*Proc. Natl. Acad. Sci. USA*
Vol. 79, pp. 5494–5498, September 1982
Biochemistry

# Reconstruction of the chloroplast noncyclic electron transport pathway from water to NADP with three integral protein complexes

(photosystem I/photosystem II/cytochrome $b_6$–$f$)

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*Communicated by Warren L. Butler, June 7, 1982*

**ABSTRACT** **Reconstruction of photosynthetic noncyclic electron transport from water to NADP has been accomplished by using three integral protein complexes isolated from chloroplast thylakoid membranes: photosystems I and II and the cytochrome $b_6$–$f$ complex. This system shows an absolute dependence on the presence of all three protein complexes for NADP reduction, in addition to plastocyanin, ferredoxin, and ferredoxin-NADP reductase. The reconstructed system was found to be sensitive to low concentrations of known inhibitors of noncyclic electron transport. Depletion of the Rieske iron–sulfur center and bound plastoquinone from the cytochrome $b_6$–$f$ complex resulted in an inhibition of the photoreduction of NADP.**

The electron transport system in chloroplast photosynthesis is widely accepted to involve the cooperation of two light reactions, photosystems I and II, in the noncyclic transfer of electrons from water to NADP (1, 2). The two light reactions are connected by a series of electron carriers (the secondary electron carriers: plastoquinone, the Rieske iron–sulfur center, cytochrome $f$, and plastocyanin) through which electron transfer is accompanied by proton translocation across the thylakoid membrane (3). The established proton gradient is used to drive the synthesis of ATP during noncyclic electron flow (4).

Recent studies have supported the concept that the electron carriers are organized into three integral protein complexes in the chloroplast membrane. Photosystems I and II are localized in separate complexes that contain the essential components involved in the primary photosynthetic reactions (reaction center chlorophyll and primary electron acceptors) as well as associated antenna pigments (5–7). A chloroplast cytochrome complex, the cytochrome $b_6$–$f$ complex, which contains many of the secondary electron carriers, has recently been isolated and characterized (8). Our current picture of the organization of these complexes is that electrons released from water pass through photosystem II to reduce plastoquinone. The cytochrome $b_6$–$f$ complex functions as a plastohydroquinone-plastocyanin oxidoreductase in transferring electrons from reduced quinone to plastocyanin. Plastocyanin, a water-soluble protein, links the cytochrome complex to photosystem I (9). Electrons then pass through the photosystem I complex to NADP in the presence of ferredoxin and the ferredoxin-NADP reductase.

The organization of the electron transfer complexes in the membrane is an area of current interest. Studies of the lateral organization of the photosystem I and II complexes in the thylakoid membrane have resulted in a model in which these two complexes are spatially separated, photosystem I being located in nonappressed stromal lamellae and photosystem II in the appressed granal membranes (10). The consequences of this physical separation of the two photosystems in terms of electron transfer mechanisms have been discussed by Anderson (11).

In order to study the interaction of the chloroplast integral protein complexes, we have employed the techniques of resolution and recombination. This approach has been used extensively in the study of the organization of the four electron-transport complexes of the mitochondrial membrane (12). In this communication, we report the reconstruction of the chloroplast noncyclic electron transport chain from water to NADP with three purified, highly resolved integral protein complexes. In the presence of photosystem I, photosystem II, and the cytochrome $b_6$–$f$ complex, as well as soluble electron transfer proteins (plastocyanin, ferredoxin, and ferredoxin-NADP reductase), it has been possible to produce a system that photoreduces NADP with water as the electron donor. This electron transport pathway is sensitive to well-known inhibitors of chloroplast noncyclic electron transport and shows a requirement for specific electron transfer carriers (the Rieske iron–sulfur center and plastoquinone) that are components of the cytochrome $b_6$–$f$ complex.

## METHODS

Freshly picked greenhouse-grown spinach was used for all preparations. The oxygen-evolving photosystem II preparation of Berthold *et al.* (13) was made as described by these authors and assayed for $O_2$ evolution activity with potassium ferricyanide as the electron acceptor. Estimation of the amount of photosystem I in this preparation based on the $P_{700}$ concentration as determined by the procedure of Melis and Brown (14) gave a value of about 1 $P_{700}$ per 20,000 chlorophyll molecules. The chloroplast cytochrome $b_6$–$f$ complex was prepared by using octyl glucoside plus cholate according to the procedure of Hurt and Hauska (8) as modified by Malkin (15) to eliminate Triton X-100 from the final sucrose gradient step. This material was assayed with durohydroquinone as electron donor and either algal cytochrome $c$ or spinach plastocyanin as electron acceptor. The photosystem I preparation of Mullet *et al.* (16) (PSI-110) was prepared as described by these authors and assayed with reduced plastocyanin as electron donor and either methyl viologen or NADP as electron acceptor.

