounts for ascorbate's stimulatory on chloroplasts' oxygen uptake.

Ferredoxin is an autoxidisable electron acceptor, and it mediates a similar overall reaction (3). Its reported ability to generate superoxide (4) might lead one to suppose

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Abbreviations Fd; ferredoxin. SOD; superoxide dismutase. MV; methyl viologen or 1,1-dimethyl-4,4'-bipyridylium dichloride.

that ferredoxin-mediated oxygen uptake proceeds in the same way as the methyl viologen-mediated reaction, but evidence that this is not the case will now be described.

In the experimental results of table I addition of superoxide dismutase was found to inhibit the rate of ferredoxin-mediated oxygen uptake by isolated chloroplasts. A similar observation has been made by Elstner and Heupel (5). This effect clearly cannot be explained only by the operation of reactions such as those described in equations (i) and (ii), and indeed SOD does not by itself affect the rate of methyl viologen-mediated oxygen uptake (2). Table I also shows that ascorbate had only a small stimulatory effect on ferredoxin-mediated oxygen uptake. In contrast, ascorbate is able to treble the rate of oxygen uptake in the methyl viologen-mediated reaction; it does this by replacing spontaneous dismutation with reduction of superoxide (2). The effect of catalase was to inhibit ferredoxin-mediated oxygen uptake, which confirms that the overall reaction with ferredoxin is nevertheless similar to the overall reaction with methyl viologen (equation (iii)). An inhibition of only catalase by azide but of both catalase and SOD by cyanide is also apparent from table I.

An alternative mechanism by which the ferredoxin-mediated reaction could give the required overall stoichiometry and yet show both inhibition by SOD and insensitivity to ascorbate is as follows;

$$Fd_{red} + O_2 \rightarrow Fd_{ox} + O_2^- \qquad \text{(iv)}$$

$$Fd_{red} + O_2^- + 2H^+ \rightarrow Fd_{ox} + H_2O_2 \qquad \text{(v)}$$

overall: $2Fd_{red} + O_2 + 2H^+ \rightarrow 2Fd_{ox} + H_2O_2$ (vi)

Although the overall reactions of equations (iii) and (vi) are similar, a replacement of superoxide dismutation by ascorbate oxidation would have an effect only on equation (iii), and if the affinity of ferredoxin for superoxide (equation (v)) were greater than that of ascorbate for superoxide then no effect of ascorbate on ferredoxin-mediated oxygen uptake would be observed. The rate constant of the oxidation of ascorbate by superoxide has recently been estimated to be 2.7×10^5 M^{-1} sec^{-1} (6).

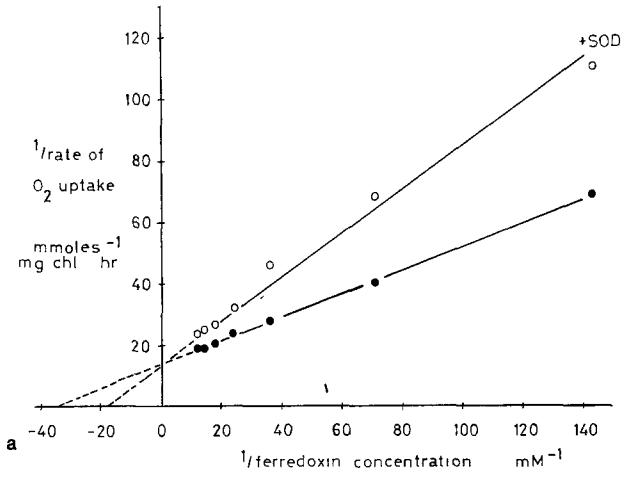
The rate constant of catalytic dismutation of superoxide

(7) of about 2 x 10^9 M^{-1} sec^{-1} would make it likely, however, that addition of SOD would replace reaction (v) by reaction (ii). If this were to occur when ferredoxin concentration limits the rate of the overall reaction, the effect of SOD would be to inhibit the rate of regeneration of oxidised ferredoxin, and hence addition of SOD would competitively inhibit both oxygen uptake and electron transport.

