Photophosphorylation Associated with Photosystem II

III. CHARACTERIZATION OF UNCOUPLING, ENERGY TRANSFER INHIBITION, AND PROTON UPTAKE REACTIONS ASSOCIATED WITH PHOTOSYSTEM II CYCLIC PHOTOPHOSPHORYLATION

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ABSTRACT

A number of uncouplers and energy transfer inhibitors suppress photosystem II cyclic photophosphorylation catalyzed by either a proton/electron or electron donor. Valinomycin and 2,4-dinitrophenol also inhibit photosystem II cyclic photophosphorylation, but these compounds appear to act as electron transport inhibitors rather than as uncouplers. Only when valinomycin, KCl, and 2,4-dinitrophenol were added simultaneously to phosphorylation reaction mixtures was substantial uncoupling observed. Photosystem II noncyclic and cyclic electron transport reactions generate positive absorbance changes at 518 nm. Uncoupling and energy transfer inhibition diminished the magnitude of these absorbance changes. Photosystem II cyclic electron transport catalyzed by either p-phenylenediamine or N,N,N',N'-tetramethyl-p-phenylenediamine stimulated proton uptake in KCN-Hg,NH₄OH-inhibited spinach (Spinacia oleracea L.) chloroplasts. Illumination with 640 nm light produced an extent of proton uptake approximately 3-fold greater than did 700 nm illumination, indicating that photosystem II catalyzed electron transport was responsible for proton uptake. Electron transport inhibitors, uncouplers, and energy transfer inhibitors produced inhibitions of photosystem II-dependent proton uptake consistent with the effects of these compounds on ATP synthesis by the photosystem II cycle. These results are interpreted as indicating that endogenous proton-translocating components of the thylakoid membrane participate in coupling of ATP synthesis to photosystem II cyclic electron transport.

These experiments have led to the hypothesis that noncyclic electron transport in isolated chloroplasts traverses two distinct sites of energy conservation. One site (site II) is presumed to be associated with PSI, while the other (site I) is proposed to function near PSI. Experiments with electron and proton/electron donors to PSI in NH₄OH-EDTA-inhibited chloroplasts by Izawa and Ort (6) strongly suggest that proton liberation near the oxidizing side of PSIII represents the energy-coupling reaction ascribed to site II.

Yocum and Guikema (23) and Yocum (21, 22) have described conditions under which appropriately inhibited chloroplasts will catalyze cyclic photophosphorylation dependent upon PSI activity. This reaction is unlike PSI cycles catalyzed by DAD or reduced PMS in that electron donors (TMPD, ferrocyanide) will support ATP synthesis which is completely sensitive to inhibition by DCMU, and partially sensitive to DBMIB and antimycin A. These findings suggest that optimal coupling of ATP synthesis to PSI cyclic electron transport involves the activity of endogenous electron carriers in the chloroplast membrane. The results presented in this communication demonstrate that PSI cyclic photophosphorylation is susceptible to uncoupling and energy transfer inhibition. The combination of valinomycin and KCl is shown to be an inhibitor of the PSI II cycle, but this inhibition does not appear to be due to the collapse of a membrane potential. Data are also presented which show that PSI cyclic electron transport promotes proton uptake. These results are interpreted as support for the hypothesis that PSIII cyclic photophosphorylation requires the operation of membrane-bound electron transport carriers to support proton accumulation and ATP synthesis.

MATERIALS AND METHODS

Chloroplasts, KCN-Hg-inhibited chloroplasts, and KCN-Hg,NH₄OH-inhibited chloroplasts from spinach (Spinacia oleracea L.) were prepared as described earlier (23). PSIII cyclic and noncyclic photophosphorylation reactions were assayed at 25°C in a reaction mixture (1.6 ml) which contained 50 mM Tricine (pH 8), 50 mM NaCl, 3 mM MgCl₂, 1 mM ADP, 5 mM NaH₂PO₄ (10⁶/cpm/ml), and 20 to 30 μg Chl (21, 23). In addition, the noncyclic reaction mixtures contained 0.25 mM PD plus 2.5 mM ferricyanide. Noncyclic electron transport was measured polarographically with a Clark electrode (YSI). All reaction mixtures were illuminated with white light (10⁶ ergs·cm⁻²·sec⁻¹) for 1 min. Reactions were terminated by the addition of 0.2 ml of 30% trichloacetic acid, and ATP synthesis was determined by gas flow counting of dried samples from which unreacted phosphate had been extracted. Uncou-
ples, energy transfer inhibitors, and ionophores were dissolved in the appropriate solvent (H₂O, ethanol, or dimethylsulfoxide) at concentrations which permitted subsequent additions of inhibitors to reaction mixtures in small volumes so that the concentration of organic solvent never exceeded 2% (v/v) in the final volume of the reaction mixtures.

