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In Vitro Cyclic Electron Transport in Barley Thylakoids follows Two Independent Pathways[1]

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In vitro cyclic electron transport around PSI was studied in thylakoids isolated from barley (*Hordeum vulgare* L.). Redox poising was obtained by using anaerobic conditions, preillumination, and the addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Postillumination rates of $P700^+$ re-reduction of 1 to 5 electrons s^{-1} were observed, depending on the conditions. The thylakoids supported two parallel paths of cyclic electron transport that were distinguishable by differences in antimycin sensitivity, saturation characteristics, and substrate specificity. The pathway most sensitive to antimycin was not saturated at ferredoxin concentrations up to 50 μM, whereas the more insensitive pathway was saturated at 5 μM ferredoxin. At the lower concentration of reduced ferredoxin, the antimycin-sensitive rate of $P700^+$ re-reduction was lower than the antimycin-insensitive rate. The lower range of reduced ferredoxin concentrations are closer to in vivo conditions. Flavodoxin is shown to mediate cyclic electron transport. Flavodoxin was less efficient in mediating the antimycin-sensitive pathway but mediated the antimycin-insensitive pathway as efficiently as ferredoxin. Antibodies raised against ferredoxin:$NADP^+$ oxidoreductase had no effect on either pathway for re-reduction of $P700^+$. However, the ferredoxin:$NADP^+$ *oxidoreductase inhibitor 2′-monophosphoadenosine-5′-diphosphoribose* was able to inhibit the antimycin-sensitive as well as the antimycin-insensitive pathway.

Traditionally, cyclic electron transport has been considered a means of providing ATP for CO_2 fixation and other energy-requiring processes in addition to the ATP provided by linear electron flow. The importance of cyclic electron transport in ATP synthesis is rather clear in the bundle-sheath cells of C_4 plants (Bendall and Manasse, 1995). In contrast, the contribution of cyclic electron transport to overall photophosphorylation has been reported to be very small in C_3 plants and cyanobacteria (Maxwell and Biggins, 1976; Myers, 1986, 1987; Herbert et al., 1990; Yu et al., 1993; Bendall and Manasse, 1995). According to a recent view, the real significance of cyclic electron transport may be to prevent photoinhibition under conditions of stress by maintaining a sufficiently low pH in the thylakoid lumen to allow dissipation of excess light energy (Heber and Walker, 1992).

Ultimately, the significance of cyclic (and pseudocyclic) electron transport must be studied in whole organisms. However, knowledge of the pathways for cyclic electron transport is very limited, and an increased understanding of these pathways is a prerequisite for the design of conclusive in vivo experiments. There is general agreement that cyclic and linear electron transport share components from plastoquinone to PSI. However, the unique components of the cyclic electron transport chain are those catalyzing the transfer of electrons from the PSI acceptors to plastoquinone, and these have so far not been identified. Fd has long been known to catalyze cyclic electron transport around PSI in vitro (Tagawa et al., 1963). This strongly suggests that Fd is also involved in the in vivo pathway, but it has not been excluded that other stromal proteins could be involved. The common textbook view of cyclic electron transport shows donation of electrons by Fd to Cyt *b*. The fact that cyclic electron transport can be suppressed by antimycin A was thought to indicate the involvement of Cyt *b*, but unlike mitochondrial Cyt *b*, the chloroplast homolog has turned out to be insensitive to antimycin A (Moss and Bendall, 1984; Rich et al., 1992). Thus, the antimycin-sensitive component in thylakoid membranes has not been identified.

FNR is bound to the thylakoid membrane, and several workers have suggested the involvement of FNR in cyclic electron transport (Shahak et al., 1981; Hosler and Yocum, 1985; Cleland and Bendall, 1992). These suggestions have been based on the effect of inhibitors, but the specificity of the inhibitors for FNR has been difficult to prove. Antibodies to FNR have not been able to inhibit cyclic electron transport (Böhme, 1977; Shahak et al., 1981). Also, FNR is not sensitive to antimycin. An indirect role of FNR in cyclic electron transport as a producer of NADPH that could in turn reduce plastoquinone through an NADPH:plastoquinone oxidoreductase may also be envisioned (Ravenel et al., 1994). However, the activity of such a reductase in higher plant thylakoids has not been demonstrated, although it is clear that proteins homologous to subunits of mitochondrial NADH:ubiquinone oxidoreductase (complex 1) are present in the chloroplast (Berger et al., 1993).