Such a competitive inhibition of photosynthetic oxygen uptake is supported by the results shown in figure 1(a); in this experiment SOD appeared to increase the ferredoxin concentration required for half maximum oxygen uptake from 30μM to 50μM, while the maximum rate itself remained unchanged at about 70 μmoles oxygen (mg chl)$^{-1}$ hr^{-1}. The inhibitory effect of SOD seen in table I can therefore be understood as an inhibition of photosynthetic electron transport itself, with the stoichiometry of oxygen uptake to electron transport being determined by equation (vi) and consequently remaining unchanged.

This interpretation contrasts with the mechanism by which SOD inhibits the ascorbate-stimulated rate of methyl viologen-mediated oxygen uptake; here the effect of the enzyme is on the stoichiometry of oxygen uptake to electron transport rather than on electron transport itself (8). Hence the inhibition by SOD of ascorbate-stimulated oxygen uptake by methyl viologen should be non-competitive in character. Figure 1(b) suggests that this is so; while the methyl viologen concentration required for half maximum velocity remained constant at about 3μM, the maximum rate of oxygen uptake was diminished by SOD from 670 to 280 μmoles (mg chl)$^{-1}$ hr^{-1}. These values are derived from the extrapolation shown in figure 1(b), and ignore the departure from linearity caused by a practical limitation of oxygen uptake by some factor other than methyl viologen concentration.

Evidence that both steps (equations (iv) and (v)) of the reduction of oxygen to hydrogen peroxide involve ferredoxin itself is presented in table II. Purified ferredoxin having been chemically reduced, its rate of re-oxidation on the introduction of oxygen was measured spectrophotometrically. The presence of SOD appeared to decrease the rate of ferredoxin oxidation, while addition of cyanide removed this inhibition. The results of this experiment are at least consistent with the



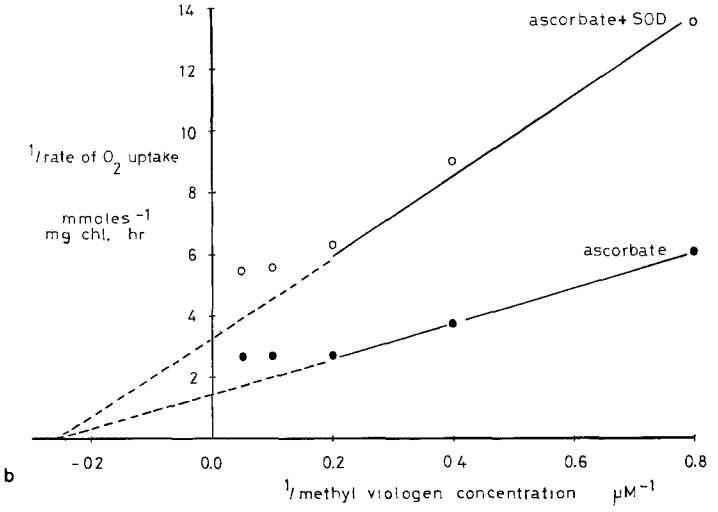


FIGURE 1 (a) A double reciprocal plot of the effect of ferredoxin concentration on chloroplast oxygen uptake in the presence and in the absence of SOD (400 units). The reaction conditions were those described in table I.

(b) A similar plot of the effect of methyl viologen concentration on chloroplast oxygen uptake. Apart from the omission of ferredoxin, these reaction conditions were also those of table I. SOD (400 units) was added to inhibit the rate obtained in the presence of ascorbate (1mM).

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proposition that reduced ferredoxin is by itself sufficient for the two-step oxygen reduction to occur.

