Photophosphorylation Associated with Photosystem II

I. PHOTOSYSTEM II CYCLIC PHOTOPHOSPHORYLATION CATALYZED BY p-PHENYLENEDIAMINE

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ABSTRACT

Incubation of spinach chloroplast membranes for 90 minutes in the presence of 50 mM KCN and 100 μM HgCl₂ produces an inhibition of photosystem I activity which is stable to washing and to storage of the chloroplasts at −70°C. Subsequent exposure of these preparations to NH₄OH and ethylenediaminetetraacetic acid destroys O₂ evolution and flow of electrons from water to oxidized p-phenylenediamine, but two types of phosphorylating cyclic electron flow can still be observed. In the presence of 3-(3,4-dichlorophenyl)-1,1'-dimethylurea, phenazinemethosulfate catalyzes ATP synthesis at a rate 60% that observed in uninhibited chloroplasts. C-Substituted p-phenylenediamines will also support low rates of photosystem I-catalyzed cyclic photophosphorylation, but p-phenylenediamine is completely inactive. When photosystem II is not inhibited, p-phenylenediamine will catalyze ATP synthesis at rates up to 90 μmol/hr·mg chlorophyll. This reaction is unaffected by anaerobiosis, and an action spectrum for ATP synthesis shows a peak at 640 nm. These results are interpreted as evidence for the existence of photosystem II-dependent cyclic photophosphorylation in these chloroplast preparations.

A large body of experimental evidence supports the hypothesis that the noncyclic pathway of electron transport in chloroplasts contains two sites of photophosphorylation. Noncyclic electron transport from water to photosystem I acceptors such as ferricyanide is inhibited by KCN, which inactivates plastocyanin (15), or by the quinone antagonist DBMIB (10, 17). Electron transport in inhibited chloroplasts is restored by addition of p-phenylenediamines or p-benzoquinones, and the accompanying phosphorylation has a P/6₂ approaching 0.5 (15, 17). This partial reaction, involving photosystem II, is termed site II. When photosystem II activity is blocked by addition of DCMU, proton/electron donors such as DAD will catalyze photosystem I (site I)-dependent electron transport to an acceptor such as MV. The P/6₂ of this reaction is about 0.5 when assays are carried out in the presence of superoxide dismutase to correct for apparent O₂ uptake due to the formation of superoxide ion (4, 14). Summation of the efficiencies (P/6₂) at sites I and II obtained by assay of site-specific partial reactions produces a value near 1.0, which is the value observed for noncyclic electron transport from water to ferricyanide, MV, or ferredoxin/NADP in uninhibited chloroplast preparations (16).

In addition to noncyclic pathways of energy-conserving electron flow, cyclic electron transport involving photosystem I will support ATP synthesis. In the presence of DCMU, p-phenylenediamines, PMS, or ferredoxin catalyze cyclic photophosphorylation (6). Recent experiments in several laboratories have provided evidence that ATP synthesis may also occur by means of photosystem II cyclic reactions. Izawa (8) reported that addition of catechol to chloroplasts inhibited by DBMIB and EDTA plus NH₄OH produced a low rate of ATP synthesis which was ascribed to a photosystem II cyclic reaction. Ben-Hayyim et al. (2) investigated the properties of diaminobenzidine as a photosystem II donor in tris-washed chloroplasts, and found that it supported ATP synthesis which was partially insensitive to KCN inhibition of photosystem I activity. Although the DCMU sensitivity of this reaction was not reported, the data of Ben-Hayyim et al. (2) suggest the occurrence of a photosystem II cyclic reaction. Yocum (19) has shown that PD restored ATP synthesis in chloroplasts inhibited with DBMIB and NH₄OH plus EDTA. At low concentrations of PD (<65 μM), the reaction was completely sensitive to DCMU, while at higher catalyst concentrations, DCMU sensitivity was partially lost. These results were interpreted to indicate the existence of an artificial cyclic pathway of ATP synthesis involving only photosystem II at low PD concentrations.

