Photosystem II - Mediated Cyclic Photophosphorylation

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SUMMARY: DCMU-sensitive synthesis of ATP can be shown to continue in KCN-treated chloroplasts after cessation of O_2 evolution. The catalyst for this reaction, p-phenylenediamine, also stimulates synthesis of ATP in NH_2OH-treated chloroplasts, but at much higher rates. This ATP synthesis can be observed in the presence of the quinone antagonist dibromothymoquinone, and under the appropriate conditions it is completely sensitive to DCMU. Since neither uptake nor evolution of O_2 can be observed during illumination, these results are interpreted as evidence for catalysis of cyclic photophosphorylation by photosystem II.

INTRODUCTION. The p-phenylenediamines are versatile mediators of electron flow in isolated chloroplasts. Under conditions where electron transport to photosystem I is interrupted by KCN (1) or by the quinone antagonist dibromothymoquinone (DBMIB)[1] (2), oxidized p-phenylenediamines can be shown to catalyze a DCMU-sensitive oxygen evolution which requires only photosystem II activity. This reaction is coupled to ATP synthesis, yielding approximately half the ATP (per electron pair) obtained when noncyclic electron transport from water to either ferricyanide or methyl viologen is assayed (3). In the presence of DCMU, ascorbate, and methyl viologen, the p-phenylenediamines also catalyze non-cyclic oxygen uptake and phosphorylation associated with photosystem I (4), while in the absence of ascorbate and methyl viologen, they catalyze a photosystem I (5) cyclic photophosphorylation reaction.

In the course of experiments to measure the stoichiometry of electron flow and ATP synthesis using KCN-treated chloroplasts and p-phenylenediamine plus ferricyanide as the electron acceptor system, it was noted that synthesis of ATP continued after cessation of O_2 evolution. Further exploration of

[1] Abbreviations: DBMIB, dibromothymoquinone; PD, p-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

this observation has provided evidence, described in this communication, for photophosphorylation mediated by photosystem II cyclic electron flow.

MATERIALS AND METHODS. Isolated chloroplasts were prepared by homogenizing 100 gm of spinach leaves in 250 ml of cold Tricine buffer (20 mM, pH 8.0) containing sucrose (0.4 M), NaCl (15 mM), and bovine serum albumin (2 mg/ml) for 15 sec. The resulting slurry was passed through four layers of cheesecloth and centrifuged (1 min) at 1,000xg. The supernatant from this step was centrifuged (10 min) at 5,000xg, the pellet was resuspended in the homogenizing medium, and the centrifugation was repeated. The washed pellet was then resuspended in homogenizing medium, and the chlorophyll concentration was determined (6). These chloroplasts were used for either KCN- or NH_2OH-inhibition treatments (1, 7) and then stored in homogenizing medium at -70°C until assay. p-Phenylenediamine (PD) was obtained from Eastman and recrystallized after charcoal treatment to remove impurities. The white crystals were dissolved in water to produce a 20 mM stock solution. DBMIB was a kind gift of Dr. N.E. Good. A 2mM stock solution was prepared in ethanol for use in assays. ADP was obtained from Sigma, and all other chemicals were of the purest grade available.

When oxygen evolution or uptake was monitored, assays were done in a thermostatted cuvette (25°C) fitted with a Clark electrode (YSI). The reaction mixture (1.6 ml) contained 20-40 ug chlorophyll, Tricine (50mM, pH 8.0), NaCl (60 mM), ADP (1.0 mM), $MgCl_2$ (3.0 mM), PD and ferricyanide at varying concentrations, and inorganic phosphate (5.0 mM) plus ^{32}P (10^6 CPM). When cyclic photophosphorylation was measured the same reaction mixture was placed in 5 ml test tubes for illumination. Saturating white light (10^6 ergs $cm^{-2}sec^{-1}$) was provided either by a microscope lamp or by photoflood lamps. Reactions were terminated after illumination by addition of 0.2 ml of 30% TCA, and ATP synthesis was determined by gas flow counting after extraction of unreacted phosphate by Avron's procedure (8).

RESULTS. Figure 1 shows the time course of O_2 evolution and ATP synthesis in KCN-treated chloroplasts in the presence of PD (250 μM) plus ferricyanide (250 μM). Under these conditions of assay, O_2 evolution stops after approximately 1 min of illumination, but ATP synthesis continues for up to 5 min. Since KCN-treated chloroplasts are unable to transfer electrons beyond cytochrome f (9), the results in Figure 1 were interpreted to indicate catalysis of cyclic photophosphorylation by photosystem II. When ferricyanide was omitted from the reaction mixture, O_2 evolution was no longer observed, but low rates of ATP synthesis (9-12 μmoles/hr/mg chlorophyll) were still obtained and this reaction was completely inhibited in the presence of 10 μM DCMU. These results showed that PD was the catalyst of electron flow rather than ferricyanide, and strongly suggested that ATP synthesis in this assay system was arising from a photosystem II cyclic reaction.

