Evidence for two cyclic photophosphorylation reactions concurrent with ferredoxin-catalyzed non-cyclic electron transport

Jonathan P. Hosler * and Charles F. Yocum **

Division of Biological Sciences and Department of Chemistry, The University of Michigan, Ann Arbor, MI 48108 (U.S.A.)

Addition of ferredoxin to isolated spinach chloroplast thylakoid membranes reconstitutes phosphorylating electron transfer characterized by elevated P/O ratios (1.6). When oxygen is the terminal electron acceptor for reduced ferredoxin, the P/O value is lowered by antimycin A or low concentrations (10 μM) of heparin (an anionic macromolecule), but not by inhibition of the activity of membrane-bound ferredoxin-NADP reductase. When NADP is present as the terminal electron acceptor for reduced ferredoxin, the elevated P/O value (again 1.6) is unaffected either by antimycin A or low concentrations (10 μM) of heparin. When ferredoxin-catalyzed cyclic or Q-loop activity is sensitive to antimycin A, the ferredoxin pool and P-700 are both present in a largely reduced state. The opposite result is obtained for antimycin-A-insensitive activity in the presence of NADP. Our results show that conditions exist whereby ferredoxin-catalyzed cyclic electron transport is insensitive to a classical inhibitor of the cytochrome *b* function. We suggest that the antimycin-A-insensitive pathway of ferredoxin-catalyzed cyclic electron transport may involve the activity of ferredoxin-NADP reductase.

Introduction

Ferredoxin reconstitutes at least three electron-transport reactions in thylakoid membranes. After reduction by PS I, ferredoxin can be oxidized by ferredoxin-NADP reductase [1-3] or by molecular oxygen (pseudocyclic electron flow) [4,5]. In addition, electrons from reduced ferredoxin may also be diverted to PQ, presumably via some intermediate carrier, in order to initiate cyclic electron

flow around PS I [6,7]. All three electron-transport pathways catalyze photophosphorylation in vitro, and all three are now considered to be important in vivo [8-1].

Although most of the electron carriers of the cyclic pathway are shared by non-cyclic electron transport, cytochrome *b*-563 appears to be unique to the cyclic pathway, a conclusion which is based primarily upon the fact that antimycin A specifically inhibits cyclic photophosphorylation [12]. Cytochrome *b*-563 has been regarded as the carrier mediating electron flow from reduced ferredoxin to plastoquinone, but this role is now thought to be unlikely, since *b*-563 is only slowly reduced by hydrophilic reductants, including ferredoxin [13,14]. In addition, plastosemiquinone has been implicated as both the oxidant and the reductant of cytochrome *b*-563 [15-20], which is more con-

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* Present address: Department of Botany, Duke University, Durham, NC 27706, U.S.A.
** To whom correspondence should be addressed.

Abbreviations: PS, Photosystem; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; EPR, electron paramagnetic resonance; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; P_i, inorganic phosphate.

sistent with Q-cycle schemes in which b-563 mediates electron transfer across the thylakoid membrane. The evidence for this type of cyclic pathway has recently been reviewed [21].

While direct measurement of the steady-state rate of cyclic electron flow is impossible, it is possible to measure the rate of photophosphorylation associated with cyclic flow. While rapid rates of cyclic photophosphorylation can be attained by supplying a pool of reduced ferredoxin to chloroplast thylakoid membranes under anaerobic conditions [22], we have chosen to study cyclic photophosphorylation concurrently with non-cyclic photophosphorylation catalyzed by electron transport from water to ferredoxin/O_2 or to ferredoxin/NADP. These measurements are closer approximations to the situation in vivo than measurements of ATP synthesis by an independently poised cycle, and yield novel results on the pathway of cyclic electron flow.

As we show here, cyclic photophosphorylation occurs during electron transport to both ferredoxin/O_2 and ferredoxin/NADP, but several differences between these cyclic reactions lead us to propose the existence of two independent pathways of cyclic electron flow. Based upon their sensitivity to antimycin A only one pathway appears to involve electron flow through cytochrome b-563; of the two pathways, one depends upon a pool of reduced ferredoxin, while the other may require the activity of ferredoxin-NADP reductase. Each cycle appears to have a unique site of ferredoxin oxidation on the thylakoid membrane.

Methods

Spinach thylakoid membranes were prepared by the method of Robinson and Yocum [22], with the exception that all resuspensions were performed with a No. 8 camel's hair brush. Thylakoid membranes prepared by this procedure routinely exhibited rates of 300–400 μmol O_2/h per mg Chl for gramicidin-uncoupled electron transport to MeV, and non-cyclic photophosphorylation efficiencies (P/2e) between 1.2 and 1.3. For some experiments, the membranes were treated with N-ethylmaleimide according to the procedure of Mills et al. [23] with the following modifications: thylakoid membranes at 1 mg Chl/ml were in-

cubated in the dark for 2 min at 25°C with 2 mM NADPH plus 10 mM N-ethylmaleimide. The reaction was quenched with 5 mM dithiothreitol, and the inhibited thylakoids were placed on ice for subsequent use. For other experiments, thylakoid membranes at 1 mg Chl/ml were incubated in the dark with 0.5 mM p-chloromercuribenzene sulfonate and 2 mM NADPH for 5 min at 25°C. These membranes were centrifuged at 4°C in an Eppendorf microfuge to give a soft pellet, washed once in 1 ml 20 mM Tricine (pH 8.0)/15 mM NaCl/0.4 M sucrose/0.2% bovine serum albumin (buffer 1), resuspended in buffer 1 to a chlorophyll concentration of 1 mg/ml, and transferred to ice. Neither the N-ethylmaleimide nor the p-chloromercuribenzene sulfonate-treated thylakoids showed reversal of the inhibition during the course of the experiment.