Proton uptake was measured following procedures outlined by Dilley (2). A combination electrode was immersed in a 2-ml thermostatted (25°C) cuvette. The electrode was connected to a Radiometer model 25 pH meter whose output was fed through a zero offset circuit to a Varian G-2000 chart recorder which required an input of 2 mV for full scale deflection. This deflection corresponded to a change of 0.1 pH unit on the pH meter. The reaction mixtures for proton uptake contained, in 1.6 ml, 40 to 50 μg Chl, 60 mM NaCl, and 3 mM MgCl₂. When solutions of the hydrochloride salts of PD or TMPD were added to the cuvette, 0.1 n NaOH was added simultaneously to offset acidification. A pH of 7.5 to 7.55 in the reaction mixture was achieved by additions of small amounts of dilute HCl or NaOH. Reaction mixtures were stirred for 10 min prior to illumination. White (1 × 10⁶ ergs·cm⁻²·sec⁻¹) or red (>600 nm, 600 kergs·cm⁻²·sec⁻¹) light from an Oriel model 6325 light source filtered through 5 cm of a 0.2% CuSO₄ solution was used to activate reaction mixtures. Monochromatic illumination was performed as described earlier (13). Extents of proton uptake were calculated from recorder traces that were calibrated by observing the deflections which occurred when a measured amount of standard acid was injected into illuminated reaction mixtures.

The 518 nm absorbance change was measured with an Aminco DW-2 dual wavelength spectrophotometer. Samples were activated by side illumination with light (1 × 10⁶ ergs·cm⁻²·sec⁻¹) that was passed through 7 cm of H₂O and an Oriel LP-63 cut-on filter before striking the cuvette. The photomultiplier tube was protected from the actinic light by combination of a Corning 4-96 blue filter with a green plastic sheet to produce a cut-off at 590 nm. The monochromator slit width was set at 2 nm. The actinic path length in the sample cuvette was 0.5 cm; the measuring beam path length was 1 cm. The reaction mixtures (1 ml) contained chloroplasts (35-40 μg Chl), 50 mM NaCl, 50 mM Tricine (pH 8), 3 mM MgCl₂, and the other additions noted in Figure 1 and Table V.

Electron transfer catalysts were recrystallized as described previously (22, 23). Antimycin A, valinomycin, and CCCP were purchased from Sigma. Gramicidin was obtained from Schwarz/Mann; DCCD was from Aldrich. Phlorizine, DBMIB, and triphenyltin chloride were generous gifts from N. E. Good and J. M. Gould. All other chemicals used in these studies were of the purest grades commercially available.

**RESULTS**

Inhibition of PSII Cyclic Photophosphorylation by Uncouplers and Energy Transfer Inhibitors. Table I shows the results of experiments using the uncouplers methylamine, CCP, atebrin and gramicidin. These compounds, which uncouple other cyclic and noncyclic photophosphorylation reactions (5), produced a similar effect on the PSII cyclic reaction. Table II shows that the energy transfer inhibitors phlorizine, DCCD and triphenyltin chloride also inhibit PSII cyclic photophosphorylation. There is no apparent difference in the susceptibility of either TMPD- or PD-catalyzed activity to uncoupling or energy transfer inhibition.

Valinomycin plus KCl and DNP are potent uncouplers of oxidative phosphorylation, but not of photophosphorylation (5, 12). When the effect of these compounds on PSII cyclic photophosphorylation was examined, the results shown in Table III were obtained. Valinomycin, in the absence of KCl, produced an inhibition (65%) of ATP synthesis which was not increased when KCl was present. In fact, a small but reproducible decrease in the extent of inhibition was noted when valinomycin and KCl were added together (Table III). Addition of a low concentration of DNP also produced substantial inhibition of ATP synthesis, and addition of valinomycin, KCl, and DNP abolished ATP synthesis completely, in agreement with earlier results (8). The effects of KCl, DNP, and valinomycin on PSII noncyclic photophosphorylation were also examined since this reaction permits one to assess uncoupling by measuring P/e values. A summary of the results of these experiments is shown in Table IV. Valinomycin plus KCl produced weak uncoupling (17% inhibition of ATP synthesis) while DNP inhibited both ATP synthesis and electron transport. Valinomycin, KCl, and DNP together inhibited ATP synthesis completely. These results with valinomycin, and those obtained from experiments with PSII cyclic reactions indicate that valinomycin plus KCl is probably not acting to uncouple photophosphorylation in KCN-Hg-NH₄OH-inhibited chloroplasts. Rather, the fact that valinomycin and NaCl produce a slightly stronger inhibition suggests that the inhibitory action of valinomycin is not directly associated with the energy-conserving mechanism.