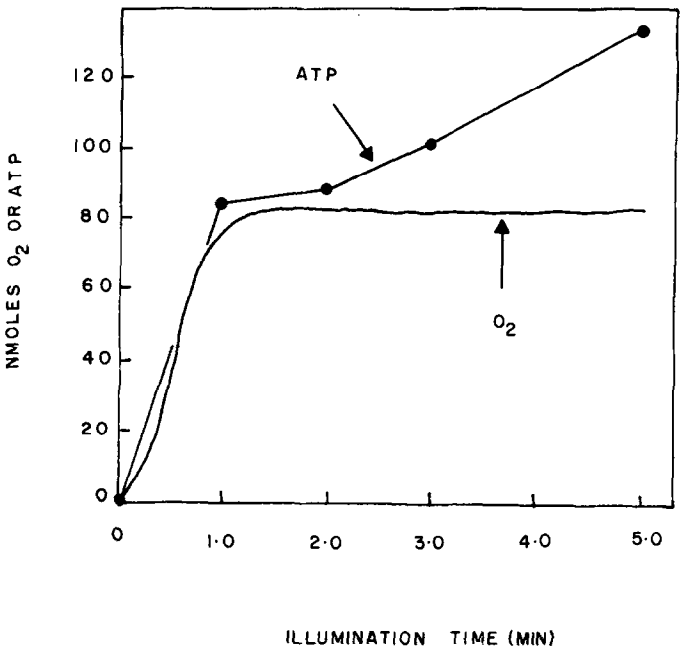

Figure 1. ATP Synthesis and O_2 Evolution Catalyzed by KCN-Treated Chloroplasts in the Presence of PD (250 μM) plus Ferricyanide (250 μM).

Attempts to improve the yield of ATP in KCN-treated chloroplasts by increasing the concentration of PD were without success, implying that some process in photosystem II was limiting the rate of cyclic electron transport or photophosphorylation. In order to see whether water photooxidation was limiting the cycle, photophosphorylation rates were determined using NH_2OH-treated chloroplasts which cannot photooxidize water, and the results were compared to those obtained with KCN-treated chloroplasts. Representative data from these experiments are shown in Table I. It is clear from these results that deletion of O_2 evolution produced more efficient photophosphorylation when PD was the catalyst, but at the same time this reaction was not completely blocked by addition of DCMU, which indicated that under the conditions shown in Table I, photosystem I was contributing to electron flow and phosphorylation.

Since NH_2OH-treated chloroplasts gave higher rates of ATP synthesis

Table I
ATP Synthesis in KCN- and NH_2OH-Treated Chloroplasts*

Chloroplast Preparation	Addition	ATP Synthesis (μmoles/hr/mg chlorophyll)
KCN-Treated	None	0
" "	PD (250 μM)	9
" "	PD (250 μM) + DCMU (10 μM)	0
NH_2OH-Treated	None	2
" "	PD (250 μM)	156
" "	PD (250 μM) + DCMU (10 μM)	16

*Conditions of assay are described in "Materials and Methods." The illumination time was 1 min.

with PD than did KCN-treated preparations, it was of interest to see whether the photosystem I component of photophosphorylation could be eliminated from the NH_2OH-treated preparations to produce photosystem II cyclic activity. When the concentration of PD was decreased in the assay system, ATP synthesis became progressively more sensitive to DCMU, but a complete inhibition of activity could not be achieved. If, however, DBMIB (10 μM) was added to assays along with concentrations of PD not exceeding 65 μM, conditions were created whereby ATP synthesis was completely sensitive to DCMU. Table II presents the results of these experiments along with data showing the rates of ATP synthesis both in the absence of and in the presence of high concentrations of PD. These results show that DBMIB alone does not appreciably stimulate ATP synthesis. The combination of 10 μM DBMIB and 62.5 μM PD, however, produced a photophosphorylation reaction which was completely inhibited by DCMU. A higher concentration of PD (1300 μM), on the other hand, produced a rate of ATP synthesis which could not be completely inhibited by either DBMIB or DCMU alone, or when added together. This activity must therefore involve a substantial contribution from photosystem I.

It has been shown that DBMIB is oxidized by oxygen (10), and it is thus possible that the activities shown in Table II were a product of non-cyclic electron flow to O_2 via DBMIB. This consideration was eliminated by experi-

Table II

Effect of DBMIB and DCMU on PD-Catalyzed ATP Synthesis in NH_2OH-Treated Chloroplasts*

PD Concentration (μM)	Additions	ATP Synthesis (μmoles/hr/mg chlorophyll)
0	None	2
"	DBMIB (10 μM)	1
"	DCMU (10 μM)	0
"	DCMU (10 μM) + DBMIB (10 μM)	0
62.5	None	106
"	DBMIB (10 μM)	25
"	DCMU (10 μM)	7
"	DCMU (10 μM) + DBMIB (10 μM)	0
1300	None	196
"	DBMIB (10 μM)	108
"	DCMU (10 μM)	68
"	DCMU (10 μM) + DBMIB (10 μM)	59

* Assay conditions are given in "Materials and Methods." Illumination time was 1 min.

ments with an oxygen electrode; under none of the conditions in Table II was oxygen uptake observed. This finding showed that the appropriate combination of PD (62.5 μM) and DBMIB (10 μM) created a condition under which photosystem II cyclic photophosphorylation occurs, by virtue of the fact that electron transfer between photosystem II and photosystem I is blocked by the DBMIB, and the photophosphorylation observed under these conditions is completely sensitive to DCMU.

