Photophosphorylation Associated with Photosystem II

IV. KINETIC ANALYSES OF PHOTOSYSTEM II CYCLIC PHOTOPHOSPHORYLATION ACTIVITY: EVIDENCE FOR TWO CYCLIC REACTIONS

JAMES A. GUIMEKÁ and CHARLES F. YOCUM
Department of Cell and Molecular Biology, Division of Biological Sciences, The University of Michigan, Ann Arbor, Michigan 48109

ABSTRACT

Photosystem II-dependent cyclic photophosphorylation activity produced by addition of p-phenylenediamines to KCN-Hg-NH₂OH-inhibited chloroplasts is the product of two separate reactions when a proton/electron donor is the catalyst. The activity observed with an electron donor as catalyst consists of a single reaction. One of the cyclic reactions, evoked by low (≤40 micromolar) concentrations of a proton/electron donor is sensitive to dibromothymoquinone and to perturbation of membrane organization by sonication. The second reaction, requiring higher catalyst concentrations, is less sensitive to either dibromothymoquinone or membrane perturbation. These results indicate that at low concentrations, proton/electron or electron donor catalysts act to produce a photosystem II cyclic reaction which is dependent on membrane-bound electron carriers. High concentrations of proton/electron donors, on the other hand, can produce a phosphorylation reaction in which the catalyst itself is largely responsible for cyclic activity.

Although thylakoid membranes exposed sequentially to KCN plus HgCl₂ and NH₂OH plus EDTA are unable to carry out reactions associated with water oxidation or electron donation to PSI, addition of lipophilic p-phenylenediamines to such membranes produces photophosphorylation activity. The sensitivity of this reaction to DCMU, its lack of sensitivity to anaerobiosis and its action spectrum have been taken as evidence for the occurrence of PSII-dependent cyclic photophosphorylation activity (18). In contrast to the PSI cyclic activities supported by lipophilic mediators such as DAD³ or PMS, the PSII cyclic reaction does not depend upon the capacity of the added catalyst to liberate protons upon oxidation, since TMPD and ferrocyanide (which are one-electron donors) will support PSII cyclic photophosphorylation reactions (19). In addition, TMPD and PD both support proton-uptake reactions to a comparable extent, and the photophosphorylation activities produced by these catalysts are sensitive to inhibition by DBMIB (19, 20), as well as to commonly used uncouplers and energy transfer inhibitors (20).

The ability of an electron donor catalyst (TMPD) to stimulate the PSII cyclic photophosphorylation reaction to rates comparable to those observed with a proton/electron donor catalyst raises the question of how the latter acts to promote cyclic activity. In the reaction observed with TMPD, the added catalyst is a one-electron donor which does not liberate protons upon oxidation (12). For other lipophilic catalysts (PD, DAT, or DAD) of PSII cyclic activity, the situation is less clear; all of these compounds can undergo either one-electron oxidations to a semiquinone form (no proton liberation) or a two electron oxidation to the quinonediimine form, thereby releasing two protons (12). Either oxidation reaction is possible in theory, and Babcock and Sauer (3) have demonstrated, by polarographic techniques, the formation of the one-electron oxidation product of PD in Tris-washed chloroplasts subjected to single turnover flashes. These considerations leave open the possibility that proton-donating p-phenylenediamines could function in the PSII cyclic reaction in a manner similar to that of TMPD (as one-electron donors), or alternatively in a fashion analogous to the action of DAD in PSI cyclic reactions, where a two electron oxidation reaction liberates the protons from the catalyst to support photophosphorylation activity (9). In this communication we report the results of experiments which detail the differences between the PSII cyclic reactions catalyzed by TMPD and by proton/electron donors (PD, DAD, DAT). Kinetic analyses of the response of these reactions to varied light intensities and catalyst concentrations, as well as the effects on activity of inhibition by DBMIB or of membrane disruption by sonication, reveal the presence of two separate PSII cyclic reactions when a proton/electron donor compound is present at assay: This finding is in contrast to the observation of a single photophosphorylation reaction when TMPD is the catalyst of activity.

