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Donation of Electrons to Plastoquinone by NAD(P)H Dehydrogenase and by Ferredoxin-Quinone Reductase in Spinach Chloroplasts

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The reduction of plastoquinone by NADPH was detected as an increase in the dark level of Chl fluorescence in osmotically ruptured chloroplasts of spinach. This activity was observed only when the chloroplasts were ruptured in a medium containing a high concentration of $MgCl_2$. The activity was suppressed by inhibitors of the respiratory NADH dehydrogenase (NDH) complex in mitochondria, capsaicin and amobarbital, suggesting that the activity was mediated by chloroplastic NDH complex. Antimycin A, an inhibitor of ferredoxin-quinone reductase (FQR), and the protonophore nigericin also inhibited the increase in Chl fluorescence by NADPH. By contrast, *N*-ethylmaleimide (NEM), an inhibitor of ferredoxin-NADP$^+$ reductase (FNR), did not suppress the fluorescence increase, showing that FNR is not involved in this reaction. When the osmotically ruptured chloroplasts were washed by centrifugation, a further addition of ferredoxin as well as NADPH was required for an increase in fluorescence. This ferredoxin-dependent activity also was suppressed by antimycin A, but only partly inhibited by capsaicin or amobarbital, suggesting that this is mediated mainly by FQR. These findings suggest that the NADPH-binding subunit of NDH complex is easily dissociated from the thylakoid membranes during the process of the washing the thylakoids by centrifugation.

Key words: Antimycin A — Cyclic electron transport — Ferredoxin-NADP$^+$ reductase (FNR, EC 1.6.7.1) — Ferredoxin-quinone reductase (FQR) — NAD(P)H dehydrogenase (NDH, EC 1.6.99.3) — *Spinacia oleracea* (Spinach).

The presence and expression of chloroplastic *ndh* genes which encode homologues of subunits of mitochondrial NADH dehydrogenase complex (NDH) have been demonstrated (Ohyama et al. 1986, Shinozaki et al. 1986, Nixon et al. 1989, Berger et al. 1993), and it has been suggested that chloroplasts contain complex I with the putative activity of NAD(P)H-plastoquinone oxidoreductase (Ohyama et al. 1988, Marder and Barber 1989, Kubicki et al. 1996). However, this activity has not been demonstrated in chloroplasts. In cyanobacteria, an NADPH-specific NDH complex in the thylakoid membranes donates electrons to the plastoquinone pool; thus, it mediates not only the respiratory electron flow but also the cyclic electron flow around PSI (Mi et al. 1992a, b, 1994, 1995). The addition of NADPH to the thylakoid membranes of *Synechocystis* PCC 6803 induced the reduction of plastoquinone as judged by an increase in Chl fluorescence. Since no similar increase in Chl fluorescence by NADPH was found in NDH complex-defective mutants (Ogawa 1991a, b), the reduction of plastoquinone was attributed to the NDH complex (Mi et al. 1995). Based upon the close evolutionary and genetic relationships of cyanobacteria and chloroplasts, a similar function of NDH complex in chloroplasts may be assumed. However, no study has indicated the participation of NDH complex in the reduction of plastoquinone in chloroplasts. Instead, Mills et al. (1979) reported a different type of NADPH-dependent plastoquinone reducing activity in spinach chloroplasts which required ferredoxin. This activity was inhibited by the antibody against ferredoxin-NADP$^+$ reductase (FNR) and by *N*-ethylmaleimide (NEM), showing the involvement of FNR in this reaction. A ferredoxin-quinone reductase (FQR), a putative mediator between ferredoxin and plastoquinone in the ferredoxin-dependent cyclic electron transport around PSI, which is sensitive to antimycin A (Moss and Bendall 1984, Bendall and Manasse 1995), was also suggested to be involved in the final step of the plastoquinone reduction. Thus the following electron transport pathway was assumed:

NADPH → FNR → ferredoxin → FQR → plastoquinone.