Ferredoxin was purified from spinach as described by Petering and Palmer [24], with the modifications of Yocum [25]. The isolated protein produced a single band on an overloaded 12% acrylamide gel.

All assays, with the exception of the measurements of EPR Signal I, were performed in a reaction mixture containing 50 mM Tricine (pH 8.0)/50 mM NaCl/3 mM MgCl$_2$/5 mM NaH$_2$PO$_4$/1 mM ADP/30–40 μg Chl in a total volume of 1.5 ml. Ferredoxin, NADP, methyl viologen and all other substrates were added as noted in the figure legends. Rates of oxygen evolution and uptake by the electron-transport reactions were measured with a Clark-type electrode fitted to a thermostatted (25°C) cuvette. Saturating light (10^3 J·m^{-2}·s^{-1}) was provided by an Oriel model 6325 light source filtered through 5 cm of an 0.2% solution of CuSO$_4$ and a red cut-on filter (longer than 600 nm). The ATP synthesis rate was determined by the incorporation of ^{32}P$_i$ into ATP. Unreacted phosphate was extracted by the method of Avron [26], and the remaining solution was analyzed for Cerenkov emission by the method of Gould et al. [27].

The rate of electron transport to ferredoxin/O_2 (oxygen reduction) was measured by the uptake of oxygen from the reaction mixture. In the presence of 0.1 mM KCN, which was used to inhibit any residual catalase activity, this reaction proceeded with an overal stoichiometry of 1 O_2 molecule

consumed for every four electrons transported from water. Similar to experiments presented by Telfer et al. [28], an excess amount of catalase was added to the reaction mixture after the illumination period, and the resulting release of oxygen from the breakdown of hydrogen peroxide was equal in magnitude to the amount of oxygen uptake observed in the light (data not shown). A number of control experiments (not shown) established that the accumulation of hydrogen peroxide during illumination had no effect on the rates of electron transport or photophosphorylation.

The rate of NADP photoreduction was measured by oxygen evolution in the presence of 840 units of catalase. This method was compared to actual NADPH formation by measuring the concentration of NADPH by oxidation with phenazine methosulfate, and the expected ratio of 2 NADPH produced per O_2 evolved was confirmed. The rates of concurrent NADP reduction and oxygen reduction were measured by the following method: residual catalase activity associated with thylakoid membranes was inhibited by 0.1 M KCN during illumination, then excess catalase (840 units) was added after the light was turned off. Total oxygen evolution (NADP reduction) was calculated by adding the amount of oxygen evolved in the light to the amount evolved from the breakdown of hydrogen peroxide by catalase. The magnitude of the catalase-induced oxygen release alone is equal to the amount of oxygen reduced in the light. Both NADP reduction and oxygen reduction have the same stoichiometry of 1 O_2 evolved or consumed per four electrons transported, and therefore the rates of oxygen uptake and evolution can be compared directly. This method gave results similar to those obtained by others [29].

The concentration of reduced ferredoxin present during steady-state electron flow was measured from the decrease in absorption seen upon illumination, using the wavelength pair 497–540 nm with an Aminco DW-2 spectrophotometry; saturating continuous illumination was filtered with a Corning 640 nm cut-on filter. The photomultiplier was protected with a broad-band interference filter from 450 to 600 nm (OCLI); the reaction cuvette was stirred and thermoregulated (25°C). The extinction coefficient for the change in light absorption upon photoreduction of fer-

redoxin was derived from absorption spectra of oxidized and photochemically reduced ferredoxin (Robinson, H.H., personal communication). The extinction coefficient at 540 nm was subtracted from that at 497 nm to give the differential extinction coefficient of 1.74 $mM^{-1} \cdot cm^{-1}$ for the 497–540 nm wavelength pair. The accuracy of this value was confirmed by reducing a known concentration of ferredoxin under anaerobic conditions; the calculated concentration agreed with the known concentration. No light-induced absorbance decrease was seen in thylakoid membranes in the absence of ferredoxin, or during electron flow to methyl viologen, and the 497–540 nm signal was eliminated by the addition of 5 μM DCMU.

Measurements of EPR Signal I were made with a Bruker ER 200D spectrometer, using a modulation amplitude of 10 gauss peak-to-peak and a microwave power of 20 mW. Continuous white light was supplied by a microscope illuminator, and chloroplast membranes were used at a concentration of 3 mg Chl/ml in buffer 1 containing 3 mM $MgCl_2$.

All chemicals were obtained from Sigma, except for methyl viologen (from Aldrich), DCMU (from K & K Labs), and $NaH^{32}PO_4$ from New England Nuclear.