The clearest assignment of a component specific for cyclic electron transport has been obtained using *Synechococcus* sp. PCC 7002 with an inactivated *psaE* gene for the

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FNR, ferredoxin:$NADP^+$ oxidoreductase; FQR, ferredoxin:quinone oxidoreductase; PADR, 2′-monophosphoadenosine-5′-diphosphoribose.

PSI-E subunit of PSI (Yu et al., 1993; Zhao et al., 1993). Photoautotrophic growth of the mutant was normal at intermediate light intensity, but compared to the wild type, growth was very slow at low light intensity (Zhao et al., 1993). The mutant did not grow photoheterotrophically in the presence of DCMU and glycerol. Compared to the wild type, the mutant was not much affected in its ability to carry out linear electron transport, whereas it was severely inhibited in cyclic electron transport (Yu et al., 1993). These results would seem to identify PSI-E as a component of the hitherto unidentified FQR. PSI-E itself carries no redox centers (Scheller et al., 1990; Falzone et al., 1994) but, interestingly, PSI-E has been shown to bind FNR in barley (Andersen et al., 1992).

This report characterizes cyclic electron transport under anaerobic conditions in thylakoids isolated from barley (*Hordeum vulgare* L. cv Svalöfs Bonus). Under these experimental conditions, two parallel pathways with different antimycin sensitivity, saturation characteristics, and substrate specificity are shown to operate. In addition to Fd, flavodoxin is shown to be a good mediator of cyclic electron transport. The FNR inhibitor PADR is shown to efficiently inhibit both pathways of cyclic electron transport, but antibodies against FNR were not able to inhibit the reactions.

MATERIALS AND METHODS

Preparation of Thylakoids and Proteins

Seedlings of barley (*Hordeum vulgare* L. cv. Svalöf's Bonus) were grown at 20°C in vermiculite on a 12-h light, 12-h dark cycle. Fluorescent tubes provided a PPFD of 80 μmol m^{-2} s^{-1}. After 7 d, the seedlings were harvested at the onset of the light cycle and the thylakoids were isolated essentially according to Cleland and Bendall (1992). The thylakoids were resuspended to a final concentration of 2 mg Chl/mL in homogenization buffer containing 20% glycerol, quickly frozen in liquid nitrogen, and stored at −80°C. Prepared and stored in this way, the thylakoids remained fully functional in linear and cyclic electron transport for at least 1 year.

Fd was isolated from barley according to Buchanan and Arnon (1971). Flavodoxin from *Anabaena* sp. PCC 7120 was expressed in *Escherichia coli* from a plasmid kindly provided by Drs. J. Zhao and D.A. Bryant (Pennsylvania State University, University Park) and isolated by chromatography (J. Zhao and D.A. Bryant, unpublished data).

Measurement of $P700^+$ Re-reduction

The turnover of $P700^+$ was measured in an anaerobic cuvette containing 50 mM Tricine (pH 7.5), 5 mM $MgCl_2$, 6 mM Glc, 2 mM ammonium chloride, 400 units/mL catalase, 4 units/mL Glc oxidase, and thylakoids corresponding to 25 μg of Chl in a total volume of 0.5 mL. Fd or flavodoxin was included as indicated in "Results." Prior to addition of Glc oxidase and thylakoids, the mixture was thoroughly flushed with argon.

The cuvette was illuminated with a Schott (Cologne, Germany) KL1500 halogen lamp. The actinic light was passed through a Schott KG3 filter to reduce UV and IR light. PPFD at the surface of the cuvette was 1000 μmol m^{-2} s^{-1}. After illumination for 30 s to establish redox poise, DCMU was added to a concentration of 10 μM. The rate of $P700^+$ re-reduction was measured as first described by Maxwell and Biggins (1976). Actinic light was applied for 1.2 s at a frequency of 0.1 to 0.2 hertz, and the redox state of P700 was determined from the changes in A_{834}. A diode laser (Melles Griot [Irvine, CA] model 06GIC108) connected to a laser driver (Melles Griot model 06DCD201) provided the measuring beam, which was expanded to a size of 5 × 3 mm. The detector was a 1-cm^2 photodiode (Melles Griot model 12DSI011) operated with 9 V of reverse bias and a 500- to 1000-Ω parallel resistor. The detector was positioned 2 cm from the cuvette and protected from actinic light and fluorescence by a 780-nm cut-on filter. The signal was passed to a TDS420 oscilloscope (Tektronix, Willsonville, OR) operated in DC mode with DC offset. The changes in A from 20 flashes were averaged and transferred to a computer. For each condition, three separate averages were acquired from the sample. Thus, it could be ascertained that there was no change in the performance of the sample during the experiment. The postillumination change in A was resolved into exponential decays by a Levenberg-Marquardt nonlinear regression procedure (Press et al., 1989). Plastocyanin makes a minor contribution to changes in A_{834}, but the redox state of plastocyanin follows light-induced redox changes in P700 and the two components can therefore be considered together (Klughammer and Schreiber, 1991; Heber et al., 1992).

