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OXYGEN EXCHANGE ASSOCIATED WITH ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION IN SPINACH THYLAKOIDS

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O_2 uptake in spinach thylakoids was composed of ferredoxin-dependent and -independent components. The ferredoxin-independent component was largely 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) insensitive (60%). Light-dependent O_2 uptake was stimulated 7-fold by 70 μM ferredoxin and both uptake and evolution (with O_2 as the only electron acceptor) responded almost linearly to ferredoxin up to 40 μM. $NADP^+$ reduction, however, was saturated by less than 20 μM ferredoxin. The affinity of O_2 uptake for for O_2 was highly dependent on ferredoxin concentration, with $K_{1/2}(O_2)$ of less than 20 μM at 2 μM ferredoxin but greater than 60 μM O_2 with 25 μM ferredoxin. O_2 uptake could be suppressed up to 80% with saturating $NADP^+$ and it approximated a competitive inhibitor of O_2 uptake with a K_i of 8–15 μM. Electron transport in these thylakoids supported high rates of photophosphorylation with $NADP^+$ (600 μmol ATP/mg Chl per h) or O_2 (280 μmol/mg Chl per h) as electron acceptors, with ATP/2e ratios of 1.15–1.55. Variation in ATP/2e ratios with ferredoxin concentration and effects of antimycin A indicate that cyclic electron flow may also be occurring in this thylakoid system. Results are discussed with regard to photoreduction of O_2 as a potential source of ATP in vivo.

Introduction

Photoreduction of oxygen by the chloroplast electron-transport chain was first recognised over thee decades ago [1] and has been termed the 'Mehler reaction' after its discover. It is clear from an examination of subsequent literature that a number of different reactions involving the photoreduction of oxygen have been studied under this label. These include: (i) direct reduction of O_2 by the electron-transport chain [2,3]; (ii) artificial acceptor (e.g., methyl viologen) -mediated O_2 reduction [3]; and (iii) ferredoxin-mediated O_2 reduction [4,5]. The ferredoxin-mediated reaction is likely to be the most physiologically relevant with respect to both quantitative rates in vivo and the potential for this pathway to participate in whole-chain electron flow independent of $NADP^+$ reduction.

The fact that this electron flow may be coupled to ATP synthesis has stimulated considerable interest. Such pseudocyclic photophosphorylation, as it has been termed, could increase ratios of ATP to NADPH production to approx. 1.5 to 1 required for the operation of the photosynthetic carbon reduction cycle [6,7]. Measurements of O_2

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Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Chl, chlorophyll; PS, photosystem.

uptake in chloroplasts and cells have produced evidence which suggests that O_2 photoreduction is [7–9] and is not [10] quantitatively important as far as capacity for ATP production in vivo is concerned. To assess the potential for this reaction to occur in vivo, a reasonable understanding of the reaction in vitro is desirable. Interactions between ferredoxin, the electron-transport chain, and O_2 have, however, been the least well characterized of all the 'Mehler reactions'. This study examines ferredoxin-mediated O_2 uptake in washed spinach thylakoids particularly with respect to their capacity for O_2 uptake, response to ferredoxin concentration and the ability to support ATP synthesis.

Materials and Methods

Thylakoids were prepared from leaves of glass house-grown *Spinacia oleracea* L. (var. Yates 102) by first preparing intact chloroplasts according to the method of Lilley and Walker [11] and osmotically shocking the chloroplasts in medium A: 0.033 M sorbitol, 5 mM Hepes, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2% bovine serum albumin adjusted to pH 7.6 with KOH. The envelope-free chloroplasts were centrifuged for 5 min at $1500 \times g$ then resuspended in medium B: 0.33 M sorbitol, 50 mM Hepes, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2% bovine serum albumin adjusted to pH 8.0 with KOH, and centrifuged as above. The pellet of washed thylakoids was resuspended in medium C (medium B with dithiothreitol omitted) and stored on ice.

O_2 exchange by the thylakoids was measured using a Varian MAT GD150/4 magnetic sector mass spectrometer as previously described [7], continuously monitoring $^{18}O_2$ (mass 36), $^{16}O_2$, (mass 32) and argon (mass 40) as an internal reference gas (this machine has four collectors enabling each mass to be collected separately and simultaneously). Thylakoids were placed in a glass cuvette (similar in design to that of an O_2 electrode) in medium C depleted of O_2 by bubbling with argon. The cuvette was stoppered and a bubble of $^{18}O_2$ allowed to dissolve into the aqueous medium until the desired O_2 concentration ($^{18}O_2 + {}^{16}O_2$) was reached. The bubble was removed and the experimental measurements of $^{16}O_2$ and $^{18}O_2$ changes began. Gases were admitted to the analyser across a polythene membrane at the base of the cuvette. Calibration of mass signals with regard to concentration of species in solution was made by bubbling liquid in the cuvette with known gas concentrations (i.e., 100% argon and 21% $^{16}O_2$ in air – as the signal response of the mass-spectrometer is linear, then a single-point calibration can be made). Temperature was controlled through a water jacket maintained at 25°C. Calculations of O_2 uptake and evolution were carried out as described previously [7] with corrections based on the change in the argon signal for consumption by the mass spectrometer. Illumination was with a quartz iodide projector lamp giving a light intensity of 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$. Reactions were initiated by switching on the light and rates were measured in the linear portion of the assay after 30 s of illumination.