Previous experiments on photosystem II cyclic reactions have relied on DBMIB, which may participate in these reactions, to block activity between the photosystems. In addition, the DCMU sensitivity of reactions in tris-washed, KCN-inhibited chloroplasts has not been reported. We have, therefore, continued our investigations into cyclic phosphorylating electron transport reactions involving photosystem II with a view toward establishing the mechanism of this reaction, and assessing its utility in examining the over-all mechanism of photosynthetic energy transduction. In this communication, we report the methods for preparation of chloroplasts strongly and irreversibly inhibited at or near plastocyanin, which have been further inhibited by deletion of O₂ evolution. In spite of these drastic conditions of inhibition, such chloroplast preparations retain cyclic photophosphorylation activity with PMS and p-phenylenediamines, and under the appropriate conditions catalyze ATP synthesis which appears to be due entirely to a photosystem II cyclic reaction.

MATERIALS AND METHODS

Broken chloroplasts were isolated from market spinach by grinding depetiolated leaves in STN according to the procedure of Guikema and Yocum (5). Chlorophyll concentrations were determined by Arnon's (1) procedure. Electron transport and photophosphorylation assays were carried out in a 1.6-ml reac-
tion mixture containing 50 mM Tricine (pH 8), 50 mM NaCl, 3 mM MgCl₂, 1 mM ADP, 5 mM NaH₂PO₄ (10⁶ cpn/ml) and 20 to 30 μg Chl. Noncyclic activities were assayed in a thermostat-
ted cuvette fitted with an O₂ electrode. White light (5 x 10⁶ ergs
per cm²·sec⁻¹) was provided by a microscope lamp. The illumination
time was 1 min unless otherwise noted. When electron transport
from water to ferricyanide was assayed, the concentration of
ferricyanide was 2.4 mM; PD (0.25 mM) was also present when
site I alone was assayed. When site I activity was assayed, Asc
(3.0 mM), DAD (0.25 mM), MV (0.125 mM) and DCMU (9 µM)
were added to the reaction mixture. Correction for supra-
oxidation of ion transport during photoinduced electron transfer in
site I assays was done by computing the ratio of electron trans-
port activities in the presence and absence of 200 µg/ml of
bovine erythrocyte supernoxide dismutase. The average ratio
(+dismutase/-dismutase) determined from several experiments
was 0.69, and agreed with results obtained in other laboratories
(S. Izawa, personal communication). When site I activity was
assayed in the absence of supernoxide dismutase, the rate of
electron transport obtained in such experiments was multiplied
by 0.69.

Cyclic ATP synthesis was assayed in test tubes placed in a
thermostatted (25°C) water bath illuminated with white light
(10⁶ ergs cm⁻²·sec⁻¹) from photoflood lamps. The illumination
time, unless otherwise indicated, was 1 min. The reaction mix-
ture was the same as that described above for noncyclic activity
and the concentrations of electron carriers used in these assays
are noted in the tables and figures under “Results.” All reactions
(cyclic and noncyclic) were terminated by addition of 0.2 ml of
30% trichloroacetic acid. The amount of light-induced ATP
synthesis was determined by gas flow counting after extraction
of unreacted phosphate as described earlier (19).

The effect of anerobiosis on photosystem II cyclic phos-
phorylation was examined by purging cyclic reaction mix-
tures with either air or O₂-free helium for 1 or 5 min; at the end
of these time periods, the reaction tubes were quickly stopped
and subjected to illumination. For the measurement of action
spectra, the intensity of light from an Oriel model 6325 light
source was adjusted to 4,000 ergs cm⁻²·sec⁻¹ through each of a
series of Oriel and Balzers interference filters (575, 600, 640,
700, and 720 nm, all with half-bandwidths of 10 nm). Light
intensity was varied with a rheostat, and measured by means of
a YSI model 65 radiometer.

All chemicals used in these studies were of the purest grades
commercially available. Crystalline bovine supernoxide dismutase
was a gift of J. Fee. Tricine, HEPES, ADP, PD, and PMS were
obtained from Sigma. NaH₂PO₄ was purchased from New
England Nuclear. DAD was obtained from Research Organics/
Inorganics, and DAT was purchased from Eastman Organic
Chemicals. All of the p-phenylenediamines were recrystallized
according to the procedure of Gould (4), and stock solutions
(20 mM) in water were prepared immediately prior to assay.