If the ATP synthesis seen in NH_2OH-treated chloroplasts under the conditions just described was in fact due to a cyclic process, this reaction should be inhibited by conditions which would interrupt cyclic electron flow. Since PD was added in reduced form, addition of ferricyanide, which oxidizes PD, should affect ATP synthesis. The ferrocyanide formed by PD oxidation might also affect the cycle, since ferrocyanide has been shown to donate electrons to photosystem II in a reaction which does not support phosphorylation (11). Table III shows the effect of ferri-and ferrocyanide on PD-catalyzed photophosphorylation with NH_2OH-treated chloroplasts in the presence and absence of DBMIB. Ferricyanide inhibits ATP synthesis 77% in the absence of DBMIB and

Table III
Effect of Ferri- and Ferrocyanide on PD-Catalyzed Photophosphorylation*

Additions	ATP Synthesis (μmoles/hr/mg chlorophyll)
None	100
Ferricyanide (125 μM)	23
Ferrocyanide (125 μM)	96
DBMIB (10 μM)	23
DBMIB (10 μM) + Ferricyanide (125 μM)	0
DBMIB (10 μM) + Ferrocyanide (125 μM)	8

*The concentration of p-PD was 62.5 μM throughout. Illumination time was 1 min.

100% in the presence of DBMIB. Ferrocyanide at equivalent concentrations has little effect in the absence of DBMIB. In the presence of DBMIB, however, a 65% inhibition of ATP synthesis is observed.

The nature of this inhibition was further explored with an O_2 electrode. In the presence of DBMIB, slow O_2 uptake was observed (5 μmoles/hr/mg chlorophyll) under the conditions shown in Table III when either ferri- or ferrocyanide was added. It is therefore likely that the strong inhibitions of ATP synthesis seen in the presence of DBMIB when ferri- or ferrocyanide was present is due to oxidation of PD (by ferricyanide) as well as to photo-oxidation of ferrocyanide by photosystem II with transfer of electrons to O_2 via DBMIB. This inhibition provides further evidence for the contention that the ATP synthesis obtained in the presence of DBMIB and PD is due to photosystem II cyclic photophosphorylation.

DISCUSSION. The results in this communication provide evidence for a new pathway of ATP synthesis in isolated chloroplasts, namely photosystem II cyclic photophosphorylation. This cycle requires an artificial catalyst (PD) along with inhibitors (KCN, DBMIB) to block electron transport between the two photosystems and the ATP yield of the cycle is increased more than twofold by destruction of O_2 evolution. A preliminary report has appeared, suggesting another PSII cycle which requires catechol and DBMIB (12). It is

thus apparent that under the appropriate conditions, ATP-yielding reactions via cyclic electron flow are not unique to photosystem I. It is also apparent, from the conditions necessary to elicit the photosystem II cycle, that this reaction probably does not represent a naturally occurring path of ATP synthesis such as that seen with ferredoxin in photosystem I.

The experiments reported here cannot give a true assessment of the efficiency of ATP synthesis catalyzed by the cycle since concentrations of PD in excess of 65 μM produce a reaction which is not completely inhibited by DBMIB plus DCMU. The requirement for DBMIB in NH_2OH-treated chloroplasts in fact presents an added complication. Since this compound is capable of oxidation-reduction (10), it is quite likely that it is an active participant in the cycle. It is probable, however, that oxygen is not reacting in the cycle. All attempts to demonstrate O_2 uptake under conditions favoring ATP synthesis were unsuccessful. Only in the presence of 125 μM ferrocyanide plus 10 μM DBMIB was any O_2 uptake observed, and under these conditions ATP synthesis was more than 60% inhibited.

The discovery of conditions for the assay of photosystem II cyclic photophosphorylation is a consequence of the finding that there is a phosphorylation site associated with photosystem II. The fact that the catalyst for the cyclic reaction, PD, liberates protons as well as electrons upon oxidation supports the proposal set forth by Izawa and Ort (11) that a proton generating system, either water or an artificial donor, is required for coupling of ATP synthesis to photosystem II electron transport. The photosystem II cyclic reaction provides a convenient means for further studies on ATP synthesis associated with this light reaction in the absence of water oxidation. Experiments are currently under way to prepare chloroplasts treated with both NH_2OH and KCN so that the photosystem II cycle may be assayed without interference from photosystem I-mediated reactions.

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