Although the molecular identity of FQR has long remained unclear, Miyake et al. (1995) recently demonstrated the participation of a low redox potential (menadiol-reducible) Cyt b_{559} in the antimycin-A sensitive cyclic elec-

Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; FNR, ferredoxin-NADP$^+$ reductase; FQR, ferredoxin-quinone reductase; Fo, level of Chl fluorescence induced by weak measuring light; NDH, NAD(P)H dehydrogenase; NEM, *N*-ethylmaleimide.

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tron transport in the mesophyll thylakoids of maize.

In this report, we show an NADPH-plastoquinone reductase activity determined by an increase in the Chl fluorescence level in the dark by the addition of NADPH to osmotically ruptured chloroplasts of spinach which is not dependent on FNR and thus differs from that reported by Mills et al. (1979). This activity was observed only when the chloroplasts were ruptured in the medium containing a high concentration of $MgCl_2$. Since inhibitors of the NDH complex in mitochondria suppressed the increase in fluorescence, this activity is attributable to the NDH complex. The activity was lost when the thylakoids were washed by centrifugation. However, when ferredoxin was added in the presence of NADPH, a reduction of plastoquinone was observed which was attributable to a series of electron transfers through FNR and FQR. The washing process might dissociate the NADPH-binding subunit of NDH. The presence of high concentrations of $MgCl_2$ during the washing did not retain the NADPH-dependent activity, suggesting that not only high Mg but also an unknown factor is required for retention of the NDH activity. Possibly because of this labile nature, chloroplastic NDH activity has not been demonstrated so far.

Materials and Methods

Spinach leaves were obtained from local markets. Intact chloroplasts were prepared from mature leaves with Percoll gradient centrifugation (Asada et al. 1990). The intact chloroplasts were osmotically ruptured in either a high or low Mg medium. The low Mg medium contained 2 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, 30 mM KCl, 0.25 mM KH_2PO_4 and 50 mM HEPES (pH 7.6). The high Mg medium contained 30 mM instead of 2 mM $MgCl_2$. The suspensions of ruptured chloroplasts were diluted with the same medium to 10 μg Chl ml^{-1} and immediately used for measurements of Chl fluorescence with a PAM Chl fluorometer (Walz, Effeltrich, Germany). Details for the fluorometer setup were described previously (Schreiber et al. 1995). Ferredoxin from spinach was purchased from Sigma.

Results

Plastoquinone reduction in the osmotically ruptured chloroplasts—Intact chloroplasts from spinach were ruptured in the sorbitol free medium containing 30 mM $MgCl_2$ and immediately used for measurements of Chl fluorescence. Addition of NADPH induced an increase in the fluorescence level, indicating the reduction of the plastoquinone pool in the dark (Fig. 1). The gradual decline of the fluorescence after the increase might be due to activity of PSI excited by a weak measuring light or a putative terminal oxidase in the thylakoids, as shown later. Subsequent addition of ferredoxin also induced a small increase in Chl fluorescence, but no increase was observed without the prior addition of NADPH. Addition of NADH did not induce increases in the fluorescence, showing that the plastoquinone-reducing enzyme has a much higher affinity to NADPH than to NADH. Antimycin A, an inhibitor of FQR, suppressed the fluorescence increase by NADPH. Amobarbital (amytal) and capsaicin, inhibitors of the mitochondrial NDH complex (Yagi 1990, Singer and Ramsay 1992, Mi et al. 1995), also suppressed the fluorescence increase by NADPH. Interestingly, these inhibitors did not inhibit the fluorescence increase induced by ferredoxin. The protonophore nigericin also suppressed the increase in fluorescence by NADPH, which is in agreement with the results by Mano et al. (1995), who showed that the protonophore inhibits electron donation to plastoquinone from photoreduced stromal components in intact chloroplasts of spinach. In the presence of some of inhibitors shown above, addition of NADPH slightly decreased the level of Chl fluorescence for some unknown reason. This decrease in the fluorescence level by NADPH was most evidenct in