RESULTS

Inhibition of Photosystem I Activity with KCN and Hg. The
mechanism by which KCN inhibits photosystem I is known to
involve inactivation of plastocyanin (15). We discovered that
centrifugation and resuspension of KCN-inhibited chloroplasts
in STN containing 0.2% BSA caused a partial restoration of
photosystem I activity. An attempt was made to modify the KCN
inhibition procedure to prevent the subsequent restoration of
activity by either washing of inhibited chloroplasts or cold storage
at −70°C for long periods of time. It has been reported that
HgCl₂ is a potent inhibitor of photosystem I activity (12), and
that this inhibition is caused by inactivation of plastocyanin. A
series of preliminary experiments were done to see whether
HgCl₂ could be combined with KCN to produce an irreversible
inhibition of photosystem I activity. Since Hg is known to dam-
age photophosphorylation, it was necessary to determine the
minimum concentration of HgCl₂ which would, in combination
with KCN, produce the desired inhibition. A concentration of
HgCl₂ (1.0 mol/3 mol Chl) which, by itself was incapable of
producing a strong inactivation of photosystem I (12), would,
when included in an incubation mixture with the appropriate
concentration of KCN, lead to an irreversible inhibition of pho-
otosystem I activity.

Based on these findings, a modified photosystem I inhibition
protocol was devised which consisted of incubating chloroplasts
(300 µg Chl/ml) for 90 min at 4°C in 0.1 M sucrose, 50 mM
HEPES (pH 8), 15 mM NaCl, 50 µM ferricyanide, 0.2% BSA,
and 50 mM KCN (pH 8) plus or minus 100 µg HgCl₂. Incuba-
tion was terminated by centrifugation of the suspensions at
5,000g and resuspension of the pellets in STN plus 0.2% BSA
to a final Chl concentration of 2 to 3 mg/ml. These suspensions
were assayed for activity and then frozen in 0.5-ml aliquots at
−70°C for assay at a later time (48 hr). The conditions of
incubation reported here led to maximal photosystem I inhibi-
tion in 60 to 90 min, and cold storage of the resuspended
material for periods up to six weeks did not affect the extent of
inhibition.

The results of a typical series of incubation experiments with
KCN alone and in the presence of HgCl₂ are presented in Table I. Incubation with KCN in the absence and presence of HgCl₂
inhibits activities which require either ferricyanide (sites II and I)
or Asc, DAD and MV (site I) for electron transport activity. The
extent of these inhibitions is on the order of 90%. Assays of site
II (H₂O → PDₒ) show substantial retention of activity in agree-
ment with the results of other investigators (15). Examination
of Table I shows that when the incubation mixture was centrifuged,
resuspended, and reassayed, the preparation incubated in KCN
alone regained some site I (Asc, DAD → MV) electron trans-
port activity. The activity of the preparation incubated in the
presence of HgCl₂ did not show a comparable increase. Data are
also included in this table which indicate that both preparations
retained site I inhibition and site II activity after freezing and
thawing. Although the data in Table I do not exclude the possi-
bility that HgCl₂ has damaged the energy-conserving apparatus
in these chloroplasts, the rates of electron transport and associ-
ated photophosphorylation observed in the presence of HgCl₂

<table>
<thead>
<tr>
<th>Table I</th>
<th>Inhibition of Photosystem I by KCN Alone or in Combination with HgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation Conditions</td>
<td>Activity</td>
</tr>
<tr>
<td>H₂O → Fe(CN)₆⁻³</td>
<td>H₂O → Pₒ</td>
</tr>
<tr>
<td>E₄ '.', I ATP² P₆/₂</td>
<td>E₄ '.', I ATP² P₆/₂</td>
</tr>
<tr>
<td>KCN, Hg</td>
<td>600 292 0.97 1000 248 0.50 1446 332 0.46</td>
</tr>
<tr>
<td>+KCN, Hg</td>
<td>40 20 1.00 820 156 0.38 155 40 0.51</td>
</tr>
<tr>
<td>+KCN, Hg</td>
<td>50 18 0.72 921 164 0.36 106 35 0.66</td>
</tr>
</tbody>
</table>