Photophosphorylation was measured by the estimation of ATP in 50-μl aliquots taken from the mass spectrometer cuvette reaction mixture at 1-min intervals following illumination. ATP synthesis was linear for at least 5 min under these conditions. The aliquots were injected into perchloric acid (3.5%), neutralized and the ATP content estimated by the luciferase assay system, measuring bioluminescence in a fluorometer (Turner and Assoc., CA, U.S.A.). Chlorophyll concentration ranged from 5 to 15 μg/ml in a 5 ml volume. (chlorophyll was estimated by acetone extraction as described previously [7].) Superoxide dismutase and catalase (1000 U/ml) were present in all assays unless otherwise indicated. Ferredoxin was prepared from spinach leaves by the method of Rao et al. [12] and its concentration assayed spectrophotometrically and by protein estimation according to the method of Lowry et al. [22]. All chemicals were obtained from Sigma with the exception of $^{18}O_2$ (99% enriched) which was obtained from Norsk-Hydro (Oslo).

Results

The nature of oxygen uptake in thylakoid preparations

Oxygen uptake in NH_4Cl-uncoupled thylakoid preparations was observed under a number of conditions that indicate the existence of at least

TABLE I

OXYGEN EXCHANGE IN SPINACH THYLAKOIDS WITH O_2 AS AN ELECTRON ACCEPTOR

O_2 concentration was 250 μM throughout; DCMU used was 1.0 μM. Catalase and superoxide dismutase (1000 U/ml) were included. Light intensity was 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$. NH_4Cl (10 mM) was present in all assays.

[Ferredoxin] (μM)	Substrate	O_2 exchange (μmol O_2/mg Chl per h)		
		Gross uptake	Gross evolution	Net uptake
Zero (dark)	O_2 only	12	–	12
Zero	O_2 only	50	30	20
Zero	O_2 + DCMU	29	7	22
60 (dark)	O_2 only	9	–	9
60	O_2 only	158	122	36
60	O_2 + DCMU	34	14	20

three separate possible routes for oxygen reduction (Table I). Firstly, in the dark both in the presence and absence of ferredoxin, there was a small rate of O_2 uptake not associated with O_2 evolution. It is not clear what this is due to or whether it proceeds at the same rate in the light. This uptake was also observed in a net manner in separate O_2-electrode experiments (data not shown). In the light (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$) two types of oxygen uptake were observed; that which existed in the absence of added ferredoxin, and oxygen uptake stimulated by ferredoxin. In the absence of ferredoxin rates of O_2 uptake between 30 and 50 μmol/mg Chl per h were observed (Table I and Fig. 1). This uptake of oxygen was not balanced in a net sense by the evolution of oxygen as would be expected if O_2 were being reduced to superoxide followed by conversion to peroxide then water and oxygen in the presence of added catalase and superoxide dismutase. This net uptake of oxygen appeared to be due to the presence of a portion of O_2 uptake which was insensitive to 10^{-6} M DCMU, and hence independent of whole-chain electron transport. This DCMU-insensitive portion of the uptake was similar in both the presence and absence of ferredoxin. The mechanism behind this O_2 uptake is unclear but probably includes the observed dark O_2 uptake. With the addition of 60 μM ferredoxin, a large increase in O_2 uptake is observed. As can be seen in Fig. 1, this stimulation is very dependent upon the concentration of ferredoxin added.

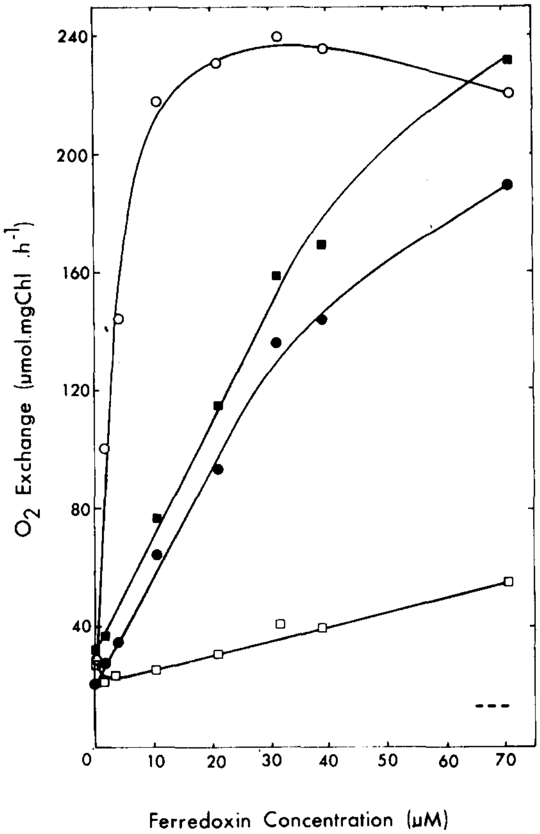

Fig. 1. the response of O_2 uptake to ferredoxin concentration in the presence (□——□) and absence (■——■) of 0.5 mM $NADP^+$. Circles indicate the corresponding O_2 evolution with $NADP^+$ (○——○) or O_2 only (●——●). Broken line indicates the dark rate of O_2 uptake. Thylakoids were uncoupled with 10 mM NH_4Cl. Catalase and superoxide dismutase were included and light intensity was 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$.