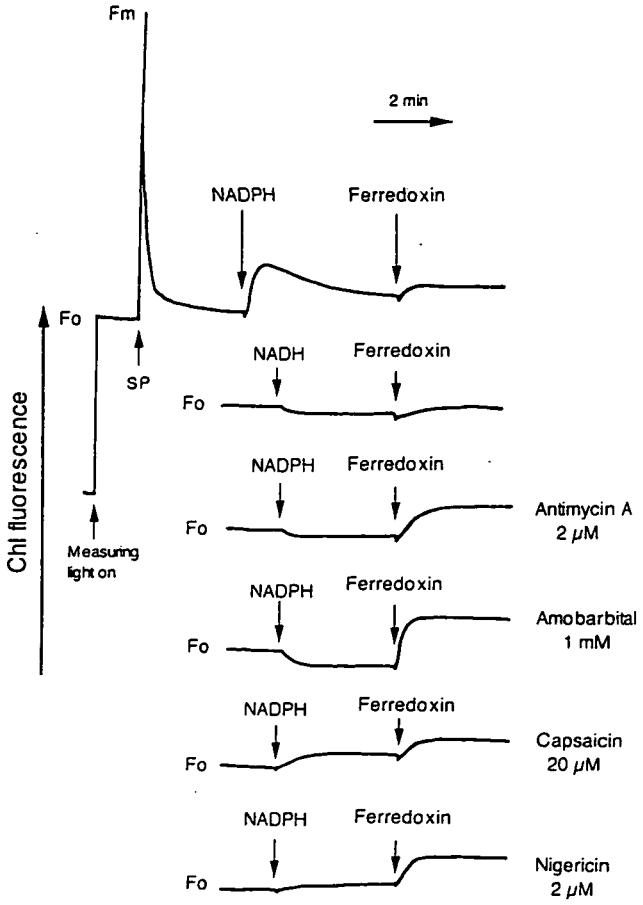

Fig. 1 Increases in fluorescence by NAD(P)H (0.25 mM) and ferredoxin (5 μM) under the weak measuring light. Osmotically ruptured chloroplasts in high Mg medium (10 μg Chl ml^{-1}) were incubated for 2 min with inhibitors prior to the fluorescence measurements. Fo indicates the level of Chl fluorescence induced by the measuring light. Fm is the yield of the maximum fluorescence induced by a saturating pulse (SP, 1 s, 3,000 μE m^{-2} s^{-1}).

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the presence of 1 mM amobarbital. In the presence of other inhibitors (antimycin A, capsaicin and nigericin), the reduction of fluorescence by NADPH was not as evident as in the presence of amobarbital, suggeting imperfect inhibition of the NADPH-dependent quinone reduction by these inhibitors. Higher concentrations of capsaicin and nigericin did not lead to further inhibition of the NADPH-dependent quinone reduction (data not shown). However, higher concentrations of antimycin A caused further inhibition (see Fig. 6).

Effects of N-ethylmaleimide—If a sufficient amount of ferredoxin was bound to the thylakoid membranes in the ruptured chloroplasts, the electron transfer from NADPH to plastoquinone sequentially catalyzed by FNR and FQR was possible without the addition of exogenous ferredoxin. To test whether FNR is involved in the observed reduction of plastoquinone by NADPH in the osmotically ruptured chloroplasts, effects of the FNR inhibitor NEM were examined (Fig. 2). To avoid oxidation of plastoquinone by PSI excited with a weak measuring light and by a putative terminal oxidase, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), an inhibitor of cytochrome *b/f* complex at the quinol oxidation site, was added prior to the measurement. This addition of DBMIB suppressed the gradual declines of fluorescence after the rapid increase by NADPH. The addition of NEM did not suppress the increase of fluorescence by NADPH, but rather slightly stimulated the fluorescence increase and inhibited an increase of fluorescence after the addition of ferredoxin. Thus, FNR does not participate in the quinone reduction by NADPH. The linear electron flow from H_2O to $NADP^+$ in the presence of added ferredoxin was reported to be inhibited by 5 mM NEM, showing that FNR is inhibited at this concentration (Mills et al. 1979). Another specific inhibitor of FNR, heparin (Hosler and Yocum 1985) at the concentration of 40 μM, showed effects identical to those of NEM (data not shown).