DCMU, antimycin A, and DBMIB were added in small volumes (2–5 μL) from ethanol solutions. For the FNR-antibody inhibition experiments, thylakoids were preincubated with 150 μg of polyclonal rabbit antibodies (Andersen et al., 1992) or an equivalent amount of BSA for 30 min at 0°C in the dark. The incubation was carried out in an anaerobic chamber (85% N_2, 10% CO_2, approximately 5% H_2) to avoid introducing oxygen into the cuvette by adding the fairly large volume (62.5 μL) of antibody/thylakoid mixture. After incubation, the reaction mixture was added to the cuvette inside the anaerobic chamber.

Photoreduction of $NADP^+$ was measured as previously described (Naver et al., 1995). Antimycin A and Glc oxidase were obtained from Sigma. BSA, also from Sigma, was the highest grade available. Catalase was from Boehringer. All other reagents were analytical grade.

RESULTS

Thylakoids were illuminated prior to addition of DCMU to reduce the added Fd or flavodoxin. When the illumination was carried out in the presence of air, it was not possible to obtain a stable redox poise and to measure re-reduction of $P700^+$ at significant rates. However, when the thylakoids were kept anaerobic by using Glc oxidase and catalase, a stable redox poise of the system could be achieved and the rate of re-reduction of $P700^+$ did not change over the course of the experiment. Longer poising times or lower concentrations of DCMU than routinely used resulted in over-reduction of the Fd pool. This was

also the case when less than 3 μM Fd was used. Over-reduction of the acceptors is easily detected by the appearance of the rapid back reaction of electrons from the terminal iron-sulfur clusters F_A and F_B in PSI, which takes place with a typical time constant of 30 ms. Therefore, it is concluded that the poising resulted in nearly total reduction of the Fd pool but left a sufficient amount of oxidized Fd to allow the photooxidation of P700 during flashes. A typical data set obtained with 25 μM Fd is shown in Figure 1. The change in *A* after the illumination period could be fitted with a single exponential curve with a time constant of 350 ms. When 5 μM antimycin A was added, the decay in *A* was slowed down and a time constant of 870 ms was calculated. Addition of 5 μM DBMIB resulted in a calculated time constant of 1180 ms. This residual reaction was always present, even in the presence of oxygen. The residual rate is similar to the rate observed with isolated PSI (data not shown) and is therefore likely to reflect the ability of $P700^+$ to extract electrons from surrounding molecules or an artificial cycle catalyzed by DBMIB itself.

The result of experiments carried out in the presence of different concentrations of Fd and flavodoxin are shown in Figure 2. Fd stimulated the reaction rate in proportion to the concentration, and even at the highest concentration

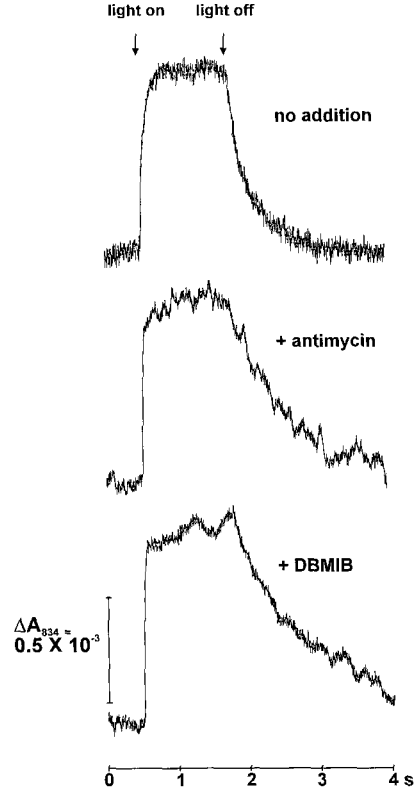

Figure 1. Flash-induced changes in *A* of P700 observed in thylakoids. The samples contained 25 μM Fd. Each curve is an average of 20 recorded transients. Time constants of 350 ms, 870 ms, and 1180 ms were calculated by fitting the changes in *A* (ΔA_{834}) with single exponential functions.