MATERIALS AND METHODS

The procedures for isolation of chloroplasts and subsequent inhibition of activity with KCN plus HgCl₂ and NH₂OH plus EDTA were carried out as previously described (18); inhibited preparations were stored frozen (−70 °C) in 0.5-ml aliquots, which were thawed at room temperature immediately before use. All of the lipophilic catalysts used in photophosphorylation reactions were recrystallized before use; stock solutions (16–20 mM in distilled H₂O) were prepared immediately before assay, and discarded after use. Photophosphorylation activity was determined at 25 °C using the method of Avron (2). The reaction mixture contained 50 mM Tricine (pH 8.0), 50 mM NaCl, 3 mM MgCl₂, 1 mM ADP, 5 mM NaH₂32PO₄ (10⁶ cpm/ml), KCN-Hg-NH₂OH-inhibited chloroplasts equivalent to 24 to 36 μg Chl, and a lipophilic catalyst in a final volume of 1.6 ml. The complete reaction

¹ This research was supported by Grant PCM 78–7909 from the National Science Foundation.
² Present address: Division of Biological Sciences, University of Missouri, Columbia, MO 65211
³ Abbreviations used are; DAD, 2,3,5,6-tetramethyl-p-phenylenediamine; PMS, phenazinemethosulfate; TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; PD, p-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone; DAT, diaminotoluene; Irel, relative light intensity; I₅₀, concentration of inhibitor required for 50% inhibition of activity.
mixtures were preincubated for 10 min in the dark at 25 C to enhance the photophosphorylation activity (19), after which they were illuminated with white light (10^6 ergs/cm^2·s) from an Oriel 6350 source which was heat-filtered by passage through 2.5 cm of an 0.2% CuSO_4 solution. In some experiments, light intensity was varied by placing wire screens between the source and the samples; light intensity at the surface of the reaction tubes was determined using a YSI model 65 Radiometer.

For sonication experiments, the procedure developed by Nelson et al. (13) was employed, using a Branson sonic cleaner. A test tube held by clamps was submerged in the bath, and the effect of sonication of native chloroplasts on PMS-catalyzed PSI cyclic activity was examined. The depth of immersion of the tube was varied to find the position which produced maximal inactivation of PMS-catalyzed activity with minimal sonication times, and this position was used for sonication of KCN-Hg-NH_2OH-inhibited chloroplasts. The buffer used for sonication was that previously described (13); the bath contained ice to maintain a lowered temperature (5–7 C) during sonication.

The catalysts used in these studies were from Eastman (PD, TMPD), Aldrich (DAT) or Research Organics/Inorganics (DAD); DBMIB was a gift from N. E. Good. All other chemicals used in these investigations were of the purest grades commercially available.

RESULTS

Kinetic Analyses of PSII Cyclic Photophosphorylation Reactions. If all lipophilic catalysts of PSI cyclic activity act only as one-electron donors to the reaction, then the proton translocating events which support ATP synthesis must arise from electron transport associated with the oxidation of endogenous membrane-bound electron carriers such as plastoquinone. One method for assessing the extent to which a photosynthetic reaction is rate-limited by the activities of membrane-bound electron carriers is to examine the response of the reaction under investigation to varied light intensity. For example, the PSI cyclic reaction catalyzed by ferredoxin, methylviologen or anthraquinone-2-sulfonate have been shown to attain rate saturation at moderate light intensities (1, 16). This finding, and the demonstration that these same reactions are sensitive to DBMIB and/or antimycin A (16), is taken to indicate that components of the electron transport chain other than the PSI photosystem itself are involved in such cyclic reactions. The PSI reaction catalyzed by PMS, on the other hand, requires high light intensities for maximum rates of ATP synthesis (11), suggesting that this reaction is rate-limited by the activity of the photosystem itself. The PSIII cyclic photophosphorylation activities catalyzed by PD and TMPD were therefore examined to determine if any differences in these reactions would be revealed by analyses of their responses to light intensity. Both reactions required light intensities above 600,000 ergs cm^{-2} s^{-1} to achieve (TMPD) or approach (PD) saturated rates of ATP synthesis, a finding similar to other observations (14) on PSIII noncyclic activity supported by lipophilic catalysts.