**Absorbance Changes at 518 nm Induced by PSII Cyclic and Noncyclic Electron Transport.** Witt (20) has shown that a positive absorbance change in the region of 515 to 520 nm in illuminated chloroplasts can be correlated with photosynthetic energy conservation. Inhibition of this change by uncouplers and electron transport inhibitors have been observed (7). Figure
Table IV. Inhibition of photosystem II non-cyclic electron transport and photophosphorylation catalyzed by 250 μM PD plus 2.5 mM ferricyanide. The KCl concentration was 25 mM in all assays.

<table>
<thead>
<tr>
<th>Inhibitor Added</th>
<th>Electron Transport</th>
<th>ATP Transport</th>
<th>Synthesis</th>
<th>P/e₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25 μM Valinomycin</td>
<td>250 μM DNP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(μequiv/hr/μg Chl)</td>
<td>(μmol/hr/μg Chl)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-</td>
<td>74.6</td>
<td>114</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>732</td>
<td>95</td>
<td>0.26</td>
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<tr>
<td>++</td>
<td>316</td>
<td>0</td>
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1 shows the extents of the 518 nm absorbance change observed in KCN-Hg- and KCN-Hg-NH₂OH-inhibited chloroplasts while noncyclic and cyclic PSII electron transport was occurring. Although these chloroplast preparations synthesize ATP at approximately the same rates (95-105 μmol/hr·mg Chl), the absorbance change elicited by noncyclic activity is approximately three times greater than that observed with PSII cyclic electron transport. Table V shows the effect of uncoupling and energy transfer inhibition on these absorbance changes. Gramicidin and triphenyltin chloride decreased the 518 nm absorbance changes substantially, while valinomycin plus KCl showed little effect on the absorbance change induced by noncyclic electron transport. This same combination did, however, diminish the absorbance change observed in the PSII cyclic reaction.

Proton Uptake Catalyzed by PSII Cyclic Electron Transport. Figure 2 shows the results of proton uptake experiments with KCN-Hg-NH₂OH-inhibited chloroplasts. In the absence of any artificial catalyst, some proton uptake occurred. If TMPD or PD was added to the reaction mixture, the extent of uptake was stimulated more than 3-fold. The extent of uptake was independent of the catalyst used; both PD, a proton/electron donor, and TMPD, an electron donor, produced similar extents of uptake.

If the proton uptake shown in Figure 2 is due to PSII cyclic electron transport, then light absorbed preferentially by PSII would promote uptake. Figure 3 shows the effects of illumination of reaction mixtures with 640 nm and 700 nm light of equivalent intensities (9 kergs·cm⁻²·sec⁻¹). In the presence of PD or TMPD, 640 nm light produced an extent of proton uptake about three times greater than did illumination at 700 nm. This figure also shows that addition of DCMU (3 μM) strongly inhibited uptake, as would be expected if PSII activity was responsible for uptake.

Table VI summarizes the results of experiments on proton uptake by PD- and TMPD-catalyzed PSII cyclic electron transport in the presence of a variety of inhibitors. DCMU, antimycin A, and DBMIB inhibited uptake, with DCMU showing the strongest inhibition. Uncoupling by gramicidin or CCCP also decreased the extent of proton uptake. Valinomycin plus KCl and DNP were slightly inhibitory when added separately; when added together, they produced nearly complete inhibition of uptake. DCCD and triphenyltin chloride also caused substantial inhibitions of proton uptake. This behavior is in variance with reports (3, 10) showing that proton uptake by PSI cycles catalyzed by PMS or pyocyanin is stimulated by DCCD and triphenyltin chloride.

In the course of these experiments, an unusual behavior of TMPD-catalyzed proton uptake was noted. Upon cessation of illumination of the reaction mixture, the pH of the medium did not return to the initial value, but instead decayed to a slightly more alkaline pH value. This phenomenon is shown in Figures 2 and 4. When gramicidin was added to abolish reversible proton uptake, illumination produced a slight alkalinization of the medium. Figure 4 also shows that when TMPD was added to untreated chloroplasts poisoned with DCMU, to elicit PSI activity, a rapid alkalinization of the medium was observed. Gramicidin uncoupling did not affect the irreversible alkalinization, which may be due to the formation of secondary products of autoxidation of the TMPD radical (9). It is not clear at present whether irreversible alkalinization in the presence of TMPD requires PSI activity, or whether it is also caused by PSI activity. It is apparent, however, that the extent of irreversible alkalinization catalyzed by KCN-Hg-NH₂OH-inhibited chloroplasts in the presence of TMPD is much smaller than that observed in untreated chloroplasts poisoned with DCMU, where TMPD is an ineffective catalyst of ATP synthesis.

