Regulation of Cyclic Photophosphorylation during Ferredoxin-Mediated Electron Transport

EFFECT OF DCMU AND THE NADPH/NADP⁺ RATIO

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ABSTRACT
Addition of ferredoxin to isolated thylakoid membranes reconstitutes electron transport from water to NADP⁺ and to O₂ (the Mehler reaction). This electron flow is coupled to ATP synthesis, and both cyclic and noncyclic electron transport drive photophosphorylation. Under conditions where the NADPH/NADP⁺ ratio is varied, the amount of ATP synthesis due to cyclic activity is also varied, as is the amount of cyclic activity which is sensitive to antimycin A. Partial inhibition of photosystem II activity with DCMU (which affects reduction of electron carriers of the interphotosystem chain) also affects the level of cyclic activity. The results of these experiments indicate that two modes of cyclic electron transfer activity, which differ in their antimycin A sensitivity, can operate in the thylakoid membrane. Regulation of these activities can occur at the level of ferredoxin and is governed by the NADPH/NADP⁺ ratio.

cyclic photophosphorylation, catalyzed by electron transport from water to ferredoxin/O₂ or to ferredoxin/NADP⁺. These experiments, performed under aerobic conditions using saturating light, provide a closer approximation of the activity of PSI cyclic photophosphorylation in vivo than does measurement of a cyclic reaction poised independently of noncyclic electron transport. Our previous studies on this reconstituted electron transport system have shown that electron transport to either ferredoxin/O₂ or to ferredoxin/NADP⁺ produces elevated P/O ratios (1.6 versus 1.25 with MeV as the acceptor); the increased P/O ratio is due to the induction, by ferredoxin, of a cyclic or Q-loop electron transfer mechanism (10). This work also suggests the existence of two separate pathways of cyclic photophosphorylation: one pathway is characterized by sensitivity to antimycin A and the requirement for a substantial pool of reduced ferredoxin, while a second pathway is insensitive to antimycin A and is consistently associated with the turnover of FNR. The results presented here provide further evidence for two cyclic photophosphorylation reactions which are regulated quite differently by both DCMU and the NADPH/NADP⁺ ratio. A new interpretation of the regulation of cyclic and noncyclic photophosphorylation by the NADPH/NADP⁺ is presented.

MATERIALS AND METHODS
Spinach thylakoid membranes were prepared by the method of Robinson and Yocum (20) and routinely gave rates of 300 to 400 μmol O₂.h⁻¹·mg⁻¹ Chl for gramicidin-uncoupled electron transport to MeV, and noncyclic P/O ratios between 1.2 and 1.3. Spinach ferredoxin was purified by the procedure of Petering and Palmer (19) with the modifications of Yocum (26). Electron transport and photophosphorylation rates were measured as described previously (10) in a reaction mixture containing 50 mM Tricine (pH 8.0), 50 mM NaCl, 3 mM MgCl₂, 5 mM NaH₂¹⁵PO₄, 1 mM ADP, and 30 to 40 μg Chl. All chemicals were obtained from Sigma, except for DCMU from K and K Labs and NaH₂¹⁵PO₄ from New England Nuclear.

The initial NADPH/NADP⁺ ratio of the assay medium was varied during measurement of electron transport to ferredoxin/NADP⁺ by increasing the percentage of NADPH present in a constant total concentration of NADPH plus NADP⁺ (2 mM). It is important to note that the increase in the concentration of NADPH during illumination changed this initial value by only 6% at the most rapid rate of NADP⁺ reduction (i.e. with 100% NADPH initially) over the course of the experiment. The individual rates of NADP⁺ reduction and O₂ reduction were measured as described previously (10), the former by net O₂ evolution and the latter by the amount of O₂ evolved after the addition of 840 units of catalase following the illumination period. Addition of antimycin A to these reaction systems produced no discernible

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3 Abbreviations: PQ, plastoquinone; MeV, methylviologen; FNR, ferredoxin-NADP reductase.
effect on the rates of electron transfer; the effect of the inhibitor was to decrease the rate of photophosphorylation, as shown in Figures 2 and 3.