Following a variation in the plotting method used to determine quantum yields for photosynthetic reactions in Scenedesmus (4), we plotted the data from the experiments described above on PD- and TMPD-catalyzed PSI cyclic reactions on an Eadie-Scatchard plot (17). This plot (Fig. 1) is also a sensitive test for the presence of more than one reaction (e.g., two different enzymes acting on the same substrate) in the system under study (17). The PSI cyclic activity catalyzed by TMPD is comprised of a single reaction (linear kinetic plot). The reaction catalyzed by PD, on the other hand, produces a biphasic kinetic plot, which is evident for the occurrence of two separate cyclic photophosphorylation reactions, with differing responses to varied light intensity. The unexpected appearance of these biphasic kinetics prevented attempts to estimate the quantum yield (v/Irel) of the PD-catalyzed system for use in comparison with the TMPD data, inasmuch as the linear portions of the plot for PD may not represent the true kinetics of either reaction (17).

The data shown in Figure 1 suggest that proton/electron donor activity can support two PSIII cyclic photophosphorylation reactions, whereas electron donor activity supports a single reaction. As a further test of this hypothesis, we next examined the kinetics of PSI II cyclic activity supported by varied concentrations of proton/electron donors and of TMPD. For these experiments, the light intensity was fixed at 10^6 ergs cm^{-2} s^{-1} throughout (Fig. 2). Although the reaction catalyzed by TMPD is seen to attain rate saturation with increasing concentrations of the catalyst, the reactions elicited by increasing levels of proton/electron donors (DAD, PD, DAT) are not rate-saturated in the range of concentrations examined here. When the data shown in Figure 2 were transformed to an Eadie-Scatchard plot, the results shown in Figure 3 were obtained. Here again, the proton/electron donor-catalyzed activities produce biphasic kinetic plots, which indicate the presence of two reactions whose kinetic parameters (K_m, V_m) differ, whereas the linear plot of the TMPD-catalyzed reaction is indicative of a single reaction. All of the activities shown are
DCMU sensitive (data not shown), which eliminates the possibility that the biphasic kinetic plots obtained with proton/electron donor catalysts arise from a mixture of reactions catalyzed by PSII and PSI.

The results in Figures 1 to 3 support the hypothesis that proton/electron donors can support two PSII-dependent cyclic photophosphorylation reactions, whereas a single reaction is produced by addition of the electron donor TMPD. These differences may arise from a similar interaction of each of these catalysts with the native electron transport chain near PSII, while the second reaction, seen only at higher concentrations of DAD, DAT, or PD might be produced by the ability of these compounds to donate protons to the photophosphorylation reaction. Alternatively, these differences in kinetics could reflect as many as three separate reactions, two of which would require the one- and two-electron donor capacities of DAD, PD, or DAT, as well as a third reaction pathway which would require an obligatory one-electron donation step unique to TMPD. This latter possibility was tested in an experiment where PSII cyclic photophosphorylation was assayed in the presence of increasing concentrations of TMPD plus or minus a fixed concentration of PD (10 μM); the PD concentration was selected to represent a level of this catalyst likely to generate only one reaction (Fig. 2). The resulting rates of ATP synthesis were corrected by subtraction of the contaminating rate of photophosphorylation catalyzed by PD alone (in those experiments where this compound was present): the corrected rates of ATP synthesis thus obtained are presented on a Lineweaver-Burk plot along with data from a control (minus PD) experiment (Fig. 4). The competitive interaction seen here suggests that PD and TMPD are acting as electron donors to the same reaction, eliminating the possibility of two separate reactions (at low concentrations of lipophilic catalysts) arising from differences in redox chemistry.