Results

Analysis of ferredoxin-catalyzed electron transport and photophosphorylation

In the absence of NADP, addition of ferredoxin to thylakoid membranes reconstitutes an O_2 reduction reaction whose rate is dependent upon the ferredoxin concentration, but is always much slower than the rates of O_2 uptake which can be observed with an efficient PS I acceptor such as methyl viologen (data not shown). This O_2 uptake reaction is slow due to the rate-limitation imposed by autoxidation of ferredoxin [30], and cannot be overcome by the use of artificial donors (such as ascorbate/DAD) to overcome possible rate-limiting steps in electron transfer between the two photosystems (data not shown). (In the absence of any ferredoxin, some O_2 uptake activity is also observed (see Fig. 1A), but this activity, which presumably arises from autoxidation of the PS I
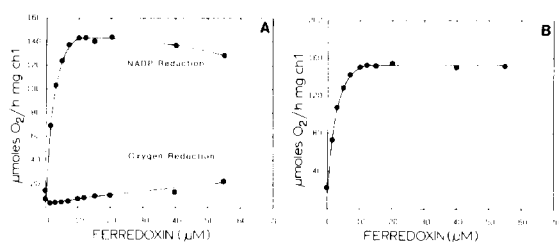
Fig. 1. Concurrent oxygen and NADP reduction mediated by ferredoxin (A). Electron-transport rates to NADP (1 mM) and oxygen were measured as described under Materials and Methods. (B) The total electron-transport rate is derived by adding the rates shown in A.

primary acceptor complex [9,31], is a minor component of the activities we assay here owing to the low concentrations of PS I centers present in our reaction mixtures.)

The rate of ferredoxin-mediated electron transport increased with the addition of saturating NADP (from 74 (without NADP) to 162 (with NADP present) μmol O_2/h per mg Chl) using 10 μM ferredoxin, since under these conditions ferredoxin was rapidly oxidized by ferredoxin-NADP reductase/NADP. A slow rate of ferredoxin-mediated oxygen reduction has previously been shown to accompany electron transport to NADP [29], and we have measured these concurrent reactions as discussed under Methods. The rate of oxygen reduction during electron transport to NADP was small, but not insignificant (Fig. 1A). Oxygen reduction was inhibited by concentrations of ferredoxin which were subsaturating for NADP reduction (less than 10 μM), indicating that rapid electron transport through ferredoxin-NADP reductase to NADP suppressed the oxygen reduction pathway. With 10 μM ferredoxin, which saturated NADP photoreduction, oxygen reduction accounted for 5% of the total electron flow, while in the presence of 50 μM ferredoxin, oxygen reduction was increased to 15% (Fig. 1A). Thus, the rate of oxygen reduction is dependent upon the concentration of oxidized ferredoxin added to the reaction in the presence or absence of NADP. The rate of NADP photoreduction declined at higher ferredoxin concentrations (Fig. 1A), a decrease which was balanced by an increase in the oxygen reduction rate, since the addition of the two curves of Fig. 1A shows a reaction whose rate does not decline at higher ferredoxin concentra-

tions (Fig. 1B). These data are consistent with a shift of electrons from NADP reduction to oxygen reduction as the ratio of oxidized to reduced ferredoxin increases.

As shown in Table I, measurement of the rate of both NADP reduction and oxygen reduction during electron transport to ferredoxin/NADP is critical for accurate calculation of ATP/O (or ATP/2e) values. The 'apparent' P/O values of Table I were calculated using the rate of NADP reduction (oxygen evolution) only, while the 'true' P/O values were calculated from the sum of oxygen reduction and NADP reduction (oxygen uptake plus evolution). Failure to account for the rate of oxygen reduction leads to an inflated P/O value, especially at higher concentrations of ferredoxin, where oxygen reduction comprises a substantial portion of total electron transport. All of the P/O values in the experiments presented here were calculated on the basis of total electron transport, and hence, the elevated P/O values observed in the presence of NADP are not due to additional ATP synthesis associated with oxygen reduction.

Photophosphorylation associated with ferredoxin-mediated electron-transport reactions was compared to photophosphorylation associated with non-cyclic electron transport to methyl viologen (Table II). Since methyl viologen is highly au-

TABLE I

DETERMINATION OF THE CORRECT P/O VALUE WITH FERREDOXIN/NADP AS THE PS I ACCEPTOR

The concentration of NADP was 1 mM. Rates of electron transport and photophosphorylation are expressed as μmol O_2 or μmol ATP.h per mg Chl. The 'apparent' P/O was calculated using oxygen evolution (NADP reduction) only, while the 'true' P/O was calculated using oxygen evolution plus concurrent oxygen uptake (oxygen reduction), which was measured as described in the text.