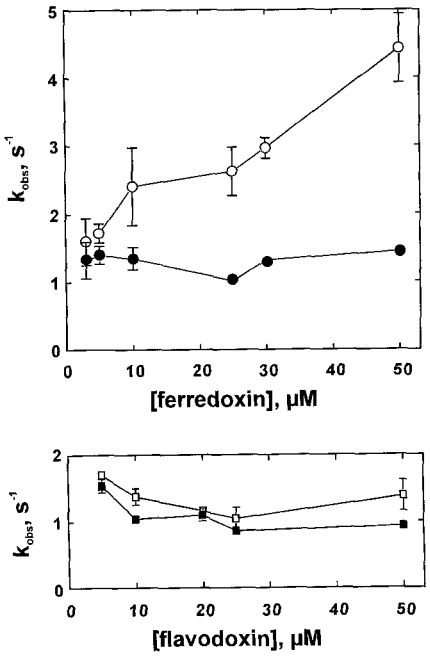

Figure 2. Re-reduction of $P700^+$ following a flash with different concentrations of Fd (upper panel) and flavodoxin (lower panel). The curves show the apparent rate constant (± SE) for $P700^+$ re-reduction with no addition (○, □) and with 5 μM antimycin (●, ■).

used there was no saturation. Antimycin A inhibited the reaction in all cases, but the residual reaction that is insensitive to antimycin was fairly independent of the Fd concentration. These results indicate the presence of two different routes for electron flow back to $P700^+$. The antimycin-sensitive route requires a high concentration of Fd with no saturation at 50 μM. The antimycin-insensitive route is already saturated at 3 to 5 μM Fd. Attempts to measure the re-reduction of $P700^+$ at concentrations of Fd lower than 3 μM were unsuccessful, since the experimental conditions used resulted in over-reduction of the acceptors. Flavodoxin was found to support cyclic electron transport (Fig. 2, lower panel). However, the reaction was largely saturated at 5 μM flavodoxin and the antimycin-sensitive reaction was much less significant than with Fd. The antimycin-insensitive reaction was similar with Fd and flavodoxin.

Figure 3 shows that Fd and flavodoxin were virtually indistinguishable in their ability to support photoreduction of $NADP^+$. In this analysis, the $NADP^+$ reduction was already close to saturation at 3 μM Fd or flavodoxin, and the apparent K_m was calculated to be 1.2 μM Fd or 1.3 μM flavodoxin. In the $NADP^+$-reduction assay, Fd and flavodoxin participate in separate reactions with PSI and FNR, and the former is likely to be limiting. However, the results indicate that there cannot be substantial differences between Fd and flavodoxin in the reaction with either PSI or FNR. The linear electron transport rates supported by 5 μM Fd or flavodoxin translate to a PSI turnover of about 75 s^{-1}. At the same concentration of Fd or flavodoxin, the rate of cyclic electron transport was only 1 to 5 s^{-1}.

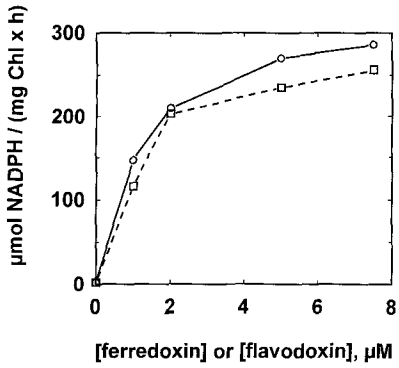

Figure 3. Photoreduction of $NADP^+$ with Fd (○) and flavodoxin (□). The reaction mixture contained 20 mM Tricine (pH 7.5), 8 mM $MgCl_2$, 0.3% decyl-maltoside, 0.5 mM $NADP^+$, 2 mM sodium ascorbate, 60 µM 2,6-dichlorophenolindophenol, 2 µM barley plastocyanin, 50 nM barley FNR, PSI corresponding to 4 µg of Chl, and Fd or flavodoxin as indicated. The light-dependent synthesis of NADPH was measured at 340 nm.