Yet another explanation for the biphasic kinetics observed with the proton/electron donors used for these studies might be that at different concentrations of these compounds, the fixed dark preincubation period (10 min) used to promote cyclic activity acts to establish different levels of redox poising in the reaction system. To test this hypothesis, the effect of dark incubation time on PD-supported PSII cyclic activity was examined at two different concentrations of the catalyst (Table 1). Although the rates of these reactions differ, as expected, Table 1 shows that no significant differences exist in the dark incubation times required to produce optimal rates of ATP synthesis, even though there is a 6-fold difference in the concentration of PD in the two experiments. Additional experiments on catalyst oxidation, in which small (0-40 μM) amounts of ferricyanide were added to reaction mixtures incubated for 10 min prior to illumination, confirmed the lack of any differences in requirements for oxidation of the catalyst. Low concentrations of ferricyanide were ineffective in promoting additional phosphorylation activity; concentrations of ferricyanide in excess of 40 μM produced some inhibition of ATP synthesis in the assay system where 40 μM PD was present (data not shown).

Effects of DBMIB and Sonication on PSII Cyclic Photophosphorylation Reactions. The preceding results show that PD, DAT, or DAD can produce two separate PSII cyclic reactions, in contrast to the single reaction observed when TMPD is the catalyst of activity (Figs. 1 to 3). The two reactions produced by proton/electron donors do not differ markedly in their requirements for a dark incubation period prior to assay of activity (Table I), and the competitive kinetics (Fig. 4) seen in experiments where PD and TMPD are both present at assay suggest that the reaction produced by low concentrations of a proton/electron donor is identical to that catalyzed by the electron donor. Since neither oxidation of TMPD nor the one-electron oxidation of PD, DAT, or DAD liberates protons (12), one would expect that at low concentrations of these lipophilic catalysts, the resulting photophosphorylation activity would be dependent on the proton translocating activity of membrane-associated electron carriers. As a test of this hypothesis, the effects of DBMIB on PSII cyclic activity catalyzed by either low (40 μM) or high (250 μM) concentrations of lipophilic catalysts was examined. The results of these experiments, presented as I0 values, are shown in Table II, where the activities supported by all lipophilic catalysts at a concentration of 40 μM are seen to be sensitive to inhibition by DBMIB. When the PSII cyclic reaction was assayed in the presence of much higher concentrations of the catalysts, on the other hand, the I0 value for DBMIB inhibition was seen to increase substantially for the proton/electron donor compounds; a much smaller increase was.

<table>
<thead>
<tr>
<th>Preincubation Time</th>
<th>Activity Catalyzed by</th>
<th>40 μM PD</th>
<th>250 μM PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>μmol ATP synthesized/h-mg Chl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>26</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>47</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>5.0</td>
<td>52</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>55</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>57</td>
<td>94</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Eadie-Scatchard plot of the data from Figure 2.

Fig. 4. Lineweaver-Burk plot of TMPD-catalyzed PSII cyclic activity assayed in the presence or absence of 10 μM PD. The lines shown were fitted by the least-squares method.

Table I. Effect of Dark Preincubation Time on PSII Cyclic Photophosphorylation Activity Catalyzed by Two Concentrations of PD

KCN-Hg-NH20H-inhibited chloroplasts were incubated with PD for the times shown and assayed for photophosphorylation activity as described in "Materials and Methods."
Table II. Effect of Catalyst Concentration on DBMIB Sensitivity of PSII Cyclic Photophosphorylation Activity

KCN-Hg-NH₂OH-inhibited chloroplasts were assayed in the presence of varying concentrations of DBMIB (0-2 μM when the catalyst concentration was 40 μM; 0-10 μM when the catalyst concentration was 250 μM). The I₀ values were estimated from plots of activity versus inhibitor concentration.

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>40 μM</th>
<th>250 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAD</td>
<td>0.3</td>
<td>≥8</td>
</tr>
<tr>
<td>DAT</td>
<td>0.4</td>
<td>≥8</td>
</tr>
<tr>
<td>PD</td>
<td>0.6</td>
<td>≥8</td>
</tr>
<tr>
<td>TMPD</td>
<td>0.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table III. Effect of Sonication on Photophosphorylation Activity in KCN-Hg-NH₂OH-inhibited Chloroplasts

Sonication was carried out as described in "Materials and Methods" for the indicated times and the chloroplasts were then assayed for photophosphorylation activity with PD or TMPD. The numbers in parentheses represent the percentage of control (no sonication) activities.