In the following experiments, the oxygen uptake specifically linked to ferredoxin reduction is examined with a view to quantifying its dependence on O_2, ferredoxin, $NADP^+$ and its contribution to ATP production.

The response of oxygen photoreduction to ferredoxin

Uncoupled thylakoids in the presence of 250 μM O_2 (21% O_2) photoreduced oxygen at a rate of less than 35 μmol O_2/mg Chl per h in the absence of added ferredoxin (Fig. 1). As discussed earlier, this basal rate of O_2 uptake is variable between preparations but in Fig. 1, O_2 uptake was stimulated approx. 7-fold by the addition of 70 μM ferredoxin. The response of O_2 uptake to ferredoxin concentration is almost linear in the range 0–40 μM, in contrast to that of $NADP^+$ reduction which is saturated by less than 20 μM ferredoxin. The reduction of $NADP^+$ in this chloroplast preparation showed half-saturation at about 4 μM ferredoxin. The O_2-uptake response is parallelled by O_2 evolution with a small net uptake resulting, stimulated slightly by high ferredoxin. As mentioned in Table I, a small amount of O_2 uptake was not light dependent (14 μmol/mg CHl per h) and a portion was light dependent but DCMU insensitive and not associated with O_2 evolution or whole-chain electron transport. If it is assumed that both these reactions proceed in the light in both the presence and absence of ferredoxin, then these may account for the rates of O_2 uptake observed in the absence of added ferredoxin. Whether a small amount of uptake remains representing a reduction of oxygen by whole-chain electron flow not mediated by ferredoxin is unclear. This certainly was the case in Table I but is less certain with the thylakoid preparation in Fig. 1.

Saturating levels of NADP (0.5 mM) greatly suppressed O_2 uptake in the presence of ferredoxin but not apparently below the basal level of O_2 uptake in the absence of ferredoxin. O_2 uptake under these conditions, however, was stimulated about 2.5-fold as ferredoxin increased to 70 μM, well above the saturation for $NADP^+$ reduction. Persistence of a basal level of O_2 uptake in the presence of $NADP^+$ and the extrapolation of the O_2-uptake response to ferredoxin (in the absence of $NADP^+$) to a value close to this basal level suggest that this O_2 uptake is independent of ferredoxin or $NADP^+$.

The absolute rate of O_2 uptake at a given ferredoxin concentration was variable between thylakoid preparations (Table I), possibly due to the absolute electron-transport capacity of the preparations, however, the general responses to ferredoxin and $NADP^+$ were similar to those reported in Fig. 1.

The affinity of oxygen uptake for oxygen

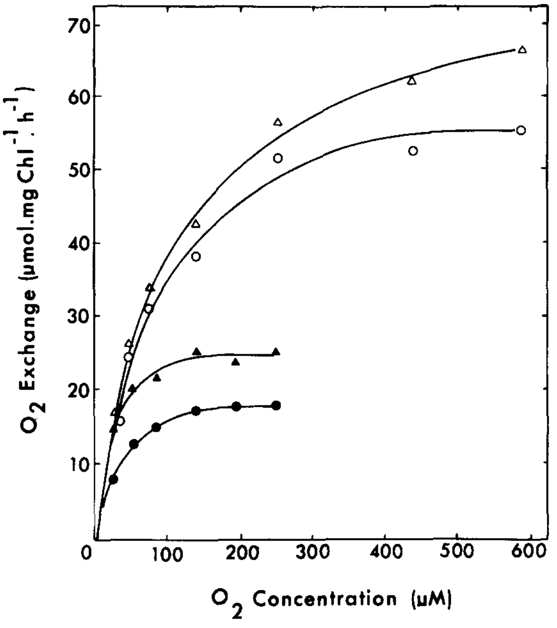

Fig. 2. The response of O_2 exchange to O_2 concentration at two ferredoxin concentrations. O_2 uptake (triangles) and evolution (circles) at 25 μM ferredoxin (open symbols) and at 2 μM ferredoxin (closed symbols). NH_4Cl (10 mM), catalase and superoxide dismutase were included. Light intensity was 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The response to O_2 of O_2 uptake in uncoupled thylakoids was determined at two ferredoxin concentrations (Fig. 2). At 2 μM ferredoxin O_2 uptake saturated by 150 μM O_2, with a $K_{1/2}(O_2)$ of less than 20 μM. At 25 μM ferredoxin, saturation occurred above 600 μM O_2 with a $K_{1/2}(O_2)$ of approx. 60 μM O_2. O_2 uptake in these experiments includes the ferredoxin-independent basal O_2 uptake discussed under Fig. 1. Thus, at 2 μM ferredoxin O_2 uptake measured will be largely comprised of this basal uptake, whilst at 25 μM ferredoxin it will largely reflect the response of ferredoxin-mediated O_2 uptake.