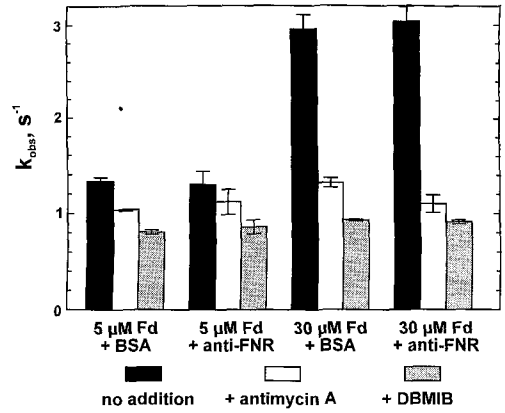

Figure 5. Effect of antibodies against barley FNR on re-reduction of $P700^+$. Prior to measurement in the presence of 5 or 30 µM Fd, the thylakoids were incubated with 150 µg of antibodies or BSA. The bars show apparent rate constants (k_{obs}) ± SE.

The effect of antimycin on P700 re-reduction was further investigated, as shown in Figure 4. Strong inhibition was obtained at 0.1 µM, as seen in the reaction with 25 µM Fd. With 5 µM Fd or with flavodoxin at either concentration, the effect of antimycin was small or absent. Higher concentrations of antimycin up to 10 µM had no additional effect (data not shown).

To investigate the possible involvement of FNR in the cyclic electron transport, the effect of antibodies raised against barley FNR was analyzed. $NADP^+$ photoreduction by thylakoids measured in the absence of added soluble FNR was completely inhibited by the FNR antibodies at a concentration of 0.3 mg/mL (data not shown). The detection limit for $NADP^+$ reduction was about 5 µmol mg^{-1} Chl h^{-1}, corresponding to a P700 turnover of 1 s^{-1}. Figure 5 shows the effect of FNR antibodies on $P700^+$ re-reduction rate. No inhibition was seen at either concentration of Fd.

The possible involvement of FNR in $P700^+$ re-reduction was further investigated by use of the inhibitor PADR. PADR was used at a concentration of 3 mM, which was shown to cause about 80% inhibition of $NADP^+$ photoreduction by PSI in the presence of added Fd and FNR (data not shown). In the assay for cyclic electron transport (Table I), PADR was as efficient as DBMIB in inhibiting $P700^+$ re-reduction and clearly both the antimycin-sensitive and -insensitive pathways were inhibited. In combination, PADR and antimycin A showed a greater tendency to inhibit than DBMIB. A likely explanation is that DBMIB, although inefficiently, supports an artificial cycle in a manner similar to that of many different hydrophobic redox mediators, e.g. 2,6-dichlorophenolindophenol.

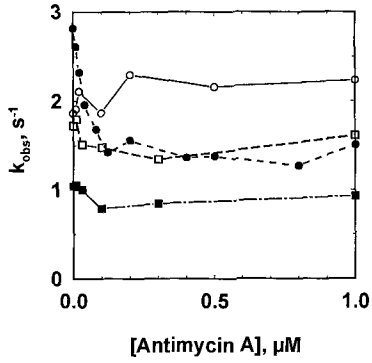

Figure 4. Effect of antimycin on re-reduction of $P700^+$. The postillumination kinetics of P700 was studied with 5 µM Fd (○), 25 µM Fd (●), 5 µM flavodoxin (□), and 25 µM flavodoxin (■).

DISCUSSION

The data presented in this paper show the presence of two parallel paths for in vitro cyclic electron transport in thylakoids under anaerobic conditions. The presence of parallel paths was first indicated by Hosler and Yocum (1985, 1987), who found a differential sensitivity to antimycin and FNR inhibitors, depending on the presence of more or less reducing conditions. Under more reducing conditions, the antimycin-sensitive pathway dominated, whereas the antimycin-insensitive pathway dominated in the presence of $NADP^+$. The concentration of reduced Fd was much higher under the more reducing conditions. Accordingly, these studies are in excellent agreement with the conclusion of the present study.