<table>
<thead>
<tr>
<th>Sonication Time (s)</th>
<th>Activity Catalyzed by</th>
<th>TMPD</th>
<th>PD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol ATP synthesized/h-mg Chl</td>
<td>40 μM</td>
<td>250 μM</td>
</tr>
<tr>
<td>0</td>
<td>78 (100)</td>
<td>93 (100)</td>
<td>52 (100)</td>
</tr>
<tr>
<td>30</td>
<td>&lt;5 (6)</td>
<td>23 (25)</td>
<td>12 (23)</td>
</tr>
<tr>
<td>60</td>
<td>&lt;5 (6)</td>
<td>15 (16)</td>
<td>&lt;5 (10)</td>
</tr>
</tbody>
</table>

seen for the TMPD-catalyzed reaction. The results in Table II indicate that in the presence of high concentrations of proton/electron donor compounds, the resulting PSII cyclic reaction is less dependent on DBMIB-sensitive electron transport for ATP synthesis to occur. One explanation for this observation is that the catalysts themselves, upon photoxidation, donate protons as well as electrons to the phosphorylation reaction. An alternative explanation would be that at high concentrations of DBMIB (which [6] block the photoreduction of p-phenyleneediamines; and [6] might permit DBMIB itself to act as a catalyst of electron transport) a new reaction is created in which the inhibitor participates in catalysis of activity.

As an alternative to DBMIB inhibition, we examined the effects of sonication, to perturb the organization of membrane-bound components of the electron transport chain, on PSII cyclic activity. The sonication procedure used here was similar to that employed for partial inactivation of PSII cyclic reactions (8, 13) (Table III). Two effects were seen in these investigations: First, there was a greater apparent damage to photophosphorylation activity if assays of activity following sonication employed added catalysts at a concentration of 40 μM. The second effect is the observation of lessened sensitivity of damage to activity if a high (250 μM) concentration of a proton/electron donor (PD) was present during assay of ATP synthesis following exposure to sonication. Although the principal effect of sonication here is probably to induce a rearrangement of membrane-bound electron carriers, it is also possible that some of the inhibition observed may also be due to an increase in membrane permeability.

Although it is difficult to control sonication to produce reproducible extents of inactivation of activity, the observation of lessened damage when high concentrations of PD were present at assay was repeatedly seen, and this suggests that PSII cyclic activity is less sensitive to perturbations of membrane organization if the catalyst can donate protons as well as electrons. To examine further the restoration of ATP synthesis in sonicated KCN-Hg-NH₂OH-inhibited chloroplasts, the experiment shown in Table IV was carried out. Here, the concentrations of catalysts (TMPD or PD) were varied in assays of the activity of the sonicated membranes. The results show that increasing concentrations of TMPD cannot restore increasing levels of ATP synthesis. For PD, however, a contrasting result is seen; increases in the concentration of this compound produce increased levels of ATP synthesis. Thus, the perturbations to membrane structure induced by sonication which prevent TMPD from restoring photophosphorylating electron transport are, in part, circumvented if the added catalyst is instead a compound (PD) capable of donating both protons and electrons to the PSII cyclic reaction.

**DISCUSSION**

The biphasic kinetics (Figs. 1 and 3) seen when lipophilic proton/electron donors produce PSII cyclic photophosphorylation activity are observed in biological systems where two catalytic processes act on a common substrate (5, 7, 15, 17). For the PSII cyclic reaction where such results were obtained, this implies that compounds such as PD, DAD, or DAT are utilized in KCN-Hg-NH₂OH-inhibited chloroplasts to generate two separate phosphorylating electron transport reactions. Our data show that one of these reactions (at low concentrations of an added mediator) is sensitive to DBMIB and to sonic perturbation of membrane organization, and that this reaction can be elicited by either a proton/electron donor or by TMPD (Fig. 4). The second reaction is unique to proton/electron donor catalysts and is observed at concentrations of these compounds in excess of those needed to saturate the first reaction. This second reaction is not sensitive to DBMIB, and is less sensitive to sonic perturbation of membrane organization than is the first reaction. Thus, the reaction observed at low concentrations of added catalysts is more dependent on the function of the native photosynthetic electron transport chain than is the second reaction.