Competition between $NADP^+$ and O_2 for reduced ferredoxin

As seen in Fig. 1, ferredoxin-dependent O_2 uptake can be considerably depressed by the addition of $NADP^+$, presumably due to competition between the $NADP^+$ reductase and O_2 for reduced ferredoxin. This suppression, however, can depend on the actual ferredoxin concentration present (Fig. 3a–d). At high light intensity (1500 $\mu E \cdot m^{-2} \cdot s^{-1}$),

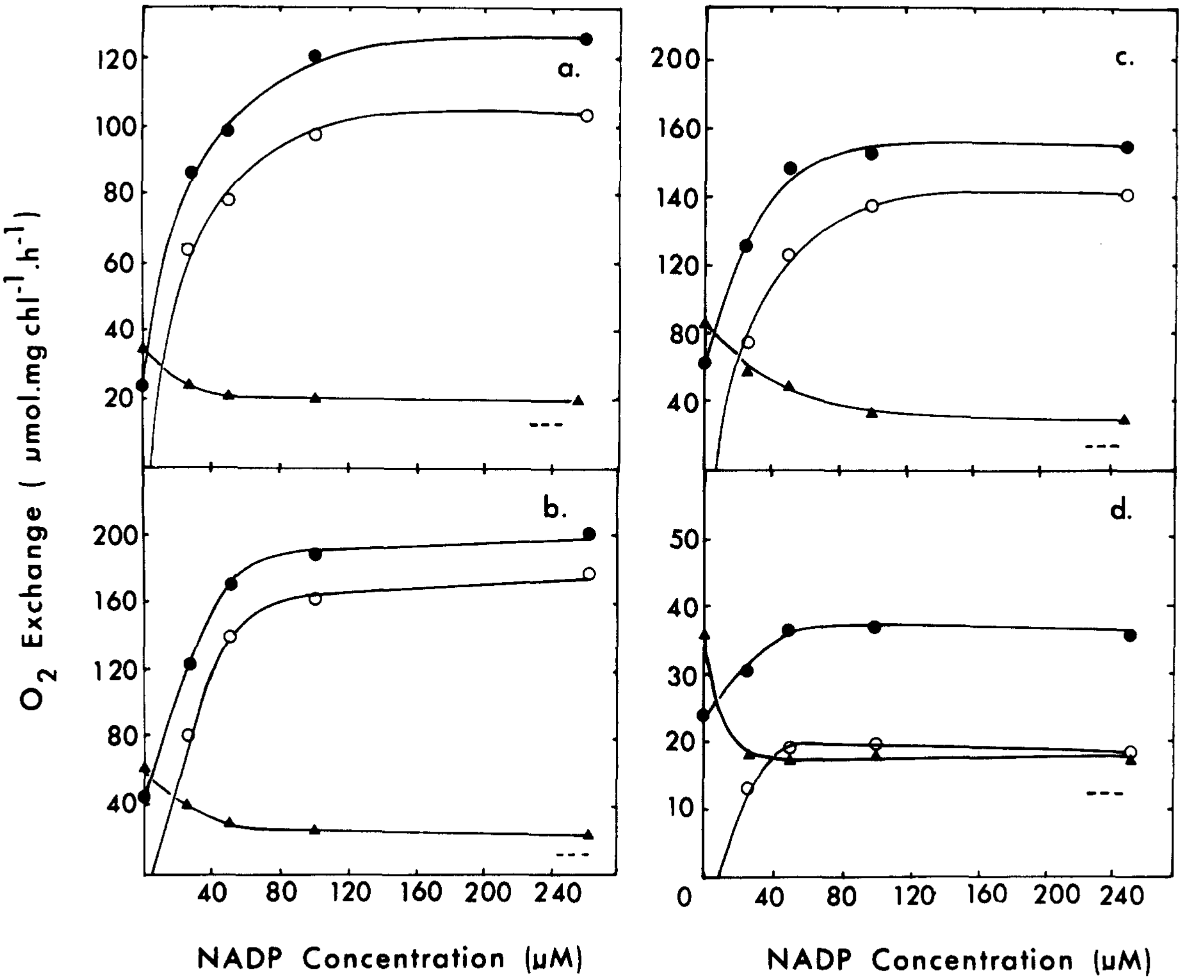

Fig. 3. The response of O_2 uptake (▲——▲), O_2 evolution (●——●) and net O_2 exchange (○——○) to $NADP^+$ concentration in uncoupled thylakoids (10 mM NH_4Cl) at (a) 3, (b) 16 and (c) 33 µM ferredoxin concentrations and 1500 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity. Panel d, 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ light intensity and 33 µM ferredoxin. Broken lines indicates dark rates of O_2-uptake. Catalase and superoxide dismutase were included.

as ferredoxin increased from 3.3 to 33 µM, O_2 uptake increased from 35 to 85 µmol/mg Chl per h. The rate at saturating $NADP^+$ levels, however, increased from only 20 to 30 µmol/mg Chl per h. This is consistent with the responses seen in Fig. 1. The rate of O_2 uptake occurring in the dark is presented for each experiment so that light-dependent O_2 uptake can be calculated.