NADP photoreduction was measured with a Gilford spectrophotometer modified for side illumination (17). Cuvettes with a 2-mm light path were used. A 340-nm blocking filter was placed over the photomultiplier tube and 680-nm light (Baird-

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Abbreviations: UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Mes, 4-morpholineethanesulfonic acid.

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Atomic interference filter, 10 mm half-bandwidth) was used for actinic illumination. Oxygen evolution or uptake was measured by using a Rank electrode with white light for actinic illumination.

Fluorescence induction curves and $P_{700}$ concentrations were measured by A. Melis, using a sensitive spectrophotometric procedure (14, 18).

Chloroplast ferredoxin, plastocyanin, and ferredoxin-NADP reductase were prepared by R. Chain of our laboratory by standard procedures. 5-*n*-Undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) was obtained from B. Trumpower and 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrodiphenyl ether (DNP-INT) from A. Trebst. Inhibitors were dissolved in dimethyl sulfoxide and added to reaction mixtures in microliter amounts. Octyl glucoside, sodium cholate, and Triton X-100 were purchased from Sigma. Durohydroquinone was purchased from K and K.

## RESULTS

**Properties of Electron Transfer Integral Protein Complexes.** The photosystem II preparation has $O_2$ evolution activity with ferricyanide as the electron acceptor. Oxygen evolution was sensitive to the photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). The preparation could not photoreduce NADP even after the addition of soluble cofactors such as ferredoxin, ferredoxin-NADP reductase, and plastocyanin. As reported by Berthold *et al.* (13), the preparation contains essentially no $P_{700}$, and a sensitive photochemical assay for $P_{700}$ gave a content of less than 1 $P_{700}$ per 20,000 chlorophyll molecules. The only cytochrome in this preparation detectable by chemical difference spectra was cytochrome $b_{559}$, which was present at a concentration of 1 per 200 chlorophyll molecules. No cytochrome *f* or cytochrome $b_6$ could be detected in the photosystem II preparation. The preparation retains a secondary electron acceptor pool, which can be detected in an analysis of the fluorescence induction curve in the absence and presence of DCMU (Fig. 1*A*). Analysis of the area over the fluorescence rise curve indicates that approximately 16 electrons can be accumulated in the secondary electron acceptor pool in the photosystem II preparation. Evidence from a variety of sources has indicated plastoquinone is the component functioning in this pool (5). On the basis of these results, it can be concluded that this photosystem II preparation is capable of transferring electrons from water to plastoquinone in a DCMU-sensitive reaction.

The cytochrome $b_6$–*f* complex is almost totally free of chlorophyll and contains a bound plastoquinone, cytochrome *f*, the Rieske iron–sulfur center, and cytochrome $b_6$ in a mole ratio of 1:1:1:2. This preparation can reduce plastocyanin with plastohydroquinone as the electron donor in a reaction that is sensitive to inhibitors interacting in the plastoquinone–Rieske iron–sulfur center region of the electron transport chain, such as 2,5-dibromo-6-methyl-3-isopropyl-*p*-benzoquinone (DBMIB) and DNP-INT (8). These two inhibitors interact with the Rieske iron–sulfur center of the complex (15), and this interaction is presumed to be responsible for the observed inhibition of catalytic activity.

The photosystem I preparation is capable of reducing NADP with reduced plastocyanin as electron donor, and this activity is totally dependent on the addition of ferredoxin, ferredoxin-NADP reductase, and plastocyanin (Fig. 1*B*). The photosystem I preparation contained the bound iron–sulfur center electron acceptors previously identified in other preparations (19), had no photosystem II activity, and contained no detectable cytochrome *f* or cytochrome $b_6$.

**Reconstruction of Noncyclic Electron Transport Activity.** The electron transport properties of the three isolated protein complexes from the chloroplast membrane indicate that each carries out a partial reaction in the overall transfer of electrons from water to NADP. Thus, a reconstruction of this pathway in the presence of all the complexes seemed a reasonable expectation.