The inhibitory effect of SOD on photosynthetic oxygen

Additions	Rate of Oxygen Uptake μmoles (mg chl)$^{-1}$ hr^{-1} without ascorbate	with ascorbate
None	21.6	25.6
SOD	16.7	11.9
SOD, KCN	20.5	28.8
Catalase	2.3	6.5
Catalase, NaN_3	23.8	27.0
SOD, catalase	1.8	2.7
SOD, catalase, NaN_3	15.8	16.2
SOD, catalase, KCN	20.3	26.2

TABLE I Ferredoxin-mediated oxygen uptake on illumination of isolated spinach chloroplasts; effects of ascorbate (1mM), SOD (400 units), catalase (4,000 units), azide (10mM) and cyanide (10mM). Isolation of broken, washed chloroplasts and details of the oxygen electrode measurements have been previously described (2). Besides the additions mentioned above the following compounds were also present; sorbitol (0.1M), $MgCl_2$ (5mM), NaCl (20mM), EDTA (2mM), NH_4Cl (5mM), HEPES (50mM) pH 7.5 and chloroplasts equivalent to 100 μg of chlorophyll, in a final volume of 2ml. Ferredoxin (10μM) had been isolated from Spirulina maxima (15) and SOD was isolated from human erythrocytes by the method of McCord and Fridovich (16).

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uptake may, therefore, be sufficiently explained by the mechanism of ferredoxin's reduction of oxygen. If this is the case then there is no necessity for the assumption that another "oxygen reducing factor" exists and confers sensitivity to SOD upon photosynthetic oxygen uptake. This assumption has been made by Elstner and Heupel (5,9), and the mechanism they proposed involves a two-step reduction of oxygen similar to that involving adrenalin (10) or dopamine (11). An important difference of the present scheme is that ferredoxin, since it is a one-electron acceptor (12), is able to be completely re-oxidised by the first step (univalent oxygen reduction) alone, and so ferredoxin-mediated oxygen uptake is not completely abolished by SOD. The mechanism of the inhibition which does occur rests on a decrease in the effective concentration of

Oxygen Injection	Initial Rate of Ferredoxin Oxidation ΔA_{420} min^{-1}	
	No Addition	Plus SOD
1st	2.84	1.74
2nd	1.63	1.06
3rd	0.94	0.48
	Plus SOD	Plus SOD, KCN
1st	2.16	3.20
2nd	1.42	1.68
3rd	0.66	0.89

TABLE II The effect of SOD on the rate of oxidation by oxygen of chemically-reduced ferredoxin. A solution of Spirulina ferredoxin (200μM) in 50mM potassium phosphate buffer pH 7.8, and at 25°C, was continually flushed with argon, the final volume in the cuvette (1cm) being 2ml. Dithionite was added to bring the A_{420} before the introduction of oxygen to 1.0. Three injections of oxygen-saturated buffer (40 nmoles O_2) were made, and the oxidation of ferredoxin was followed at 420 nm on a Unicam SP800 spectrophotometer. A second set of three oxygen injections followed re-reduction of the ferredoxin (to A_{420} = 1.0) by dithionite. SOD as in table I (100 units) and KCN (5mM) were also present where indicated. The order of accuracy in these estimates of the rates is about ± 10%

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oxidised ferredoxin, rather than on the prevention of the cyclic regeneration of some other electron-accepting species.

An inhibition by SOD of both NADPH oxidation and oxygen uptake in the presence of ferredoxin with ferredoxin-NADP reductase has also been observed by the present author (unpublished work), though a difficulty in freeing the reductase preparation of spinach SOD activity was encountered.

Misra and Fridovich have concluded that a variable proportion of oxygen reduction by ferredoxin proceeds without the involvement of superoxide (4). Instead of assuming divalent oxygen reduction to be occurring in a single step, it would be simpler to suppose that reduction of superoxide by ferredoxin (equation (v)) can compete with a superoxide-detecting reaction such as oxidation of adrenalin. The hypothesis advanced here

may also explain the absence of EPR-detectable superoxide production by plant ferredoxin in the experiment of Orme-Johnson and Beinert (13), since rapid reduction of superoxide by ferredoxin would result in a lower steady-state superoxide concentration than would be produced if spontaneous dismutation were involved.

A rapid removal of superoxide in photosynthetic oxygen reduction could also be of physiological importance; the two-step mechanism would prevent the production of cytotoxic free radicals by any pseudocyclic contribution which may be made to *in vivo* photosynthetic phosphorylation. An inability of chloroplast SOD to provide similar protection from the consequences of rapid electron flow to oxygen is presumably the basis of the herbicidal action of methyl viologen (14).

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