Measurement	$H_2O \rightarrow 10 \mu M$ ferredoxin/ NADP	$H_2O \rightarrow 55 \mu M$ ferredoxin/ NADP
O_2 evolution	143	129
O_2 uptake	8	23
Total electron transport	151	152
ATP synthesis	482	483
Apparent P/O	1.69	1.87
True P/O	1.60	1.59

TABLE II

PHOTOPHOSPHORYLATION WITH METHYL VIOLO-
GEN, FERREDOXIN/O_2 AND FERREDOXIN/NADP AS
TERMINAL ACCEPTORS

The concentrations of PS I acceptors were: methyl viologen, 67
μM; ferredoxin in the absence of NADP, 20 μM; feredoxin in
the presence of NADP, 10 μM; NADP, 1 mM. Rates of
electron transport and photophosphorylation are expressed as
μmol O_2 or μmol ATP/h per mg Chl. In the presence of
NADP, the rate of electron transport includes both NADP
reduction and oxygen reduction.

Acceptor	Electron transport rate	Photophos-phorylation rate	P/O
Methyl viologen	200	505	1.26
Ferredoxin/O_2	96	308	1.60
Ferredoxin/NADP	159	501	1.58

Fig. 2. Photophosphorylation rates (A) and ATP/O (ATP/2e)
ratios (B) for electron transport to ferredoxin/O_2 and ferredo-
xin/NADP. Conditions were as in Fig. 1; P/O ratios were
calculated by the method of Table I.

tooxidizable, it can only catalyze non-cyclic activ-
ity under aerobic conditions; the P/O value gener-
ated by this reaction was routinely observed to be
about 1.25, in agreement with others [32]. Electron
flow to both ferredoxin/O_2 and ferredoxin/NADP
yielded P/O values of approx. 1.6, substantially
greater than the pure non-cyclic P/O value. This
increased P/O value must result from an ad-
ditional proton-pumping reaction which depends
upon the presence of ferredoxin, and functions
concurrently with non-cyclic electron flow to
oxygen or NADP; this ferredoxin-dependent reac-
tion must be PS I cyclic electron transfer activity.

Electron transport to ferredoxin/NADP sup-
ported a greater rate of ATP synthesis than the
oxygen reduction pathway at all of the ferredoxin
concentrations assayed (Fig. 2A), because the rate
of non-cyclic electron transfer was greater with
NADP. The rate of ATP synthesis associated with
electron flow to ferredoxin/NADP saturated at 10
μM ferredoxin, while concentrations of ferredoxin
up to 70 μM did not saturate the rate of photo-
phosphorylation in the absence of NADP. In spite
of this difference a maximum P/O value of 1.6
was obtained for ferredoxin-mediated electron
transfer in the presence or absence of NADP (Fig.
2B).

In order to further examine the cyclic compo-
nent of photophosphorylation supported by elec-
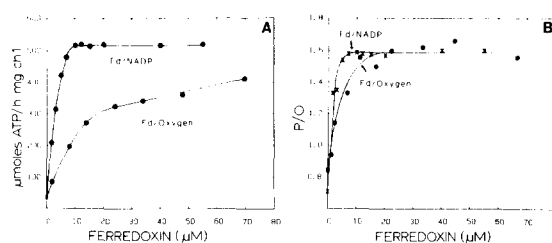tron flow to ferredoxin, the effect of antimycin A

on these reactions was examined. Previous find-
ings have shown that antimycin A strongly inhibits
cyclic photophosphorylation catalyzed by fer-
redoxin when the reaction is artificially poised
[22], and antimycin A has been used as a specific
inhibitor of the physiological cyclic reaction in
some systems [10,33]. Our data show that low
concentrations of antimycin A decrease the P/O
value of the oxygen reduction pathway from ap-
prox. 1.6 to 1.2 (Fig. 3), which is essentially the
same as the non-cyclic value generated by electron
transport to methyl viologen (Table II). This de-
crease was due to a decline in the rate of ATP
synthesis (the rate of oxygen reduction was unaf-
fected by antimycin A); furthermore, the plateau
at a P/O value of 1.2 indicates that the initial
decrease was not due to partial uncoupling by the
inhibitor. The same concentration of antimycin A
had no effect on non-cyclic photophosphorylation
(water to methyl viologen), but concentrations of
10 μM or greater began to inhibit linear electron
flow and uncouple ATP synthesis (data not shown).
The decline in the P/O value of oxygen reduction
in response to antimycin A (Fig. 3) represents a
selective inhibition of cyclic photophosphorylation
which leaves the remaining non-cyclic activity un-
affected.

The high P/O value (1.6) obtained in the pres-
ence of ferredoxin and NADP indicates that cyclic
photophosphorylation is also a substantial compo-
nent of the total rate of ATP synthesis measured
during concurrent NADP and oxygen reduction
(Table II and Fig. 2B). The synthesis of ATP
during electron flow to ferredoxin/NADP, how-
ever, was largely insensitive to low concentrations

of antimycin A, and the P/O value remained high (Fig. 3). A similar experiment by Arnon and Chain [33] led to the conclusion that cyclic photophosphorylation associated with electron flow to ferredoxin/NADP was inhibited by antimycin A. Since we did not observe this inhibition, we also assayed ferredoxin/NADP activity for sensitivity to antimycin A under a wide variety of conditions, including approximations of those used in Ref. 33. As shown in Table III, elevated Chl concentrations (up to 250 μg/ml) and decreased light intensities (as low as $4.2 \cdot 10^{-2}$ J $\cdot$ m^{-2} $\cdot$ s^{-1}) did not lead to any inhibition of ATP synthesis by antimycin A. Conditions were created which might affect the binding properties of antimycin A to the membrane; for example, thylakoids were incubated with high concentrations of antimycin A plus dithionite in the dark, followed by transfer to the normal reaction medium for assay. This procedure failed to produce antimycin A sensitivity. Finally, oxidizing conditions were created in the interphotosystem chain by partially inhibiting PS II with low concentrations of DCMU during electron transport to ferredoxin/NADP. Again, the P/O values were high, and were unaffected by antimycin A.