If it is assumed that there is a constant level of ferredoxin-independent O_2 uptake which remains unaffected by $NADP^+$ level, then this may be subtracted from the observed O_2-uptake rates to give a measure of ferredoxin-mediated O_2 uptake. For these purposes we will assume that this ferredoxin-independent rate is that obtained at saturating levels of $NADP^+$ (17–20 µmol O_2/mg Chl per h). Having done this, a Dixon plot of ferredoxin-dependent O_2 uptake vs. [$NADP^+$] [13] can be constructed for each ferredoxin concentration, and this is presented in Fig. 4. This plot emphasises the point that $NADP^+$ approximates a competitive inhibitor of ferredoxin-dependent O_2 uptake with a K_i in the 8–15 µM region. This implies the observed fact that at higher ferredoxin

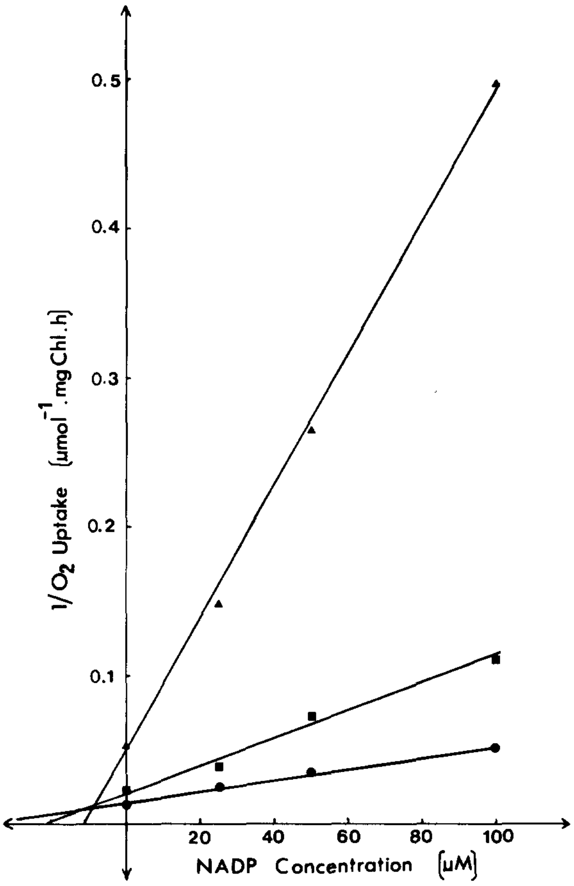

Fig. 4. A Dixon plot [13] of ferredoxin-dependent O_2 uptake vs. [$NADP^+$] as an inhibitor. Data are replotted from Fig. 3, with basal O_2 uptake subtracted (see text). Three ferredoxin concentrations are shown, 3.3 (▲——▲), 16.5 (■——■) and 33 µM (●——●), all run at 1500 µE·m^{-2}·s^{-1} light intensity. The K_i, calculated from the intersection region of the lines, was 8–15 µM $NADP^+$.

levels it takes more $NADP^+$ to suppress O_2 uptake to the basal level and less $NADP^+$ at lower ferredoxin levels. The $NADP^+$ required for suppression of O_2 uptake to the ferredoxin-independent level coincides with the $NADP^+$ required for saturation of $NADP^+$-linked O_2 evolution.

At low light intensity (150 µE · m^{-2} · s^{-1}) and 33 µM ferredoxin, light-dependent O_2 uptake was suppressed by much lower $NADP^+$ concentrations than those necessary at high light. Presumably, this is due to the lower levels of reduced ferredoxin under these conditions and the relative affinities of O_2 and $NADP^+$ for this substrate.

Photophosphorylation supported by $NADP^+$ and O_2 photoreduction

Rates of ATP formation in thylakoids supplied with ferredoxin, ADP, P_i, 250 µM O_2 and where applicable, 0.5 mM $NADP^+$ are shown in Table II. ATP synthesis in excess of 600 µmol ATP/mg Chl per h is supported by saturating levels of $NADP^+$ and ferredoxin, whilst with ferredoxin alone (25 µM) and electron flow to oxygen, the rate of ATP synthesis was about 280 µmol ATP/mg Chl per h. This clearly demonstrates that ferredoxin-dependent O_2 uptake is capable of driving the synthesis of ATP. Although the rates are less than those measured with $NADP^+$ as an acceptor, clearly as seen in Figs. 1 and 2, if both ferredoxin and O_2 were increased in concentration it would be expected that both whole-chain electron transport and ATP-synthesis rates would reach those seen with $NADP^+$ present.