Table I. *Effect of PADR and antimycin A on $P700^+$ re-reduction*
k_{obs}, *Apparent rate constant.*

Inhibitor	k_{obs} ± SE	
	5 µM Fd	40 µM Fd
	s^{-1}	
None	1.4 ± 0.1	2.0 ± 0.2
3 mM PADR	0.9 ± 0.0	0.8 ± 0.1
5 µM antimycin A	1.2 ± 0.2	0.9 ± 0.1
3 mM PADR + 5 µM antimycin A	0.7 ± 0.1	0.6 ± 0.1
5 µM DBMIB	0.9 ± 0.1	0.8 ± 0.1

Hosler and Yocum used photophosphorylation as a measure of cyclic electron transport, and it has subsequently been suggested that the results could have been influenced by a varying degree of plastoquinone cycling under the different experimental conditions used (Cleland and Bendall, 1992). However, Q cycling would not affect the P700 turnover rates measured in the present study. Hosler and Yocum controlled the redox poise by using different ratios of NADPH and $NADP^+$ in the assay. Since NADPH:plastoquinone reductase has subsequently been suggested to be involved in cyclic electron transport (Ravenel et al., 1994), it was of interest to determine if the cyclic pathways were dependent on NADPH. In the present investigation, no NADPH was present and it is therefore concluded that two parallel paths for cyclic electron transport are indeed operating, neither of them involving NADPH.

Bendall and co-workers have analyzed cyclic electron transport in an assay system very similar to the one used in the present study (Cleland and Bendall, 1992; Manasse and Bendall, 1993). However, they monitored changes in *A* due to redox changes of Fd rather than of P700 and they routinely used high concentrations of reduced Fd. With plant thylakoids, at least 90% of the Fd oxidation was antimycin sensitive in the presence of 50 μM reduced Fd (Cleland and Bendall, 1992). With 50 μM Fd in the present study, the antimycin-sensitive electron transport was 87% of the activity above the DBMIB-insensitive background (Fig. 2). A concentration of about 75 μM reduced Fd was found to be required for saturation of the Fd oxidation rate following illumination (Cleland and Bendall, 1992). This result is in good agreement with those of the present investigation.

From the physiological perspective, it is important to know the concentration of reduced Fd under in vivo conditions. The total concentration of Fd in the stroma is around 100 μM (Böhme, 1977; Lawlor, 1993). The $NADP^+$:NADPH ratio in illuminated leaves varies with the conditions but is typically close to unity (Laisk et al., 1991; Siebke et al., 1991), corresponding to a stromal redox potential of about −320 mV. Obviously, stromal components are not at thermodynamic equilibrium during photosynthesis, but with a midpoint potential of −430 mV for Fd and considering the high efficiency of FNR, it seems reasonable to assume that the Fd pool is largely oxidized. If thermodynamic equilibrium were reached, the concentration of reduced Fd would be less than 2 μM. Siebke et al. (1991) have estimated that the Fd pool is only 3% reduced under steady-state conditions in vivo. Hosler and Yocum (1987), working with isolated thylakoids, found the Fd pool to be 10% reduced when the NADPH pool was 50% reduced. Based on these considerations, it seems likely that both the antimycin-sensitive and the antimycin-insensitive pathways for cyclic electron transport would significantly contribute to the overall rate under natural conditions. With only 3% reduced Fd, the antimycin-insensitive pathway would be predicted to be the more important pathway.

This conclusion is in agreement with in vivo photoacoustic studies of *Chlamydomonas* (Ravenel et al., 1994). The antimycin-sensitive pathway will dominate under the strongly reducing conditions that are often achieved during in vitro studies with isolated membranes and chloroplasts, in particular under anaerobic conditions as in the present study. However, it is important to realize that in leaves the reduction state of the stroma is tightly controlled and over-reduction does not occur, even when linear electron flow to CO_2 is severely restricted (Heber et al., 1992). In the bundle-sheath cells of C_4 plants, cyclic electron transport is the major route of electron transport. Notably, antimycin had little effect on CO_2 fixation by bundle-sheath cells of different C_4 plants (Woo et al., 1983).

The anaerobic conditions used in the present investigation will result in highly reduced conditions, and the possibility of a back reaction to $P700^+$ from reduced Fd through F_A/F_B should be considered. Antimycin and DBMIB do not inhibit forward electron transport by PSI under Fd-limiting conditions. Also, Fd and flavodoxin are indistinguishable in the forward reaction to $NADP^+$ (Fig. 3) but are very different in mediating antimycin-sensitive re-reduction of $P700^+$ (Figs. 2 and 4). Therefore, only the antimycin- and DBMIB-insensitive rate of $P700^+$ re-reduction may reflect a back reaction through PSI. This back reaction is unlikely to take place under redox conditions prevailing in vivo but could be responsible for part of the DBMIB-insensitive residual rate in vitro. However, the back reaction would appear to be insignificant, since the DBMIB-insensitive re-reduction of $P700^+$ was also observed in the presence of oxygen.