Our data therefore show that under our assay conditions the presence or absence of NADP determines the sensitivity of ferredoxin-mediated cyclic photophosphorylation to antimycin A. The reason for this difference may lie in the fact that the presence or absence of NADP leads to fundamental changes in both the net oxidation-reduction state of the interphotosystem electron carriers and in the size of the pool of reduced ferredoxin generated by electron transport.

During oxygen reduction, the slow rate of ferredoxin autooxidation should lead to reducing conditions in the interphotosystem electron-transport chain, while rapid electron flow to NADP should maintain electron carriers on the reducing side of the plastoquinone pool in a more oxidized condition. The redox state of these interphotosystem electron carriers was estimated by measuring the relative concentration of oxidized P-700 (EPR Signal I) during steady-state electron transport. In the presence of methyl viologen, P-700 was largely oxidized (Fig. 4), indicating that electrons were being withdrawn from PS I at a faster rate than they were supplied. A similar result was seen with electron flow to ferredoxin/NADP; in both cases

TABLE III

ABSENCE OF ANTIMYCIN A INHIBITION OF PHOTOPHOSPHORYLATION DURING ELECTRON TRANSPORT TO FERREDOXIN/NADP UNDER A VARIETY OF REACTION CONDITIONS

Assay conditions are described under Materials and Methods except for the changes noted. The light intensity was varied by the insertion of wire screens between the reaction cuvette and the lamp. For the experiments using dithionite, thylakoid membranes at 1.0 mg Chl/ml were incubated for 2 min in the dark in the presence of 1 mM sodium dithionite $\pm$ 120 μM antimycin A, and then transferred to the reaction cuvette at a final concentration of 20 μg Chl/ml for assay. Antimycin A was added to 5 μM with 100 μg Chl/ml and 12.7 μM with 250 μg Chl/ml in order to keep the antimycin-A/Chl ratio constant with the control experiment. For all other reactions antimycin A was added as indicated, to a final concentration of 1.7 μM. Ferredoxin was present at 10 μM and NADP at 1 mM throughout. The rate of ATP synthesis is given as μmol ATP/h per mg Chl.

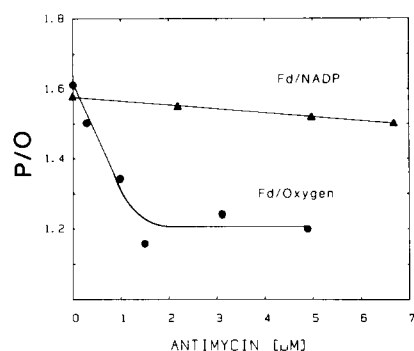| Treatment	Antimycin A absent		Antimycin A present		
	ATP	P/O	ATP	P/O	
Control	489	1.57	478	1.51	
100 μg Chl/ml	307	1.52	299	1.56	
250 μg Chl/ml	152	1.52	150	1.58	
$1.3 \cdot 10^2$ J$\cdot$m^{-2}$\cdot$s^{-1}	160	1.21	162	1.17	
$4.2 \cdot 10$ J$\cdot$m^{-2}$\cdot$s^{-1}	77	0.93	71	0.98	
Preincubation with 1 mM dithionite $\pm$ 120 μM antimycin A	450	1.50	454	1.47	
+ 0.33 μM DCMU	68	1.58	65	1.52	

Fig. 3. Effect of antimycin A on the ATP/O ratio of photo-phosphorylation during electron transport to ferredoxin/O_2 and ferredoxin/NADP. Electron acceptors were 25 μM ferredoxin alone or 10 μM ferredoxin plus 1 mM NADP.

Fig. 5. Steady-state concentrations of reduced ferredoxin during ferredoxin-mediated electron transport to O_2 or NADP. The steady-state amplitude of the 497–540 nm signal was used to calculate the concentration of reduced ferredoxin as described under Materials and Methods; NADP was used at 1 mM.

the interchain carriers must have been in a relatively oxidized condition. In contrast, during electron transport to ferredoxin/O_2 the steady-state concentration of oxidized P-700 was much lower (Fig. 4).