Centrifugation,
Resuspension of:

| KCN, Hg | 580 295 1.02 960 235 0.49 1530 325 0.43 |
| +KCN, Hg | 30 18 1.20 788 155 0.39 337 59 0.35 |
| +KCN, Hg | 46 16 0.70 740 130 0.35 157 26 0.33 |

48 hr Cold
Storage of:

| KCN, Hg | 620 300 1.03 985 240 0.51 1420 345 0.49 |
| +KCN, Hg | 42 20 0.95 920 182 0.40 333 52 0.31 |
| +KCN, Hg | 36 12 0.67 932 201 0.43 113 24 0.43 |

1 µequiv. transferred/hr·mg Chl
2 µmol synthesized/hr·mg Chl
3 Corrected for superoxide ion formation
show little deviation from the results obtained with KCN by itself. Of more importance is the observation that inclusion of HgCl₂ during incubation has produced an inhibition of photosystem I electron transport activity which is not readily reversed by centrifugation and resuspension.

**Inhibition of Oxygen Evolution in KCN-Hg-treated Chloroplasts.** Previous investigations (19) showed that elimination of O₂ evolution increased the ATP yield of a photosystem II cyclic reaction catalyzed by PD. It was therefore necessary to inhibit O₂ evolution in KCN-Hg-treated chloroplasts. Chloroplasts were first inhibited with KCN and HgCl₂ as described above, and centrifuged and resuspended (1-2 mg Chl/ml) in a medium consisting of STN, 0.2% BSA, and 2 mM MgCl₂. This suspension was then exposed at 4°C to 5 mM NH₂OH and 1 mM EDTA according to the procedure of Ort and Izawa (13). Maximal inhibition of O₂ evolution was obtained within 20 min, after which the suspension was centrifuged and resuspended in STN containing 0.2% BSA and frozen at -70°C in 0.5-ml aliquots. The results of a representative experiment (Table II) clearly show that this procedure did not appreciably reverse the photosystem I inhibition of electron transport caused by prior exposure to KCN and Hg. Data presented in Table II also show that KCN-Hg-NH₂OH-inhibited chloroplasts retain inhibitions of electron transport after cold storage at -70°C.

**ATP Synthesis by KCN-Hg-NH₂OH-inhibited Chloroplasts.** Ouitrakul and Izawa (15) showed that PMS concentrations between 100 and 200 μM could partially bypass the site of KCN inhibition in photosystem I, while concentrations of DAD as high as 0.6 mM could not. Similar experiments were carried out with KCN-Hg-NH₂OH-inhibited chloroplasts and uninhibited preparations in the presence of 9 μM DCMU. The results of a typical set of experiments are presented in Table III. While about 60% of the photophosphorylation activity catalyzed by PMS is retained in inhibited chloroplasts, the catalytic activities of DAD and DAT, even at high concentrations, are more than 95% inhibited. p-Phenylenediamine, which in untreated chloroplasts produces a rate of photophosphorylation 10% that of the corresponding PMS rate, is completely incapable of bypassing the site of KCN-Hg inhibition. These results demonstrate that the KCN-Hg inhibition of photosystem I is similar to the inhibition obtained with KCN alone, and the results with PMS show that a considerable capacity for ATP synthesis is retained by our inhibited preparations, in spite of the drastic conditions used to eliminate noncyclic electron transport activity.

The effect of DCMU on PD-catalyzed photophosphorylation in KCN-Hg-NH₂OH-inhibited chloroplasts was next examined. The results of an experiment in which the concentration of PD was varied in the absence and presence of 9 μM DCMU are shown in Figure 1. When the reducing side of photosystem II is inhibited by DCMU, ATP synthesis is inhibited at all PD concentrations shown. In the absence of DCMU, however, ATP synthesis increases sharply when the PD concentration is raised from 0 to 100 μM. Note that in the absence of DCMU and PD, there is residual activity amounting to approximately 7 to 10 μmol ATP synthesized/hr-mg Chl. The effect of increasing concentrations of PD on phosphorylation activity is complex. Levels of PD in excess of 100 μM continue to stimulate activity, but the increase in phosphorylation is much less dramatic than at PD concentrations below 100 μM. Even at a PD concentration of 1250 μM, the reaction has not reached saturation. The biphasic nature of the rate-concentration curve shown in Figure 1 suggests that a complex mechanism is involved in the PD-catalyzed reaction.