Based on the considerations set forth above, two models for alternate mechanisms of the PSII cyclic reaction induced by addition of lipophilic mediators to KCN-Hg-NH₂OH-inhibited chloroplasts may be described, as shown schematically below:

![Diagram](https://www.plantphysiol.org/plantphysiol/)

(1) P680 → Q·B → PQ → 2H⁺out

Donor H₂ ↔ Donor H₂⁺ ↔ PQH₂

ATP ↔ 2H⁺₁n

(low concentrations of added mediators)
Although tentative, these models provide for alternative mechanisms by which a single class of compounds (proton/electron donors such as PD) could elicit two separate cyclic photophosphorylation activities involving PSII. Model 2 will be recognized as the PSII analog of the mechanism proposed to explain the DBMIB-insensitive PSI cyclic photophosphorylation reactions observed with DAD or PMS (9), where these mediators, by virtue of their redox chemistry, are envisioned to produce a proton/electron shuttle across the thylakoid membrane to provide both electrons (to P700) as well as the protons necessary for ATP synthesis to occur. Model 1 is more complex; here, the mediator is proposed to act as a one-electron donor/acceptor (donor H₂⁺ is used in the model to denote the semiquinone form, or Wurster's radical of the p-phenylenediamines) which serves to connect electron transfer between endogenous components, including plastoquinone, of the electron transport chain around PSII. One-electron oxidation states exist for the p-phenylenediamines (it is the only oxidation state of TMPD) (12), and as noted above, a one-electron oxidation product has been detected in experiments where Tris-treated chloroplasts were subjected to short flashes of light in the presence of PD (3). Because of its dependence on the endogenous electron transport chain for proton translocation to occur, reaction mechanism (1) would be expected to show sensitivity to DBMIB or to disruption of membrane organization by sonication.

We have previously reported the existence of biphasic kinetics associated with PSII noncyclic reactions catalyzed by oxidized p-phenylenediamines (7). In those investigations, all p-phenylenediamines, including TMPD, produced biphasic kinetics in the PSII noncyclic reaction, a result we interpreted to indicate the existence of multiple sites of photoreduction by PSII of oxidized p-phenylenediamines. For the PSII cyclic reaction, a more likely origin of the biphasic kinetics seen with proton/electron donors is the probable existence of more than one site of electron donation to these compounds. At least three species associated with PSII exist which might serve to photooxidize added p-phenylenediamines. These are: (a) the "intermediate potential" form of Cyt b559 (ECₐ = +0.24 v) created by NH₄OH-EDTA treatment of chloroplasts (10); (b) the donor (ECₐ = 0.48 v) to signal IIc; or (c) the species giving rise to signal IIc (3). With the exception of the b559 species, which might not be reducible by PD (ECₐ = +0.36 v) at our assay pH (8.0), any of these PSII-associated electron transport components might be sites of electron donation by lipophilic catalysts of the PSII cyclic reaction.

Our observations to date on PSII cyclic activity have established the unique feature of this artificially induced reaction, namely the ability of lipophilic compounds to generate an activity which requires components of the native photosynthetic electron transport chain for support of photophosphorylation activity. Why p-phenylenediamines such as DAD, PD, or DAT should preferentially catalyze this type of activity rather than a transmembrane proton/electron shuttle reaction is puzzling, and we can offer no ready explanation for the phenomenon. One possibility may be that, at low concentrations, the lipophilic mediators we employ in our assays are not participants in cyclic activity at all, but serve only as reductants to poise the components of an endogenous pathway of cyclic electron flow around PSII. We have repeatedly observed the induction of substantial rates of PSII cyclic activity by low (5-15 μM) concentrations of DAD and TMPD, but at higher concentrations than these, it is difficult to envision a reaction where the mediators would not be undergoing oxidation/reduction themselves. Further investigations to resolve the nature and components of the PSII cyclic reaction are now in progress.

LITERATURE CITED