The method described above was complicated slightly by the addition of NADPH, which supports a slow rate of O₂ uptake in the dark (0-24 μmol O₂·h⁻¹·mg⁻¹ Chl over the range from 0-95% NADPH) catalyzed by ferredoxin and FNR. While we cannot be sure if the reaction NADPH → ferredoxin/FNR → O₂ proceeds at the same rate in the light as in the dark, we have assumed here that it does in order to apply a correction procedure to the measurements of light-driven O₂ reduction and NADP⁺ reduction. This NADPH-driven reaction was carefully characterized by incubating thylakoids plus 10 μM ferredoxin in the dark at each NADPH/NADP⁺ ratio assayed (data not shown). The addition of excess catalase halves the rate of the dark O₂ uptake, indicating that the NADPH-driven dark reaction reduces O₂ to H₂O₂. To obtain a correct rate of O₂ reduction by light-driven electron transport, one-half of the absolute value of the rate of dark O₂ uptake observed during 60 s (i.e. the same length of time as the illumination period) was subtracted from the postillumination measurement of O₂ evolution using catalase as described above. The same value was added to the rate of O₂ evolution in order to obtain the correct rate of NADP⁺ reduction. This correction procedure was employed only to obtain the closest possible estimate of the rates of electron transport to O₂ and to NADP⁺. The total rate of electron transport (NADP⁺ reduction plus O₂ reduction) remains the same whether or not the corrections are made. Since all of the P/O ratios were calculated using the total rate of electron transport, these values are unchanged by the correction procedure.

RESULTS AND DISCUSSION

Regulation of Cyclic Photophosphorylation by PSII Inhibition.

During ferredoxin-mediated O₂ reduction, addition of low concentrations of DCMU led to an increase in the P/O value (Fig. 1), because the rate of ATP synthesis was less sensitive to the inhibitor than was the rate of electron transport (Table I). The

![Graph](image)

**Fig. 1.** The effect of DCMU on the P/O (P/2e) ratio of ferredoxin-mediated electron transport; (O), data obtained with O₂ as the acceptor; (X), data obtained in the presence of NADP⁺. The P/O values presented are calculated from the data of Table I. Ferredoxin was used at 27 μM during electron transport to O₂ since this concentration supports a high rate of concurrent cyclic photophosphorylation. The effect of DCMU on the P/O ratio of electron flow to ferredoxin/O₂ was tested at several concentrations of ferredoxin between 15 and 30 μM and found to be the same.

Table I. Effect of an Increasing Concentration of DCMU upon Electron Transport and Photophosphorylation Using Either Ferredoxin/O₂ or Ferredoxin/NADP⁺ as the Electron Acceptor

<table>
<thead>
<tr>
<th>DCMU</th>
<th>Ferredoxin/O₂</th>
<th>Ferredoxin/NADP⁺</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>O₂ reduction</td>
<td>ATP synthesis</td>
</tr>
<tr>
<td></td>
<td>μmol·h⁻¹·mg⁻¹ Chl</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>97</td>
<td>277</td>
</tr>
<tr>
<td>3.3 x 10⁻¹⁰</td>
<td>96</td>
<td>275</td>
</tr>
<tr>
<td>3.3 x 10⁻⁹</td>
<td>96</td>
<td>271</td>
</tr>
<tr>
<td>3.3 x 10⁻⁸</td>
<td>83</td>
<td>276</td>
</tr>
<tr>
<td>3.3 x 10⁻⁷</td>
<td>29</td>
<td>156</td>
</tr>
<tr>
<td>3.3 x 10⁻⁶</td>
<td>0</td>
<td>19</td>
</tr>
</tbody>
</table>

P/O ratio of noncyclic electron transport alone, measured as water to MeV, was not stimulated by DCMU (data not shown); therefore the rise of the P/O value of O₂ reduction reflects an increase in the ratio of cyclic to noncyclic photophosphorylation.

The actual rate of cyclic photophosphorylation can be estimated from the data of Table I by assuming the P/O ratio of noncyclic electron transport to be 1.25. (This assumption is based upon three observations. First, electron transport to MeV, which is strictly noncyclic under aerobic conditions, yields a P/O value of 1.25 [10, 18]. Second, the addition of antimycin A during electron transport to ferredoxin/O₂ selectively inhibits cyclic photophosphorylation and the remaining noncyclic pathway retains a P/O value of 1.2 to 1.25 [10]. Finally, electron transport to either ferredoxin/O₂ or ferredoxin/NADP⁺ is coupled with equal efficiency, whether the reactions are concurrent or separate [10]). By this method, the estimated rate of cyclic photophosphorylation increased from 35 μmol ATP·h⁻¹·mg⁻¹ Chl in the absence of inhibitor to 84 μmol ATP·h⁻¹·mg⁻¹ Chl in the presence of 0.33 μM DCMU.