Effects of washing—The NADPH-dependent activity of quinone reduction was found only when the intact chloroplasts were ruptured in the medium which contained a high concentration of $MgCl_2$ (Fig. 3), suggesting that a factor involved in this reaction is easily dissociated from the thylakoid membranes in a low Mg medium. When the osmotically ruptured chloroplasts were washed by centrifugation, the NADPH-dependent reduction of plastoquinone decreased. Adding ferredoxin in the presence of NADPH induced a significant increase in Chl fluorescence. The presence of high concentrations of $MgCl_2$ throughout the rupturing and washing processes did not prevent the loss of NADPH-dependent activity (Fig. 3C), suggesting that not only high concentrations of $MgCl_2$ but also an un-

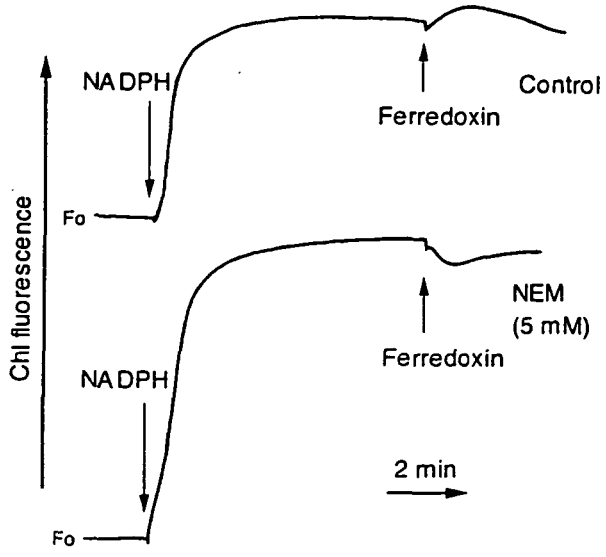

Fig. 2 Effects of *N*-ethylmaleimide (NEM) on the fluorescence increase by NADPH and ferredoxin in the osmotically ruptured chloroplasts. The procedure for the measurements was the same as Fig. 1 except that DBMIB (5 μM) was added to the reaction mixture in advance. Length of the thick upward arrow represents Fo intensity of Chl fluorescence.

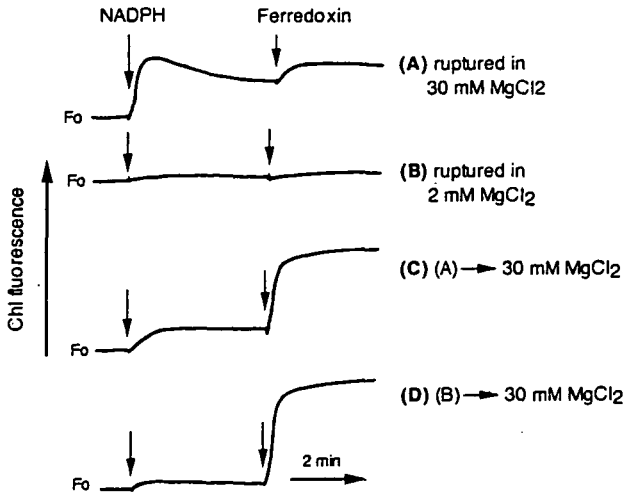

Fig. 3 Effects of the washing and $MgCl_2$ concentration on the fluorescence increase by NADPH and ferredoxin. (A), Intact chloroplasts were osmotically ruptured in the sorbitol-free medium containing 30 mM $MgCl_2$ and immediately assayed as in Fig. 1. (B), Intact chloroplasts were ruptured in the sorbitol-free medium containing 2 mM $MgCl_2$ and immediately assayed. (C), The chloroplasts ruptured in the sorbitol-free medium containing 30 mM $MgCl_2$ was centrifuged (5,000 × *g*, 5 min) and resuspended in the medium containing 0.3 M sorbitol and 30 mM $MgCl_2$ (1 mg Chl ml^{-1}). The suspension was kept on ice for 1 h, and then diluted with the sorbitol-free medium with 30 mM $MgCl_2$ (final conc. 10 μg Chl ml^{-1}) for fluorescence measurement. (D), The chloroplasts ruptured in the sorbitol free medium containing 2 mM $MgCl_2$ were treated as in (C). Length of the thick upward arrow represents Fo intensity of Chl fluorescence.