The rate of $P700^+$ re-reduction measured in the present in vitro study is 1 to 5 s^{-1} depending on the conditions. Similar rates of $P700^+$ re-reduction by cyclic electron transport have been found in vivo (Maxwell and Biggins, 1976; Myers, 1986; Yu et al., 1993). Thus, although different routes for cyclic electron transport may operate in vivo, e.g. via NADPH and NADPH:plastoquinone oxidoreductase, it can be concluded that the electron pathways observed in the present in vitro study are sufficient to account for in vivo cyclic electron transport.

Rates of cyclic electron transport are only a few percent of linear electron transport rates. Therefore, the possible physiological significance of the process has been contested (Myers, 1986, 1987). However, although such low rates may be unimportant for photophosphorylation, it is conceivable that cyclic electron transport could have a regulatory function (Heber and Walker, 1992). The physiological relevance of cyclic electron transport is indicated by the growth characteristics of the *Synechococcus psaE* mutant (Zhao et al., 1993), but further studies with different mutants and organisms are needed.

Fd has long been known to mediate cyclic electron transport in vitro. Many cyanobacteria, and some algae, synthesize flavodoxin rather than Fd under conditions of low iron supply and are able to sustain photoautotrophic growth with this enzyme. Also, as shown in this study, flavodoxin efficiently supports linear electron transport. However, it has not previously been reported whether flavodoxin can support cyclic electron transport. Presumably, cyclic electron transport would also take place under conditions characterized by preferential synthesis of flavodoxin. On the other hand, there may always be some Fd present, and

since the in vivo rates of cyclic electron transport are low compared to the linear rates, a small residual amount of Fd could be sufficient to sustain cyclic electron transport (Bendall and Manasse, 1995). The data presented here show that flavodoxin and Fd are similar in their ability to mediate the antimycin-insensitive pathway of cyclic electron transport, whereas Fd is more efficient in mediating the antimycin-sensitive pathway. In cyanobacteria, antimycin-sensitive cyclic electron transport has not been found (Manasse and Bendall, 1993), and therefore Fd and flavodoxin may perform equally well as catalysts of cyclic electron transport in these organisms. Obviously, this suggestion needs to be tested with cyanobacterial thylakoids.

The direct involvement of Fd or flavodoxin in cyclic electron transport has not been clearly established. The need for Fd in assays for cyclic electron transport may reflect the need for poising while the actual cyclic electron flow bypasses Fd. The antimycin-insensitive pathway does not change with Fd concentrations or when flavodoxin is used, and it can be speculated that this pathway is a more direct route from the PSI acceptors to plastoquinone.

Hosler and Yocum (1985) have suggested that FNR is involved in antimycin-insensitive cyclic electron transport. Since PSI-E has been shown to bind FNR in barley (Andersen et al., 1992) and since cyanobacteria that lack PSI-E are highly inhibited in cyclic electron transport (Yu et al., 1993), it is tempting to propose that the antimycin-insensitive route for cyclic electron transport includes the PSI-E-bound FNR, the latter reducing plastoquinone in the membrane. FNR bound to PSI-E is much less efficient than soluble FNR in $NADP^+$ reduction but shows an ability to reduce soluble quinones when NADPH is used as the electron donor (Nielsen et al., 1996). However, the actual reduction of plastoquinone rather than soluble quinones has not been demonstrated. The reactions of FNR bound to PSI were shown to be insensitive to antimycin (Nielsen et al., 1995). The *Synechococcus* sp. PCC 7002 studied by Yu et al. (1993) has no FNR bound to PSI-E (Schluchter and Bryant, 1992), and the manner in which PSI-E mediates cyclic electron transport is therefore not understood. Possibly, PSI-E interacts with an unidentified protein that catalyzes the reduction of plastoquinone.