The sensitivity of the reaction shown in Figure 1 to DCMU demonstrates that this activity requires electron carriers which function on the reducing side of photosystem II. It was of interest to see whether conditions affecting the oxidizing side of photosystem II would affect PD-catalyzed activity. A series of experiments were therefore done in which PD-catalyzed ATP synthesis was assayed both before and after the elimination of O₂ evolution by NH₂OH exposure. Ferriyanide was not included in

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**Table II**  
Failure of NH₂OH and EDTA to Reverse Photosystem I Inhibition Caused by KCN and HgCl₂

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity</th>
<th>H₂O-PD₅₀ₐx</th>
<th>Asc, DAD-MW</th>
<th>μeq transferred/hr-mg Chl</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+KCN, +Hg</td>
<td>1100</td>
<td>1550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+KCN, +Hg, +NH₂OH</td>
<td>800</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Same, stored 48 hr at -70°C</td>
<td>5</td>
<td>60</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

**Table III**  
Photosystem I-Dependent Cyclic ATP Synthesis by Untreated and KCN-Hg-NH₂OH Inhibited Chloroplasts

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>Untreated Chloroplasts</th>
<th>KCN-Hg-NH₂OH Inhibited Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of ATP Synthesis (μmol synthesized/hr-mg Chl)</td>
<td>% of Untreated Rate</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>PMS (125 μM)</td>
<td>800</td>
<td>475</td>
</tr>
<tr>
<td>DAT (125 μM)</td>
<td>435</td>
<td>18</td>
</tr>
<tr>
<td>PD (125 μM)</td>
<td>215</td>
<td>5</td>
</tr>
<tr>
<td>PDI (125 μM)</td>
<td>80</td>
<td>0</td>
</tr>
</tbody>
</table>
these assays, ruling out the possibility of noncyclic electron transport. The results of such an experiment are shown in Table IV. In O₂-evolving preparations, ATP synthesis is depressed; elimination of this activity stimulates the rate of phosphorylation more than 5-fold, showing that water and PD compete for a common electron donation site. It is thus apparent that PD-catalyzed ATP synthesis in KCN-Hg-NH₂OH-inhibited chloroplasts requires access to electron donor and acceptor sites on both the oxidizing and reducing sides of photosystem II, which in turn indicates that the activity observed with PD arises from cyclic electron transport.

**Effect of Anaerobiosis on PD-catalyzed ATP Synthesis by KCN-Hg-NH₂OH-inhibited Chloroplasts.** When inhibited chloroplasts were illuminated with PD in an O₂ electrode cuvette, neither evolution nor consumption of O₂ could be detected. Although this result is in accord with a cyclic reaction, we were concerned that in the presence of O₂, H₂O₂ might be formed on illumination and subsequently participate as an electron donor (7) at low concentrations during our assays. This possibility was eliminated by carrying out assays of activity on reaction mixtures which had been purged with either air or O₂-free helium. The results (Table V) of these experiments show that purging with either air or helium causes some decline in activity, but the similarity in extent of the inactivations suggests that the purging operation rather than O₂ depletion is responsible for loss of activity. In view of the results in Table I, it is extremely unlikely that H₂O₂ is participating in the photosystem II cyclic reaction.