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known factor which is easily lost during washing are required for retention of the NADPH-dependent activity. The ferredoxin-requiring activity, which significantly increased after the washing, was independent of $MgCl_2$ concentration during the rupturing process (Fig. 3C, D). However this activity was found when the washed thylakoids were suspended in high $MgCl_2$ medium but not in low $MgCl_2$ medium (see Fig. 5).

Plastoquinone reduction in the washed thylakoids—Effects of various inhibitors on the ferredoxin-dependent activity in thylakoids which had been ruptured in low Mg medium and washed in high Mg medium are shown in Fig. 4. Antimycin A is the most potent inhibitor of this activity. Amobarbital and capsaicin, which suppressed NADPH-dependent activity, partly suppressed the ferredoxin-requiring activity; the rates of the increase in Chl fluorescence in the presence of 1 mM amobarbital and 5 μM capsaicin were 63% and 57% of the control, respectively. Higher concentrations of the NDH inhibitors did not further suppress the fluorescence increase. A remaining minor activity in the presence of 2 μM antimycin A was not suppressed by the addition of either amobarbital or capsaicin. Unlike the NADPH-dependent activity, nigericin did not suppress the ferredoxin-requiring activity (data not shown). Compared with NADPH, NADH induced a much smaller increase in fluorescence (data not shown).

Dependence on $MgCl_2$—Effects of concentrations of $MgCl_2$ on the NADPH-dependent activity (in ruptured chloroplasts) and on the ferredoxin-requiring activity (in washed thylakoids) were examined (Fig. 5). The ferredoxin-requiring activity (by FNR and FQR) was saturated at about 15 mM $MgCl_2$, while the NADPH-dependent activity (by NDH) was saturated about 30–50 mM. These results suggest different molecular bases for the requirement for $MgCl_2$ in these two activities.

Antimycin A inhibition curves—Effects of concentrations of antimycin A on the NADPH-dependent activity (in ruptured chloroplasts) and on the ferredoxin-requiring activity (in washed thylakoids) were examined (Fig. 6). Both activities were inhibited similarly, with a half inhibition concentration of about 0.1 μM antimycin A. However, in the 1–2 μM range, a minor difference between the sensitivities of these two activities was found. The ferredoxin-requiring increase in fluorescence in the ruptured choroplasts shown in Fig. 1 was not clearly inhibited by antimycin A, suggesting that the ferredoxin-requiring activ-

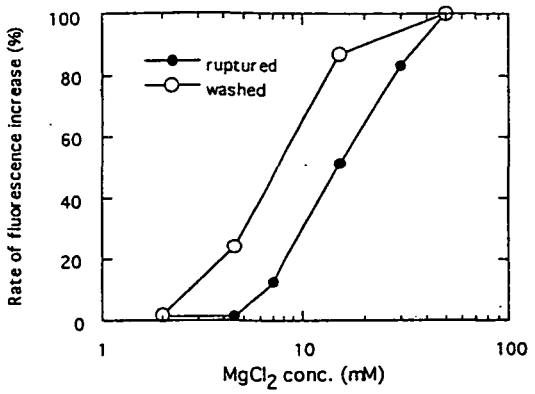

Fig. 5 Concentration effects of $MgCl_2$. Chloroplasts were ruptured in medium with different concentrations of $MgCl_2$, and the rate of increase in Chl fluorescence upon the addition of NADPH (0.25 mM) was measured (closed circles), or chloroplasts were ruptured in low Mg medium, washed by centrifugation, and resuspended in medium with a different concentration of $MgCl_2$. Then, the rate of increase in Chl fluorescence upon addition of NADPH (0.25 mM) in the presence of ferredoxin (5 μM) was measured (open circles). Values are expressed relative to those in 50 mM $MgCl_2$.