The antimycin-sensitive pathway for Fd-mediated plastoquinone reduction does not appear to include a possible quinone-reducing site on PSI. The concentration of Fd needed to saturate the reaction is much higher than the concentration needed for saturating electron transfer to FNR. Also, the differences between Fd and flavodoxin in antimycin-sensitive cyclic electron transport are not correlated with differences in electron transport at the level of PSI. Thus, the antimycin-sensitive cyclic electron transport must include a protein or protein complex in the thylakoid membrane that is not associated with PSI. The identity of this FQR has been elusive. Some data have suggested the involvement of FNR in the antimycin-sensitive reaction (Shahak et al., 1981; Cleland and Bendall, 1992), but a major objection to this hypothesis has been the inability of FNR antibodies to inhibit the reaction (Böhme, 1977; Shahak et al., 1981). The existence of two pathways of cyclic electron transport was not addressed in the previous studies, but in the present study it is evident that neither pathway is affected by FNR antibodies. Thus, there is still no conclusive evidence that FNR is involved in cyclic electron transport. However, it must be realized that the rates of cyclic electron transport are much lower than the rates of linear electron transport and consequently an almost complete inhibition of linear electron transport may still be insufficient for inhibition of cyclic electron transport. The binding site for FNR in the thylakoid membrane is controversial and several different binding proteins have been reported (Vallejos et al., 1984; Ceccarelli et al., 1985; Shin et al., 1985; Matthijs et al., 1986; Berzborn et al., 1990; Nakatani and Shin, 1991; Andersen et al., 1992). Whether FNR bound to these proteins binds antimycin or is able to reduce plastoquinone has not been reported.

PADR is reported to be a specific inhibitor of FNR (Avron, 1981), and a recent study has shown inhibition of cyclic electron transport in *Chlamydomonas* (Ravenel et al., 1994). The interpretation of the experiment was that PADR prevents the FNR-catalyzed synthesis of NADPH, causing substrate limitation for NADPH:plastoquinone reductase. The presence of this reductase is quite well established in *Chlamydomonas*, and this pathway for cyclic electron transport is likely to be operating (Cleland and Manasse, 1995). However, in the present study no NADPH was present during the measurements. Therefore, the inhibition by PADR must be a more direct effect on the plastoquinone reduction. PADR did not appear to inhibit antimycin-sensitive cyclic electron transport in *Chlamydomonas* (Ravenel et al., 1994), which is contrary to the conclusion of the present investigation. At present we cannot decide between possible explanations for this difference. Although the inhibitory effect of PADR would support the idea that FNR is involved in cyclic electron transport, it should be realized that the specificity of PADR has not been extensively studied. At the rather high inhibitor concentration used, it is likely that the specificity is low. Ravenel et al. (1994) used a concentration of 2.5 mM, i.e. almost the same as in the present study, but used whole cells subjected to electroporation, and therefore it is not known what the inhibitor concentration was inside the chloroplasts.

The isolation and characterization of the enzymes specific to cyclic electron transport have not been achieved,

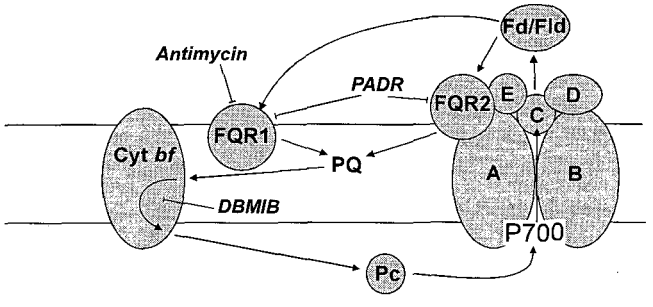

Figure 6. Model of pathways for cyclic electron transport in barley thylakoids. The sites of action of inhibitors used in the present study are indicated. FQR1 is hypothesized to be associated with the PSI-E subunit of PSI. A, B, C, D, E, Subunits of PSI; Fld, flavodoxin; Pc, plastocyanin; PQ, plastoquinone.

and therefore our understanding of this activity is remarkably incomplete compared to our understanding of the linear electron transport. A schematic model for cyclic electron transport based on the results of the present study is presented in Figure 6. The main conclusion is that there are two different pathways for cyclic electron transport in barley thylakoids. Flavodoxin was shown to catalyze in vitro cyclic electron transport. Since reduced flavodoxin is much more easily handled than reduced Fd, the conclusions of the present investigation should facilitate the identification and purification of FQR. Eventually, we may also be able to understand how the cyclic electron transport pathways are regulated in response to a variety of conditions and how this in turn may affect the overall photosynthetic process and adaptability of plants. A clear identification of the enzymes involved in cyclic electron transport is necessary for the study of the significance of the process in vivo, e.g. by suppression of genes by transformation with antisense constructs.

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