**Action Spectra of Photosystem II- and Photosystem I-catalyzed Cyclic ATP Synthesis.** If the activity catalyzed by PD in KCN-Hg-NH₂OH-inhibited chloroplasts was, in fact, due to photosystem II rather than photosystem I activity, then the action spectrum of this reaction should differ from that of a reaction dependent on photosystem I activity. We therefore compared the action spectra for PD-catalyzed activity in inhibited preparations and DAD-catalyzed activity in untreated chloroplasts in the presence of DCMU (9.0 µM). The results of these experiments are presented in Figure 2; ATP synthesis is adjusted to a value of 1.00 at the wavelength of peak activity for each assay system. The PD-catalyzed system shows peak activity at 640 nm, whereas the DAD-catalyzed reaction peaks at 700 nm. Although our results are not as precise as other action spectra determinations (11) owing to the availability and quality of interference filters, the observed differences in our action spectra clearly indicate that the PD-catalyzed reaction is associated with photosystem II, whereas the DAD-catalyzed reaction is, as expected, dependent on photosystem I activity.

**DISCUSSION**

**Inhibition of Noncyclic Electron Transport.** Incubation of chloroplasts with KCN and HgCl₂ irreversibly inhibits photosystem I electron transport. Use of this procedure in concert with subsequent exposure to NH₂OH and EDTA provides a convenient means of preparing chloroplasts in which noncyclic electron transport reactions have been eliminated. Although Braden et al. (3) and Izawa and Good (9) have shown that Hg can act as an inhibitor of photophosphorylation, and our data (Table I) cannot exclude this possibility from our preparations, comparison of site II activity before elimination of O₂ evolution in KCN and KCN-Hg-inhibited preparations reveals that inclusion of Hg has not dramatically affected the efficiency of site II energy conversion.

**Cyclic ATP Synthesis by KCN-Hg-NH₂OH-inhibited Chloroplasts.** The finding that PMS can bypass the KCN-Hg inhibition site in our inhibited chloroplasts while p-phenylenediamines cannot is in accord with the results of previous investigations (15) which used KCN alone to inhibit photosystem I activity. The observation that PD was completely inactive as a catalyst of photophosphorylation in our preparations when DCMU was
present permitted the use of this compound as a catalyst of electron transport in the absence of DCMU to demonstrate the existence of photosystem II-dependent photophosphorylation in inhibited chloroplasts. The inhibition of this reaction by DCMU or the presence of functional O₂-evolving activity, its insensitivity to anaerobiosis, and its action spectrum led us to conclude that PD-catalyzed activity in KCN-Hg-NH₄OH-inhibited chloroplasts represents photosystem II cyclic photophosphorylation.

The rate concentration curve shown in Figure 1 suggests that the process of catalysis of activity by PD is complex, consisting of two reactions, one of which saturates below 100 μM PD, and another which is not fully saturated at 1,250 μM.

The ability of PD to catalyze DCMU-sensitive photosystem II activity is a consequence of its ability to donate electrons to photosystem II, while at the same time exhibiting little affinity for electron donation past the KCN-Hg site of inhibition. Yamashita and Butler (18) used PD and Asc to restore electron transport in tris-washed chloroplasts, and their results indicated that PD was preferentially oxidized by photosystem II. Ort and Izawa (14) compared the electron donation efficiencies of several phenylenediamines to photosystem I, and found that PD was the least efficient of this class of compounds. Thus, PD alone among the p-phenylenediamines appears to be sufficiently inefficient as a photosystem I donor to permit its use as a catalyst where measurements of pure photosystem II activity are required.

Our data provide no clue as to the mechanism of PD-catalyzed photosystem II cyclic ATP synthesis. The observation that a low rate of photophosphorylation can be obtained in our preparations without added catalyst indicates that native carriers in the chloroplast membrane must be participating in activity. The higher rates of ATP synthesis obtained with PD need not, however, use the same electron transport pathway. The simplest explanation for the activity of PD is that it acts in a manner similar to PMS in photosystem I cyclic reactions, undergoing photooxidation inside the membrane and photooxidation on the outside to establish an artificial shuttle of protons and electrons from outside to inside the membrane. While this is the most reasonable explanation for the activity of PD, we cannot exclude the interesting possibility that the photosystem II cycle operates by means of native carriers in the membrane such as plastoquinone and/or Cyt b₅₆₃. Experiments are now underway to determine more precisely the pathway of electron flow which supports photosystem II cyclic ATP synthesis.

Acknowledgment — We thank H. Ikuma for many helpful suggestions in the course of this investigation.

LITERATURE CITED