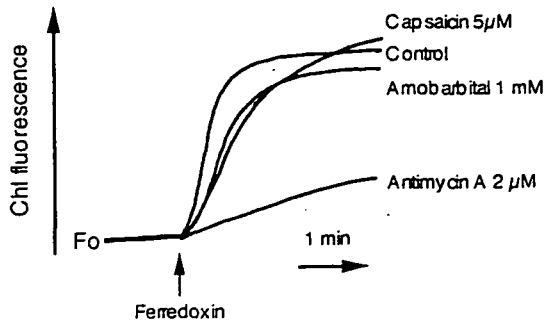

Fig. 4 Effects of inhibitors on the fluorescence increase by ferredoxin (5 μM) in the presence of NADPH (0.25 mM) in the washed thylakoids. Washing procedure was the same as in Fig. 3(D). The inhibitors were added to the suspension 2 min before fluorescence measurements. Length of the thick upward arrow represents Fo intensity of Chl fluorescence.

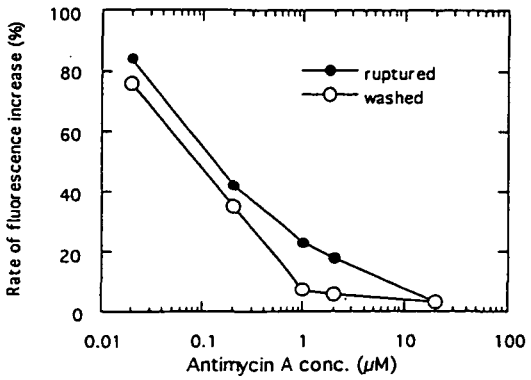

Fig. 6 Inhibition curves due to antimycin A. The ruptured chloroplasts (closed circles) and washed thylakoids (open circles) were prepared as in Fig. 5 using the high Mg (30 mM) midium, and the activities were measured as in Fig. 5 in the presence of varying concentrations of antimycin A. Values are expressed relative to those in the absence of antimycin A.

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ity found in ruptured chloroplasts and that in washed thylakoids were due to different oxidoreductases.

In general, the extent of the increase in Chl fluorescence induced by NADPH changed with the rate of the increase in fluorescence in the experiments shown in Fig. 5 and 6.

Discussion

Responses to various types of inhibitors indicate that the NADPH-dependent activity of reducing plastoquinone and the ferredoxin-requiring activity could be attributed to different enzyme systems. We propose that the ferredoxin-requiring activity is attributed to FNR+FQR (pathway (ii) in Fig. 7) and identical to the fluorescence-increasing activity reported by Mills et al. (1979), because the activity was (1) suppressed by antimycin A, (2) requiring high $MgCl_2$ and (3) insensitive to uncoupling by the protonophore. The NADPH-dependent activity is supposed to be mediated by the NDH complex, because the activity was inhibited by specific inhibitors of mitochondrial NDH, amobarbital and capsaicin, but not by the inhibitors of FNR.

Inhibition by antimycin A of the NDH-mediated reduction of plastoquinone by NADPH is indicative of sequential electron transport from NDH to antimycin A-sensitive FQR (pathway (i) in Fig. 7). It may also be explained by the presence of an antimycin A binding site in NDH complex (pathway (iii) in Fig. 7). Since the sensitivities to antimycin A in NDH complex and FNR+FQR were not clearly different (Fig. 7), it is difficult to determine which is the case. However, data supporting pathway (iii) have been obtained in a preliminary study (Endo et al. in preparation) with thylakoids from a mutant tobacco defective in its chloroplastic NDH complex (Shikanai et al. in preparation). That is, two ferredoxin-plastoquinone reducing activities have been shown in thylakoid preparations from wild-type tobacco, both of which were inhibited by antimycin A with different sensitivities. One of them was attributable to the NDH complex and the other to FQR; only one of them (FQR-dependent one) was found in the NDH-defective mutant. Thus, the NDH complex in tobacco chloroplast has