The slow autooxidation of reduced ferredoxin should also lead to its accumulation during electron flow to ferredoxin/O_2, and therefore the steady-state amount of reduced ferredoxin present during illumination was measured as described in 'Materials and Methods'. The concentration of reduced ferredoxin increased as a function of ad-
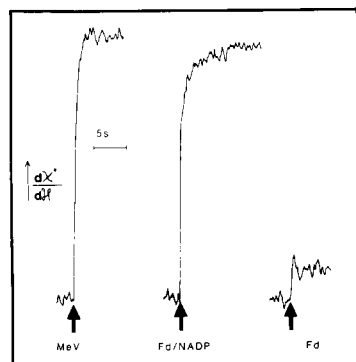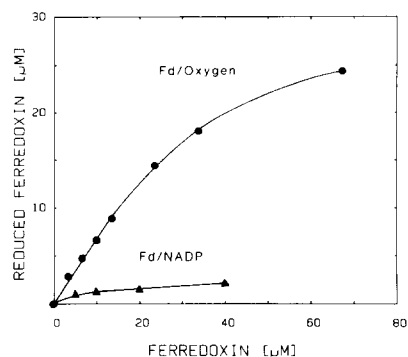

Fig. 4. Relative concentrations of P-700$^+$ during steady-state electron transport to ferredoxin/O_2, ferredoxin/NADP and methyl viologen. The onset of continuous white light is indicated by the arrow. Electron acceptors were 33 μM methyl viologen, 68 μM ferredoxin plus 2 mM NADP or 68 μM ferredoxin alone; P-700 was approx. 8.4 μM. The amplitude is a measure of the relative concentration of P-700$^+$.

ded ferredoxin (Fig. 5), and, not surprisingly, the accumulation of reduced ferredoxin was some 5–12 times greater in the absence of NADP. In the presence of NADP, ferredoxin was rapidly oxidized and little accumulation of the reduced protein was observed (Fig. 5).

The rate of cyclic photophosphorylation during electron transport to ferredoxin/O_2 at different concentrations of added ferredoxin was estimated by subtracting ATP synthesis rates measured in the absence and presence of 1.7 μM antimycin A (data not shown). A comparison of these data to the top curve of Fig. 5 showed that the rate of cyclic photophosphorylation was dependent upon the concentration of reduced ferredoxin, in agreement with measurements of the independently poised antimycin-A-sensitive cycle [22]. In contrast the rate of cyclic photophosphorylation (estimated from Fig. 2A by assuming a non-cyclic P/O of 1.25) during electron flow to ferredoxin/NADP is faster (110 vs. 33 μmol ATP/h per mg chl at 10 μM ferredoxin during electron flow to NADP and O_2, respectively) and obviously not dependent upon a large steady-state concentration of reduced ferredoxin (Fig. 5, bottom curve). In fact, the P/O ratio of this reaction remained high under conditions where the ferredoxin concentration was clearly subsaturating for NADP reduction (e.g., Fig. 2B at 5 μM ferredoxin; Table III, elevated chlorophyll concentrations).

Effects of N-ethylmaleimide, p-chloromercuribenzene sulfonate and heparin on ferredoxin-catalyzed activity

The differences in phosphorylation activity reconstituted by addition of ferredoxin plus or minus NADP to thylakoid membranes raises the question of whether ferredoxin-NADP reductase is involved in catalysis of cyclic electron-transport activity. To examine this possibility, thylakoid membranes were treated with known inhibitors of the reductase. Photophosphorylation reactions catalyzed by thylakoids treated with N-ethylmaleimide were identical to the reactions of the untreated membranes (Table IV). Non-cyclic photophosphorylation (water to methyl viologen) was unaffected, indicating that N-ethylmaleimide did not inhibit the coupling factor or uncouple electron transport under these conditions. The P/O value

TABLE IV

EFFECT OF N-ETHYLMALEIMIDE AND p-CHLORO-MERCURIBENZENE SULFONATE ON ELECTRON TRANSPORT AND PHOTOPHOSPHORYLATION

Substrates were used in the following concentrations: methyl viologen, 67 μM; ferredoxin alone, 27 μM; ferredoxin with NADP, 10 μM; NADP, 1 mM; antimycin A, 1.7 μM. Excess catalase (840 units) was added during the measurements of the inhibition of NADP reduction (ferredoxin/NADP), but not during the other measurements of electron transport. The measurements of electron flow to ferredoxin/NADP used to calculate the P/O value were obtained as described under Materials and Methods. Treatments with N-ethylmaleimide and p-chloromercuribenzene sulfonate were as described under Materials and Methods.

Acceptor	Percentage inhibition of electron transport	P/O	
		Untreated thylakoids	Treated thylakoids
Treatment with N-ethylmaleimide			
methyl viologen	3	1.23	1.25
ferredoxin/O_2	0	1.61	1.63
ferredoxin/O_2 + antimycin A	0	1.19	1.25
ferredoxin/NADP	59	1.54	1.48
Treatment with p-chloromercuribenzene sulfonate			
methyl viologen	17	1.22	1.13
ferredoxin/O_2	11	1.51	1.37
ferredoxin/O_2 + antimycin A	11	1.21	1.13
ferredoxin/NADP	85	1.63	1.39

of the oxygen reduction pathway was also unaffected, and antimycin A had the same effect in both the inhibited and uninhibited membranes. These data strongly suggest that ferredoxin-NADP reductase is not involved in cyclic activity in the absence of NADP. The possibility that the remaining 40% of reductase activity in these thylakoids was sufficient to support maximum cyclic photophosphorylation was eliminated by using membranes treated with p-chloromercuribenzene sulfonate. Treatment caused a slight decrease in the overall phosphorylation efficiency, as evidenced by the change in the non-cyclic P/O value (water to methyl viologen) from 1.22 to 1.13 (Table IV). The rate of ATP synthesis during oxygen reduction was still sensitive to low concentrations of antimycin A, and the magnitude of the antimycin A-induced decline in the P/O value was nearly equal to that of the uninhibited membranes (Table IV), which constitutes evidence for the continued operation of the ferredoxin-mediated cycle under conditions in which the activity of ferredoxin-NADP reductase is largely (85%) inhibited.