The increase in the ratio of cyclic to noncyclic photophosphorylation (Fig. 1) and the net acceleration of cyclic flow show that reduced ferredoxin is a more successful donor to the membrane-bound components of the cyclic pathway when the flow of electrons from PSII is restricted. This result is consistent with the fact that the highest rates of ferredoxin-mediated cyclic photophosphorylation are obtained in the complete absence of PSII electron flow as long as reduced ferredoxin is supplied (20).

Addition of 1 mM NADP⁺ eliminated the stimulation of cyclic photophosphorylation by DCMU (Fig. 1). Electron transport and ATP synthesis rates decline in parallel (Table I) and the constant P/O ratio indicates that the balance of cyclic and noncyclic electron flow is unchanged. The estimated rate of cyclic photophosphorylation declined from 69 to 11 μmol ATP·h⁻¹·mg⁻¹ Chl in the presence of 0 and 0.33 μM DCMU, respectively.

The differences seen in the presence and absence of NADP⁺ may be explained by the differing redox conditions imposed upon cyclic flow by noncyclic electron transport. In the absence of NADP, ferredoxin is forced to reduce only O₂, and because this reaction is quite slow (9), it must result in larger pools of reduced ferredoxin and PQ. The addition of low concentrations of DCMU will cause a partial oxidation of both pools. Since the rate of cyclic electron flow is accelerated by DCMU, the initial "overreduction" of PQ must be more limiting to cyclic flow under these conditions than the supply of electrons from ferredoxin. In the presence of NADP⁺, ferredoxin turns over rapidly to FNR.
and thus the addition of inhibitory concentrations of DCMU will cause the PQ pool to become oxidized. This alleviates any restriction of electron transfer from reduced ferredoxin to PQ due to the redox state of the PQ pool and, consistent with this argument, DCMU has no effect. Significantly, the addition of DCMU does not eliminate cyclic photophosphorylation in the presence of NADP⁺ (i.e. the P/O does not drop to 1.25) even though the steady state concentration of reduced ferredoxin in the presence of DCMU is very low (data not shown). This is consistent with the fact that cyclic photophosphorylation in the presence of NADP⁺ appears to depend upon the turnover of FNR, and not upon the accumulation of a pool of reduced ferredoxin (10).

**Regulation of Cyclic Photophosphorylation Activity by the NADPH/NADP⁺ Ratio.** Increasing the ratio of NADPH to NADP⁺ to a maximum of 95% NADPH during electron transport to ferredoxin/NADP⁺ inhibited the rate of NADP⁺ reduction by 89% (Table II), presumably because of competition between NADP⁺ and NADPH for binding to FNR. With the decline in NADP⁺ reduction, the rate of concurrent O₂ reduction quickly increased to approximately 40 μmol O₂ h⁻¹ mg⁻¹ Chl (Table II), which is the same rate of O₂ reduction obtained using 10 μM ferredoxin in the absence of NADP⁺ (data not shown). While the overall rate of ATP synthesis declined as NADPH was added, it was still 53% of the original rate in the presence of 95% NADP⁺ (Table II). The P/O ratio of electron transport therefore rose from 1.5 with 100% NADP⁺ to approximately 2.5 in the presence of 95% NADPH plus 5% NADP⁺ (Fig. 2). The major part of this increase occurred at high NADPH/NADP⁺ ratios (>80% NADPH).

The P/O ratios shown in Figure 2 are partially sensitive to a concentration of antimycin A which inhibits the antimycin A-sensitive cyclic pathway (10); the ATP which contributes to a P/O ratio greater than 1.25 in the presence of antimycin A must be supplied by an antimycin A-insensitive cyclic photophosphorylation reaction. Using these P/O ratios and the data from Table II, the estimated rate of each cyclic photophosphorylation reaction was derived (Fig. 3). The antimycin A-insensitive cyclic photophosphorylation reaction occurred at high rates with both high and low ratios of NADPH to NADP⁺, although the rate begins to decline at levels of NADPH greater than 80%. Conversely, the antimycin A-sensitive pathway contributed little to the overall rate of photophosphorylation until a high NADPH/NADP⁺ ratio was established, at which point the rate of the reaction was greatly stimulated (Fig. 3). In the presence of 95% NADPH the rates of the two cyclic pathways were equal with activities of about 70 μmol ATP h⁻¹ mg⁻¹ Chl.