DISCUSSION

The results of the experiments reported in this communication demonstrate that PSI cyclic photophosphorylation is similar to

![FIG. 1. Extent of light-induced absorbance changes at 518 nm produced by PSII-catalyzed noncyclic and cyclic electron transport. In addition to the assay conditions described under 'Materials and Methods,' the reaction mixtures contained 2.5 mM ferricyanide and 0.25 mM PD (noncyclic system) or 250 μM PD (cyclic system). The PSII cyclic reaction mixture was incubated for 10 min prior to illumination. The cuvette holder of the spectrophotometer was thermostatted at 20°C.](attachment://image.png)

![FIG. 2. Proton uptake by KCN-Hg-NH₂OH-inhibited chloroplasts: stimulation by p-phenylenediamines.](attachment://image.png)
The site of proton uptake demonstrates the presence of a negative 518 nm absorbance change, which is much smaller than that observed when PSII-specific noncyclic electron transport is occurring. Although this absorbance change is diminished or abolished by the appropriate inhibitors of energy conservation, the small extent of the absorbance change observed when PSII cyclic electron transport is occurring is difficult to explain. We have observed other absorbance changes in the 500 nm region of KCN-Hg-NH₂OH-inhibited chloroplasts, which are negative rather than positive. Whether these changes detract from the magnitude of the positive 518 nm absorbance change is unclear and requires further investigation.

The PSII cyclic electron transport obtained by addition of PD or TMPD to KCN-Hg-NH₂OH-inhibited chloroplasts supports proton uptake four times greater in extent than that observed if an electron transport catalyst is absent. The fact that 640 nm light is three times more effective than 700 nm light in promoting proton uptake demonstrates that electron transport involving PSII, rather than PSI, supports proton accumulation in the presence of PD or TMPD. This finding is in accord with earlier data (23) showing that PD-catalyzed ATP synthesis in KCN-Hg-NH₂OH-inhibited chloroplasts was 3-fold higher at 640 nm than at 700 nm. A number of inhibitors which depress PSI cyclic photophosphorylation also inhibit proton uptake. DCCD and triphenyltin chloride, which stimulate PMS-dependent proton uptake by PSI electron transport (3, 10), depress proton accumulation by PSI cyclic electron transport, but do not abolish it completely. There is no readily apparent explanation for this discrepancy.

PD- and TMPD-catalyzed PSI cyclic electron transport supports a greater extent of proton uptake (700-800 nmol/mg Chl) than is observed in a comparable PSI cyclic reaction (225-250 nmol/mg Chl) where PMS is the catalyst (C. F. Yocum, unpublished observation). It has been reported (11, 15) that low pH amines, including PD, can stimulate proton uptake in PMS- or pyocyanin-catalyzed PSI cycles. This stimulation is presumed to arise from internal buffering by the amine. The concentrations of amine required to elicit this stimulation are at least 4-fold greater than the amounts of PD or TMPD used in the present investigation. It is therefore unlikely that the proton uptake elicited by PD and TMPD in the PSI cyclic reaction is due solely to internal buffering effects.

When TMPD serves as the catalyst for PSI cyclic electron transport, an irreversible alkalinization of illuminated reaction mixtures is observed. This alkalinization accounts for no more than 15% of the extent of the reversible alkalinization catalyzed by TMPD, and must be contrasted with a TMPD-catalyzed PSI cyclic reaction, where irreversible alkalinization upon illumination is the only pH change observed. This phenomenon, which may be caused by autoxidation of a TMPD radical (9), does not represent a substantial contribution to the pH changes obtained when TMPD is the catalyst of PSI cyclic electron transport. Of more importance is the observation that TMPD is capable of catalyzing reversible proton uptake in a phosphoryl-
ating system (the PSII cycle) whereas the same compound cannot elicit this reversible reaction under conditions where it is an ineffective catalyst of ATP synthesis (PSI cyclic electron transport).

The demonstration of proton uptake catalyzed by both proton/electron and electron donor catalysts strongly implicates the operation of membrane-bound proton translocating components in the coupling of ATP synthesis to PSI cyclic electron transport. Since water oxidation is inhibited in KCl-Hg-NH₄OH-inhibited chloroplasts, site II cannot be contributing to energy conservation during cyclic electron transport. The other endogenous energy-conserving site, site I, is presumed to function between plastocyanine and Cyt f in uninhibited chloroplasts. While ATP synthesis by the PSI cycle is sensitive to inhibition by DBMIB, concentrations of DBMIB far in excess of those necessary to inhibit noncyclic electron transport by untreated chloroplasts produce only partial inhibitions of the PSI cyclic reaction (22). One cannot, therefore, arbitrarily assume that plastocyanine oxidation/reduction is an obligatory component of the energy-conserving reaction associated with the PSI cycle. An extensive characterization of sites I and II in untreated chloroplasts has been reported (4). It will be of interest to determine whether further characterization of the PSI cyclic photophosphorylation reaction, currently in progress, will permit the identification of its energy-conserving site, or sites, with those previously identified in uninhibited chloroplasts.

LITERATURE CITED

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