While the reductase does not appear to be involved in cyclic photophosphorylation during electron flow to ferredoxin/O_2, these data do not rule out its participation in the cyclic pathway during electron transport to ferredoxin/NADP, since if both the cyclic and no-cyclic (NADP reduction) pathways are inhibited to the same extent by N-ethylmaleimide or p-chloromercuribenzene sulfonate, the P/O value would not change. Indeed, Table IV shows no significant difference between the P/O values with ferredoxin/NADP or ferredoxin/O_2 in reductase-inhibited thylakoids. However, if membrane-bound reductase is involved as an electron carrier in cyclic photophosphorylation during electron transport to ferredoxin/NADP, the addition of excess exogenous ferredoxin-NADP reductase would be expected to lower the P/O ratio toward 1.25, since soluble reductase is able to catalyze NADP reduction, but it is unlikely to be able to communicate with the membrane-bound carriers of the cycle. In fact, the addition of purified reductase at a ratio of 40 per P-700 was found to have this effect (data not shown).

In contrast to the irreversible inhibition of ferredoxin-NADP reductase described above, we have

found that the polysaccharide heparin reversibly inhibits NADP photoreduction, principally by inhibiting ferredoxin binding to the reductase [34]. The data of Table V show that heparin had no effect on the P/O value of non-cyclic photophosphorylation (water to methyl viologen), indicating that heparin is neither an uncoupler nor an energy-transfer inhibitor. In the presence of 10 μM heparin, the P/O value generated by electron transport to ferredoxin/O_2 dropped from 1.54 to 1.27, a result which is identical to that observed using 1.7 μM antimycin A. The simultaneous addition of heparin and antimycin A produced no further effect (Table V). These results show that heparin, like antimycin A, selectively inhibits cyclic photophosphorylation during electron flow to ferredoxin/O_2. As a redox-inactive ionic analog of ferredoxin it is likely that heparin produced this effect by disturbing the association between reduced ferredoxin and its site of electron donation to the cyclic reaction, perhaps by binding at or near a ferredoxin binding site. The same concentration of heparin (10 μM) had little effect on the P/O value of electron flow to ferredoxin/NADP (Table V), indicating that in this case cyclic photophosphorylation was not selectively inhibited. The interpretation of the decrease of the P/O value of this reaction using 100 μM heparin is complicated, since the inhibition of ferredoxin-NADP reductase by heparin induced a substantial amount of ferredoxin-catalyzed O_2 uptake (data

not shown). Therefore some of the decrease in the P/O value is due to the inhibition of cyclic photophosphorylation which was linked to electron transfer to ferredoxin/O_2.

Discussion

Addition of ferredoxin to chloroplast thylakoid membranes reconstitutes electron-transfer reactions which photophosphorylate with elevated P/O ratios (1.6 vs. 1.25 with methyl viologen as the acceptor). This increased P/O ratio is due to the ability of ferredoxin to catalyze concurrent cyclic and non-cyclic electron transfer. The additional ferredoxin-dependent cyclic or Q-loop reaction must raise the P/O value by increasing the number of protons pumped into the thylakoid lumen. The most probable H^+/ATP ratio for thylakoid membranes is 3 [35] and non-cyclic electron transport operates with an H^+/2e value of 4 [36] which predicts a P/2e or P/O value of 1.33, very close to the measured value of 1.25. By these stoichiometries ferredoxin mediated electron transport must produce a total H^+/2e of 5, and therefore the cycle or Q-loop must pump 1 additional H^+ for every electron pair used to ultimately reduce O_2 or NADP. Using 1.25 as the measured P/O value for 'pure' non-cyclic electron transfer, we estimate that the rates of ATP synthesis by the cycle or Q-loop reactions account for 16–21% of the total observed rate of ATP synthesis during electron flow to ferredoxin/O_2 or ferredoxin/NADP.

As we show here, only the ferredoxin/O_2 reaction generates ATP synthesis which is sensitive to inhibition by antimycin A (Fig. 3). In the presence of NADP, higher rates of ATP synthesis and electron transfer are observed, but the P/O ratio of 1.6 is insensitive to antimycin A (Fig. 3). Our results are similar in this regard to those of Furbank and Badger [37], although we do not observe the inhibitory effects of antimycin A on both ATP synthesis and electron transport that they report. Our attempts to induce antimycin-A-sensitive ATP synthesis during electron transfer to NADP have been unsuccessful (Table III), and we conclude that, at least for reconstituted systems with ferredoxin/NADP, antimycin A is not a potent inhibitor of the cyclic reaction. The finding that antimycin-A-sensitive ATP synthesis correlates

TABLE V

EFFECT OF HEPARIN ON PHOTOPHOSPHORYLATION WITH METHYL VIOLOGEN, FERREDOXIN/O_2, AND FERREDOXIN/NADP AS TERMINAL ACCEPTORS

Substrates were used in the following concentrations: methyl viologen, 67 μM; ferredoxin alone, 21 μM; ferredoxin with NADP, 10 μM; NADP 1 mM, antimycin A, 1.7 μM.