The effect of the NADPH/NADP⁺ ratio on the concentration of reduced ferredoxin during electron transport to ferredoxin/NADP (H) was measured as in Hosler and Yocum (10) (data not shown). The total pool (10 μM ferredoxin) was only 10% reduced when NADPH comprised 50% or less of the total pyridine nucleotide pool. As the relative concentration of NADPH was increased above 50%, the ferredoxin pool became more reduced, reaching 70% reduction in the presence of 95% NADPH. The increased reduction of the ferredoxin pool resulted from the direct reduction of ferredoxin by NADPH/FNR, as well as increased light-driven reduction because electron transfer through FNR was inhibited.

While both DCMU and NADPH slowed linear electron transport to ferredoxin/NADP⁺, only NADPH increased the ratio of cyclic to noncyclic photophosphorylation (Fig. 2) as well as the total rate of cyclic photophosphorylation ferredoxining. 3). This change was brought about by an increase in the rate of the antimycin A-sensitive cyclic photophosphorylation reaction (Fig. 3), which resulted from the NADPH-dependent increase in the concentration of reduced ferredoxin. The rate of antimycin A-insensitive cyclic photophosphorylation did not decline in parallel with NADP⁺ reduction, as it did in the presence of DCMU (Fig. 1), but rather continued to function at its maximum rate until a high ratio of NADPH to NADP⁺ was reached. If FNR is a component of this cyclic pathway (10), the direct reduction of FNR by NADPH may catalyze this reaction in addition to the reaction produced by reduced FNR by light-driven electron transport. Other experiments in this laboratory (not presented) have shown that the addition of NADPH and ferredoxin to DCMU-inhibited thylakoids catalyzes a slow rate of cyclic photophosphorylation which is largely insensitive to antimycin A. Similarly, cyclic electron flow in bundle sheath cells of C4 plants, which appears to be totally dependent upon NADPH as a source of electrons (13), is only slightly sensitive to antimycin A (25).

The NADPH/NADP⁺ ratio in the chloroplast has been proposed to control electron flow; a low ratio directs electrons to NADP⁺ reduction, while a high ratio diverts electrons to cyclic electron flow and O₂ (1, 2, 16, 17, 21). The data of Table II provide clear evidence for the ability of the NADPH/NADP⁺ ratio to switch electrons from the noncyclic to the cyclic pathways, since noncyclic electron transport (NADP⁺ reduction plus O₂ reduction) declines by 80% as the NADPH increases from 0 to 95% while the overall rate of ATP synthesis decreases only 47%. The mechanism of this switching presumably depends upon the redox requirements of each of the cyclic pathways.

Since the antimycin A-sensitive pathway is associated with the accumulation of reduced ferredoxin (10), its regulation may be controlled by the reaction of reduced ferredoxin with PQ, as suggested above. However, unlike the effect of DCMU, the acceleration of antimycin A-sensitive cyclic flow by a high ratio of NADPH to NADP⁺ must be a result of the increased reduction

**Table II. Effect of an Increasing NADPH/NADP⁺ Ratio on Electron Transport and Photophosphorylation using Ferredoxin/NADP⁺ as the Electron Acceptor**

<table>
<thead>
<tr>
<th>NADPH/NADP⁺</th>
<th>NADPH</th>
<th>O₂ Reduction</th>
<th>NADPH Reduction</th>
<th>Total e-transport</th>
<th>ATP Synthesis</th>
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<tr>
<td></td>
<td>%</td>
<td>μmol h⁻¹ mg⁻¹ Chl</td>
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was more than no by cyclic data insensitive than A-sensitive cycle closure of PSII
FERREDOXIN experiments chloroplasts under more pathways. Under these conditions, our in vitro ATP/NADPH ratio of data obtained A are suggested from this value by calculating the fraction of the P/O ratio (minus antimycin A, Fig. 2) greater than 1.25 which is sensitive to 1.7 μM antimycin A and the fraction which is insensitive to the inhibitor. The uppermost (A) curve shows the effect of the NADPH/NADP ratio on the total rate of cyclic photophosphorylation. In these assays, antimycin A had no effect on electron transport activity.