During these experiments an absolute value for whole-chain electron flow is presumably given by the gross O_2-evolution measurements. Thus, an ATP/2e ratio can be calculated. With 5 µM ferredoxin, ATP/2e ratios with O_2 and NADP as acceptors were 1.15 and 1.23, respectively. At 25 µM ferredoxin in both cases the ATP/2e ratio increased, to give 1.39 with $NADP^+$ and 1.55 with O_2 only. This increase in ATP/2e ratio cannot be attributed to the increase in O_2 uptake which resulted as electron transport was measured directly from gross oxygen evolution. An increase in ATP/2e ratio must indicate that ferredoxin is mediating some reaction such as cyclic electron flow which can directly increase ATP production, independent of O_2 evolution [4,14]. The involvement of ferredoxin-mediated cyclic flow is further supported by the action of antimycin A [14,15]. Its inclusion with 25 µM ferredoxin decreases ATP/2e ratios with both $NADP^+$ and O_2 as acceptors, although the effect is more dramatic with oxygen only where ATP/2e decreases from 1.55 to 1.21.

With DCMU present, O_2 evolution was inhibited to almost zero, and ATP synthesis ceased. This was despite the fact that some O_2 uptake as discussed before (Table I) was still present. This O_2 uptake then appears not to be associated with reactions that partition protons accross the membrane and lead to ATP synthesis.

TABLE II

PHOTOPHOSPHORYLATION AND O_2 EXCHANGE WITH O_2 AND $NADP^+$ AS TERMINAL ACCEPTORS

Substrates used were in the following concentrations: ADP concentration (initially) was 2 mM; P_i, 5 mM; O_2, 250 μM; NADP, 0.5 mM; DCMU, 1.0 μM and antimycin A 2 μM. Rates of net and gross evolution, and uptake of O_2 are expressed as μmol O_2/mg Chl per h.

Substrate additions	[Ferredoxin] (μM)	Gross O_2 uptake	Gross O_2 evolution	Net O_2 evolution	Phosphorylation rate (μmol ATP/ mg Chl per h)	ATP/2e
$NADP^+$	5	20	239	219	587	1.23
$NADP^+$	25	29	241	212	669	1.39
$NADP^+$ + antimycin A	25	36	213	177	551	1.30
O_2 only	5	41	37	7	85	1.15
O_2 only	25	101	92	−9	284	1.55
O_2 + antimycin A	25	109	91	−18	221	1.21
$NADP^+$ + DCMU	25	24	9	−15	0	0
O_2 + DCMU	25	19	5	−14	0	0

Discussion

Ferredoxin-mediated photoreduction of oxygen is capable of supporting electron transport (O_2 evolution) at rates similar to those obtained with $NADP^+$ as a terminal acceptor (Fig. 1). To obtain these rates, however, considerably higher levels of ferredoxin are required than those necessary to saturate $NADP^+$ reduction. Whereas ferredoxin-mediated electron flow to $NADP^+$ is saturated by 20 μM ferredoxin, oxygen reduction responds linearly to ferredoxin up to at least 40 μM, and is still not saturated by 70 μM.

The reason for the different responses is not entirely clear based on a simple view of reduced ferredoxin interacting with either $NADP^+$ via $NADP^+$ reductase, or O_2, as represented in Fig. 5A. If ferredoxin accepts electrons from PS I in the same manner in both $NADP^+$ and O_2 reduction, then the rate at which it becomes reduced will be the same whether reducing O_2 or $NADP^+$. As 20 μM ferredoxin is saturating for $NADP^+$ reduction, then a step other than its capacity to accept electrons must be limiting for oxygen reduction. This presumably would be the interaction between reduced ferredoxin and oxygen. If so, then at ferredoxin concentrations which are capable of accepting electrons from PS I in excess of the measured O_2-reduction rate, there will arise a situation where reduced ferredoxin rises to a level in the system such that the rate of the bimolecular interaction between reduced ferredoxin and O_2 equals the rate of reduction of oxidised ferredoxin by PS I. As the oxygen reduction rate should be linearly dependent on the reduced ferredoxin concentration (given constant O_2), increasing total ferredoxin will lead to higher O_2-uptake rates until the maximum rate of whole-chain electron transport is reached. Thus, if 100 μM ferredoxin leads to rates of electron transport equivalent to rates of $NADP^+$ reduction, then presumably only 20 μM or less of the ferredoxin is oxidised, whilst the rest is reduced.