Additions	P/O		
	Methyl viologen	Ferredoxin/O_2	Ferredoxin/NADP
None	1.25	1.54	1.60
10 μM heparin	1.26	1.27	1.55
100 μM heparin	1.21	1.25	1.34
Antimycin A	1.24	1.28	1.60
100 μM heparin + antimycin A	–	1.23	1.27

with reducing conditions in the ferredoxin pool and the interphotosystem electron carriers can be compared to an earlier report showing that antimycin A is a more potent inhibitor of cyclic photophosphorylation in intact chloroplasts when high concentrations of ascorbate are added [38]. It seems unlikely that reducing conditions affect the actual binding of antimycin A, based upon our results (Table III) and the fact that tight binding of the inhibitor has been demonstrated in thylakoid membranes in the dark and in the absence of added reductants [39].

We view our results as suggestive evidence for the existence of two separate cyclic electron-transport pathways which can be catalyzed by reduced ferredoxin during concurrent non-cyclic electron flow. Antimycin-A-sensitive cyclic activity, presumably electron transfer through cytochrome b-563 (see Ref. 40 for a review), is reconstituted under conditions where the ferredoxin pool and P-700 are substantially reduced. An antimycin-A-insensitive reaction is catalyzed in the presence of NADP, where the pools of added ferredoxin and of P-700 are largely oxidized in the steady state. Furthermore, the results of the experiment using a low concentration of heparin (Table V) suggest a different binding site for ferredoxin for each of the cyclic pathways.

While ferredoxin-NADP reductase does not appear to be involved in the antimycin-A-sensitive cyclic pathway, it may be involved in the alternative pathway which is consistently associated with the turnover of the membrane-bound reductase. Of particular interest in this regard is that cyclic photophosphorylation in bundle-sheath cells of C_4 plants, which seems to be totally dependent upon NADPH as the source of electrons [41], is largely insensitive to antimycin A [42].

Brief mention of the possible pathways of cyclic electron transfer and their regulation is in order, since our results show that the switching of electrons into one cycle or the other must depend the redox balance of the ferredoxin pool and the interphotosystem electron carriers. The antimycin-A-sensitive pathway is consistent with current working models of electron flow through b_6-f and b-c_1 complexes, in which cytochromes b mediate transmembrane electron transfer [16,21,44–46]. During electron flow to ferredoxin/O_2, at least two fac-

tors may favor this pathway. First, if plastosemiquinone is the oxidant for reduced cytochrome b-563 toward the outside of the membrane [16,17,47] the accumulation of reduced ferredoxin in the medium would favor the initial reduction of oxidized plastoquinone. Second, the reducing conditions present in the interphotosystem chain should promote the reduction of b-563 by plastosemiquinone [15,46,48]. In the oxidizing conditions created by the addition of NADP the converse of both of these arguments is true, and we suggest an alternative pathway in which transmembrane electron transfer through b-563 is largely or completely bypassed; thus, the cycle loses sensitivity to antimycin A. Under these conditions ferredoxin may reduce plastoquinone through the activity of ferredoxin-NADP reductase (possibly via the low-potential flavin semiquinone, whose concentration may be as high as 50% of the total reductase flavin during steady-state turnover to NADP [43]). Reduced plastoquinone would then be oxidized at a site toward the inside of the membrane, presumably the Q_0 site proposed in Q-loop models [45,46], but with plastosemiquinone re-reducing the Rieseke center/cytochrome f region rather than cytochrome b-563 (see Ref. 46). It is noteworthy that the slow phase of the 518 electrochromic shift, argued to be an indicator of both antimycin-A-sensitive cyclic electron flow [39,49–52] and transmembrane electron flow involving b-563 [44,52], is not seen in the presence of ferredoxin plus NADP in isolated thylakoid membranes [51]. This result is consistent with our proposals regarding electron flow in the presence of NADP.

The results presented here also allow some conclusions about the redox poising of cycling photophosphorylation by non-cyclic electron flow. First, it is obvious that significant rates of cyclic photophosphorylation (up to 110 μmol ATP/h per mg Chl) accompany non-cyclic photophosphorylation catalyzed by high intensity light during electron transport to ferredoxin/O_2 or ferredoxin/NADP. It is also clear that oxygen alone is sufficient to poise one pathway of cyclic photophosphorylation under conditions where the cycle might be expected to be 'over-reduced', i.e., with maximum electron flow from PS II and no electron flow to NADP. The addition of NADP changes the net oxidation-reduction state of the interphotosystem

electron carriers, by bringing the carriers following plastoquinone into a more oxidized state. These conditions induce a different cyclic pathway which supports a greater rate of ATP synthesis. The existence of two cyclic pathways with different redox requirements in vivo would allow chloroplasts to maintain high rates of cyclic photophosphorylation concurrent with non-cyclic photophosphorylation under a wide range of NADPH/NADP ratios, and hence maintain a consistently high ratio of ATP to NADPH.

Acknowledgements

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