The ATP/ADP ratio drops and the NADPH/NADP ratio rises (e.g. low light intensity [14, 23]) the antimycin A-sensitive cycle may accelerate. As the additional ATP generated by these two reactions is utilized by CO₂ fixation activity, NADPH is oxidized and the rate of antimycin A-sensitive cyclic photophosphorylation is adjusted by the redox state of the NADP⁺ pool. Significantly, the P/O values in our experiments did not rise rapidly until a high ratio of NADPH to NADP⁺ was reached (Fig. 2). Since high concentrations of ATP may also be inhibitory to electron transport by increasing the proton back pressure (22), additional ATP may be generated only when necessary.

Evidence consistent with the existence of two cycles in vivo may be found in measurements of CO₂-dependent O₂ evolution in intact spinach chloroplasts. Antimycin A strongly inhibits photosynthesis driven by low intensity light, but inhibits only slightly in high intensity light (22). (Woo [24] reports a similar result, but Heber et al. [7] found the opposite effect using high and low intensity red light. The reason for this discrepancy is unclear. On the other hand, DeWolf et al. [5], using detergent-isolated PSI subchloroplast membranes which retain energy coupling, have found antimycin A-insensitive activity in agreement with our earlier report [10].) Under low intensity illumination the NADPH/NADP⁺ ratio reaches its greatest value (8). By the scheme presented above the high ratio of NADPH to NADP⁺ stimulates the antimycin A-sensitive cycle, which assumes a more important role in supplying ATP for CO₂ fixation, and thus increases the sensitivity of photosynthesis to antimycin A. Under high light intensity, which results in a lower ratio of NADPH to NADP⁺ (8, 23), sufficient ATP may be generated by noncyclic electron transport and the antimycin A-insensitive cycle; thus,

CONCLUSIONS

The possible existence of two cyclic electron transfer pathways leads to a novel explanation of the regulation of cyclic and noncyclic electron transport to optimize the ratio of ATP and NADPH in vivo. The presence or absence of 100% NADP⁺ in our in vitro system produces the extremes of possible redox conditions, at least in terms of the net oxidation-reduction state of the ferredoxin pool and the interphotosystem electron carriers.

Under these conditions electron flow through the two cyclic pathways appears to be mutually exclusive. However, with the more physiological situation of high NADPH levels (measured to be between 50 and 85% in intact photosynthesizing chloroplasts under a variety of conditions [8, 14, 23]) both cyclic reactions may be poised simultaneously (Fig. 3). Since one of the pathways, the antimycin A-insensitive reaction, is present at both low and high NADPH/NADP⁺ ratios, the P/2e ratio should not fall significantly below 1.5. However, under conditions where

**FIG. 2.** The effect of the NADPH/NADP ratio on the P/O (P/2e) value of electron transport to ferredoxin/NADP; (○), control values; (×), data obtained in the presence of 1.7 μM antimycin A. The P/O values in the absence of antimycin A are calculated from the data of Table II. (The ATP/NADPH ratio of electron transport can also be calculated from the data of Table II and ranges from 1.66 with 100% NADP to 6.4 with 95% NADPH and 5% NADP). The P/O values in the presence of antimycin A were obtained from otherwise identical experiments. Antimycin A produced no discernible effect on electron transport activity in the experiments shown.

of the ferredoxin pool rather than the partial oxidation of PQ because NADPH has been shown to reduce PQ and cause partial closure of PSII traps (16, 17). It is noteworthy that the antimycin A-sensitive cycle was not stimulated until the NADP⁺ pool was more than 80% reduced (Fig. 3); at this point the ferredoxin pool was more than 50% reduced. This suggests either that a threshold concentration of reduced ferredoxin is required, or a critical cyclic component becomes reduced at this point to allow cyclic flow, as suggested in Crowther and Hind (4). The antimycin A-insensitive cycle is well poised at a low NADPH/NADP⁺ ratio (where FNR turns over rapidly and the ferredoxin pool is largely oxidized) but begins to decline at NADPH concentrations greater than 80% (Fig. 3). This is consistent with our previous proposal that the activity of the antimycin A-insensitive pathway is inhibited by the reduction of the ferredoxin pool and interphotosystem electron carriers (10).
antimycin A has little effect on the rate of photosynthesis. A more direct effect of light intensity upon the sensitivity of the cyclic pathways to antimycin A was ruled out, because the inhibition pattern seen using our isolated thylakoids did not change over a wide range of light intensities during electron transport to ferredoxin/O₂ or ferredoxin/NADP⁺ (data not shown).

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LITERATURE CITED