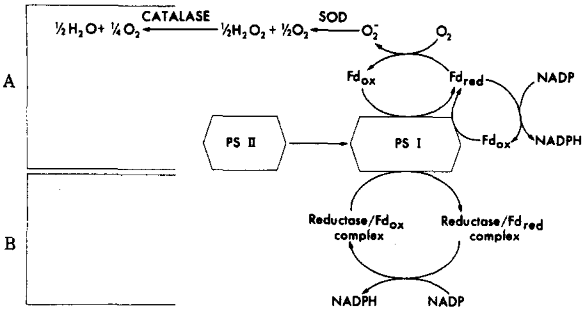

Fig. 5. Fd, ferredoxin; SOD, superoxide dismutase.

A second possible explanation for the differences in the response of O_2 uptake and $NADP^+$ reduction to ferredoxin is that ferredoxin may accept electrons in two different ways from PS I, depending on whether it is reducing $NADP^+$ or O_2. This possibility is raised by the recent suggesting [16] that a ferredoxin–ferredoxin-$NADP^+$ reductase complex may act as the electron acceptor unit in the photoreduction of $NADP^+$. This is outlined in Fig. 5B. In this scheme ferredoxin can be reduced either directly or via being associated with the membrane-bound $NADP^+$ reductase. The response of $NADP^+$ reduction to ferredoxin would be largely determined by the binding of ferredoxin to the $NADP^+$ reductase, whilst the response of oxygen reduction will be dependent on the direct interaction of oxidised ferredoxin with the PS I reductant.

The responses of ferredoxin-dependent O_2 uptake to oxygen (Fig. 2) indicates that unlike photoreduction of O_2 in the anbsence of ferredoxin [2] or methyl viologen-mediated O_2 uptake [3], oxygen in excess of 250 μM O_2 is required to saturate this reaction. The response curve is, however, dependent on the level of ferredoxin present. At low ferredoxin (2 μM) uptake is saturated by 100 μM O_2, whilst at high ferredoxin (25 μM) O_2 in excess of 500 μM is required. The oxygen consumption at low ferredoxin will include a substantial portion of ferredoxin-independent O_2 uptake as discussed in Table I, and its response to O_2 will not reflect the properties of the ferredoxin-mediated reaction. At higher ferredoxin where most of the O_2 uptake is via the ferredoxin route, a better idea can be gained of the properties of this reaction. That this reaction is not saturated at low levels of O_2 indicates that the interaction between O_2 and reduced ferredoxin may be slow under some conditions compared to the rate of reduction of ferredoxin. As discussed in Fig. 5, this will mean that under these conditions (in the absence of $NADP^+$) the ferredoxin pool under steady-state O_2 uptake could be largely in the reduced state. The oxygen response is not consistent with a rapid reduction of O_2 by ferredoxin, leading to low steady-state levels of reduced ferredoxin.

In the presence of $NADP^+$, ferredoxin-mediated O_2 uptake is greatly suppressed (Figs. 1 and 3), however, the degree of suppression is closely related to the level of $NADP^+$. As shown in Fig. 4, $NADP^+$ approximates a competitive inhibitor of O_2 uptake, with a K_i of about 8–15 μM. This K_i is similar to the K_m for $NADP^+$ as a substrate in its photoreduction by thylakoids. It is clear from both Figs. 1 and 3 that saturating $NADP^+$ does not suppress O_2 uptake to zero. A basal level of O_2 uptake remains and it is not entirely clear what this may represent. In Fig. 3, the remaining O_2 uptake is of a level which seems to be composed largely of dark uptake and ferredoxin-independent O_2 uptake (Table I). In Fig. 1, however, it is clear that under some conditions this O_2 uptake at saturating $NADP^+$ may respond to increasing ferredoxin. Allen [5] found previously that with saturating $NADP^+$, it could be estimated that increasing ferredoxin from 10 to 90 μM would lead to at least a 5-fold stimulation in ferredoxin-mediated O_2 uptake. At the highest ferredoxin concentrations more than 30% of electron transport was to oxygen as a terminal acceptor. Fig. 1 indicates that at 70 μM ferredoxin and saturating $NADP^+$, O_2 uptake is at least 23% of the total O_2 evolution, however, half of this may be ferredoxin independent. In Fig. 3 the situation is less dramatic and it appears that little ferredoxin-dependent O_2 uptake remains at saturating $NADP^+$. It is possible to see how variations in the residual rate of ferredoxin-dependent O_2 uptake at saturating $NADP^+$ may occur. If the capacity of the $NADP^+$ reductase to reduce $NADP^+$ exceeds that of whole-chain electron-transport capacity then $NADP^+$ may effectively out-compete O_2 for PS I electrons. If, however, $NADP^+$ reductase capacity is significantly less than this (perhaps due to chloroplast preparation procedures) then at saturating $NADP^+$, there will be a considerable flow of electrons available for O_2 reduction.

Ferredoxin-mediated O_2 uptake can support ATP synthesis with ATP/2e ratios equivalent to or in excess of those measured with $NADP^+$ reduction (Table II). This is important when considering this reaction as a possible extra source of ATP for the fixation of CO_2 in the intact chloroplast and indicates O_2 is a terminal electron acceptor. The contribution of this so-called pseudocyclic electron flow and photophosphorylation to ATP synthesis in vivo has been an issue of some discussion over the past decade [8,9,14]. The in vitro experiments presented here provide a basis from which to make some general statments on the potential magnitude of ferredoxin-mediated O_2 uptake under varying photosynthetic regimes.