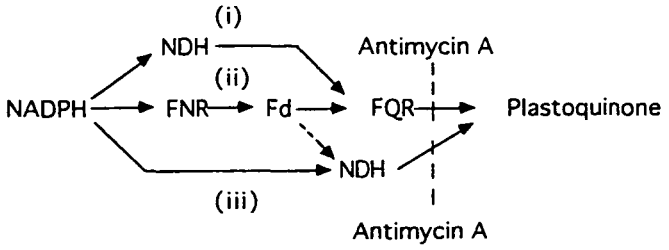

Fig. 7 Pathways of plastoquinone reduction by NADPH in thylakoid membranes of spinach. Fd, ferredoxin. Pathway (ii) is the previously proposed pathway by Mills et al. (1979).

the activity of ferredoxin-plastoquinone reductase and has an antimycin A binding site, supporting the pathway (iii). This thylakoid preparation of tobacco might correspond to the washed thylakids of spinach shown in this report, in which the NDH complex lost activity of the NADPH oxidation.

The chloroplastic NDH complex in spinach showed a much higher affinity to NADPH than to NADH, which is consistent with the characteristics found in the cyanobacterial NDH complex (Mi et al. 1995). It should be noted that, in both cases, the NDH complex is associated with photosynthetic electron transport. This suggests the possibility that the NDH complex is involved in NADPH-mediated cyclic electron transport around PSI in spinach chloroplasts, as demonstrated in cyanobacteria (Mi et al. 1992a, b, 1994, 1995).

The spinach NDH complex shown here seems to be able to accept electrons from reduced ferredoxin, because the ferredoxin-requiring activity was partly suppressed by amobarbital and capsaicin (Fig. 4). Although the antimycin A-sensitive cyclic electron transport has been considered to be mediated by FQR (Moss and Bendall 1984), the present results show that both FQR and NDH activities are included in so called antimycin A-sensitive cyclic flow. Since the thylakoidal NDH complex of *Synechocystis* PCC 6803 was insensitive to antimycin A (Mi et al. 1995), the thylakoidal NDH complex in higher plants and that in cyanobacteria appear to differ in terms of response to the inhibitor.

Recently, Guedeney et al. (1996) demonstrated the association of FNR with subunits of the chloroplastic NDH complex using immunoblot analysis and proposed that FNR functions as the NADPH-binding component of the NDH complex. However, the insensitivity of the increase in Chl fluorescence by NADPH to NEM and heparin (Fig. 2) indicates that the chloroplastic NDH complex itself includes the NADPH-binding component and that FNR is not required for the reduction of the plastoquinone by NADPH.

The activity of NADPH-dependent reduction of plastoquinone found here was lost during the washing process, indicating release of a putative NAD(P)H binding subunits or inactivation during the washing of the thylakoids. The requirement of a high concentrations $MgCl_2$ for the NADPH-dependent increase in fluorescence supports dissociation of NADPH binding subunit rather than inactivation, because Mg^{2+} can bind peripheral (stromal) proteins to the membranes (Süss et al. 1993, Ogawa et al. 1997). Dissociation of intact NDH complex from the membranes is inconceivable because the reported NDH complexes are membrane spanning and require solubilization by detergents for their dissociation.

The ferredoxin-requiring activity was not evident in the freshly ruptured chloroplasts, indicating that the (FNR+FQR) reaction was suppressed by an unknown

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mechanism. This might be in part due to the competition of NDH and FNR for NADPH, because inhibitors of NDH stimulated ferredoxin-requiring activity in the ruptured chloroplasts (Fig. 1). However, this stimulation was rather minor when compared with the ferredoxin-requiring activity after the wash. One possible explanation is that the dissociation of NADPH-binding subunits of NDH complex may facilitate the access of NADPH to FNR.

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