(i) O_2 uptake will be of potentially higher magnitude as ferredoxin increases significantly above the level apparently necessary for the reduction of $NADP^+$, due to the apparently different response of the two reactions (Fig. 1). Ferredoxin levels in chloroplasts have been estimated at anywhere between 55 μM and 1.5 mM [17,18] and a 2 : 1 stoichiometry of ferredoxin to $NADP^+$ reductase has previously been measured [19]. Thus, free chloroplast ferredoxin may be anywhere between 28 and 750 μM. Ferredoxin concentration in the chloroplasts used for thylakoid preparation in this study was determined to be approx. 860 μM (unpublished data), using rocket gel immunoelectrophoresis. These values certainly span the concentration range necessary to reduce O_2 at rates possibly approaching those of $NADP^+$ reduction.

(ii) Oxygen uptake via this route will not be saturated until quite high values of O_2, depending on the ferredoxin concentration (Fig. 2). A number of workers have assumed that a Mehler reaction such as this will saturate at quite low O_2 levels [10]. This may be true of ferredoxin-independent O_2 photoreduction [2] and methyl viologen-mediated O_2 uptake [3], but it is certainly not the case for ferredoxin-mediated O_2 uptake. It is not surprising then that many workers examining so-called Mehler reactions in vivo [10,20] find that the $K_m(O_2)$ of such O_2 uptake is in the region of 8–15% O_2, and greater than 21% O_2 is required for saturation.

(iii) The interaction of NADP reduction with the rate of O_2 photoreduction needs to be considered in relation to a number of variables. As discussed earlier, if the capacity of ferredoxin-$NADP^+$ reductase to accept electrons from PS I and reduce $NADP^+$, equals or exceeds that of whole-chain electron transport, then saturating $NADP^+$ may completely suppress ferredoxin-mediated O_2 uptake (Fig. 3). Under these conditions, it appears that the remaining O_2 uptake does not represent a significant whole-chain flow of electrons and so may be unimportant as far as ATP synthesis is concerned. If, however, potential electron transport exceeded the reductase capacity, as for example could occur at high light, then at saturating $NADP^+$ an appreciable amount of reduced ferredoxin may be available for O_2 reduction.

Saturating $NADP^+$ in vivo may not be a likely event if one considers that CO_2 fixation is limited by ATP availability. Under these conditions, the pyridine nucleotide pool will be significantly reduced and $NADP^+$ levels may be well below saturating. If this occurs then as shown by the competitive nature of the suppression of O_2 uptake, quite appreciable amounts of O_2 uptake are possible, and hence would contribute significantly to ATP synthesis. As has been pointed out by other workers [4,6], the competitive nature of interactions between $NADP^+$ and O_2 or other acceptors for electrons from ferredoxin could be envisaged to lead to a balancing mechanism for the ATP/NADPH synthesis ratio to be maintained at 3 : 2 necessary for CO_2 fixation.

(iv) The relative magnitide of the O_2-supported pseudocyclic flow of electrons, described here, compared to a cyclic flow of electrons from PS I to ferredoxin and eventually to a region around cytochrome *b*-563 [14,21] remains unclear. From the experiments in Table II it is obvious from the variation in ATP/2e ratio, in both the presence and absence of $NADP^+$, that some system such as cyclic flow of electrons around PS I, mediated by ferredoxin, must be occurring simultaneously with O_2 photoreduction. At 25 μM ferredoxin, with oxygen only as the acceptor, the variation in the ATP/2e ratio over the antimycin A-treated control suggests that over 20% of the ATP is being generated by such a cyclic flow. The significant change in the ATP/2e ratio when ferredoxin increased from 5 to 25 μM suggests, however, that this cyclic flow may respond to ferredoxin in a manner similar to O_2 uptake. This is to be expected if both processes are dependent on reduced ferredoxin levels. Only if the cyclic pathway had a much higher affinity for reduced ferredoxin would it predominate at low ferredoxin. The evidence here indicates that both pathways for extra ATP synthesis could be quantitatively significant under appropriate physiological conditions. Both would be regulated by $NADP^+$ levels in a manner previously described.

Experiments with intact chloroplasts, cells and leaves show that there is significant photoreduction of O_2 at high CO_2 concentrations [8,7,20]. Under these conditions, where ribulosebisphosphate oxygenase, the other major O_2-consuming reaction, is suppressed, electron flow to oxygen may be greater than 10% of total electron transport. As has been argued previously [7], this would be sufficient to increase the ATP/NADPH ratio to 3 : 2. That this O_2 uptake is occurring via the ferredoxin-mediated pathway is consistent with the properties of the in vitro thylakoid reactions presented here. Given that ferredoxin-mediated cyclic flow may also be occurring at the same time, there would appear to be adequate means for the chloroplast to solve its ATP/NADPH balancing problems.

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