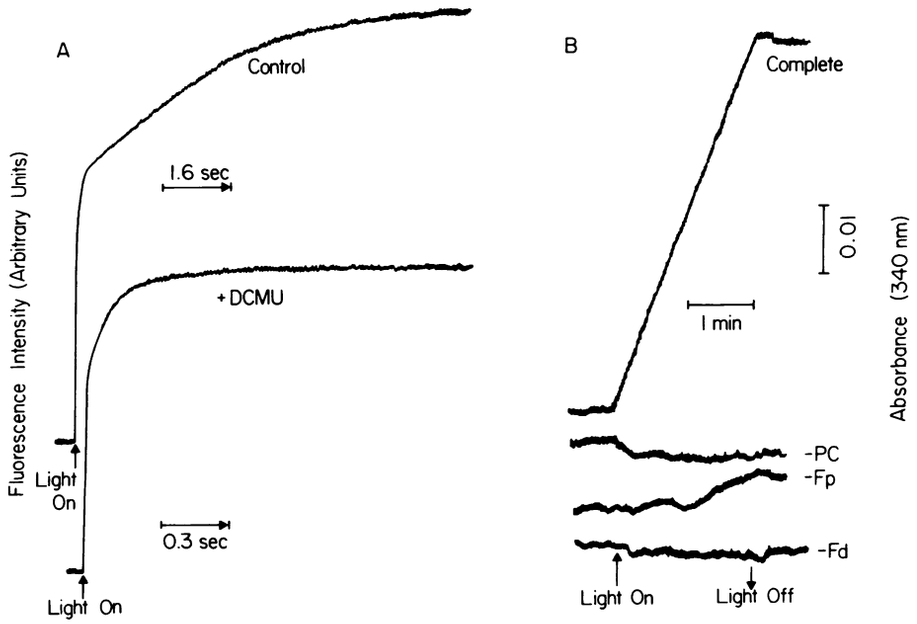


FIG. 1. Photochemical activities of photosystem I and photosystem II electron transfer complexes. (*A*) Fluorescence induction kinetics of photosystem II preparation in the presence and absence of DCMU. The reaction mixture contained photosystem II fragments (0.11 mg of chlorophyll per ml), 5 mM $MgCl_2$, 15 mM NaCl, and 20 mM 4-morpholineethanesulfonic acid (Mes) buffer (pH 6.85). Where indicated, 15 μM DCMU was also added. Fluorescence was measured as described in ref. 18. (*B*) NADP photoreduction by photosystem I. The reaction mixture contained in 1 ml: 50 mM Mes buffer (pH 6.0), 5 mM $MgCl_2$, 15 mM NaCl, 10 mM sodium ascorbate, photosystem I fragments (PSI-110, 5 μg of chlorophyll), 2 mM NADP, and, where present, 8 μM ferredoxin (Fd), 0.2 μM ferredoxin-NADP reductase (Fp, a flavoprotein), and 1.2 μM plastocyanin (PC). NADP photoreduction was measured at 340 nm.

As shown in Fig. 2, electron transport from water to NADP, measured as NADP photoreduction at 340 nm, could be restored in the presence of the three protein complexes and the soluble proteins (plastocyanin, ferredoxin, and ferredoxin-NADP reductase) known to be required for this pathway. Rates of electron transfer were calculated on the basis of the concentration of cytochrome *f* added, rather than on a chlorophyll basis, because of various antenna sizes in the photosystem I and II preparations. Such calculation gave reconstituted rates that were ≈20% of the rates observed in untreated chloroplast membranes. Also shown in Fig. 2 are results that indicate the absolute requirements in the reaction for the photosystem I complex, the cytochrome $b_6$–*f* complex, plastocyanin, and the photosystem II complex. The small amount of NADP reduced in the absence of photosystem II originates from a low concentration of plastocyanin, which is reduced in our preparation and can donate electrons to photosystem I in the absence of photosystem II. No light-induced absorbance changes at 340 nm were observed when NADP, ferredoxin, or ferredoxin-NADP reductase were omitted from the complete reaction mixture.

To confirm that the absorbance changes at 340 nm were due to the formation of NADPH in this system, NADPH was also estimated by the enzymatic procedure described by Ben-Hayyim *et al.* (20). NADPH was formed only after illumination of the complete system, and the omission of individual electron transfer complexes or carriers from the complete reaction mixture resulted in no light-dependent NADPH formation. However, attempts to measure oxygen evolution linked to NADPH formation in the reconstructed system were not successful. This is apparently related to a light-dependent uptake of oxygen that is observed in the presence of the photosystem II preparation and the cytochrome complex. Because cytochrome $b_6$ is known to be autooxidizable, it is conceivable that the oxidation of this

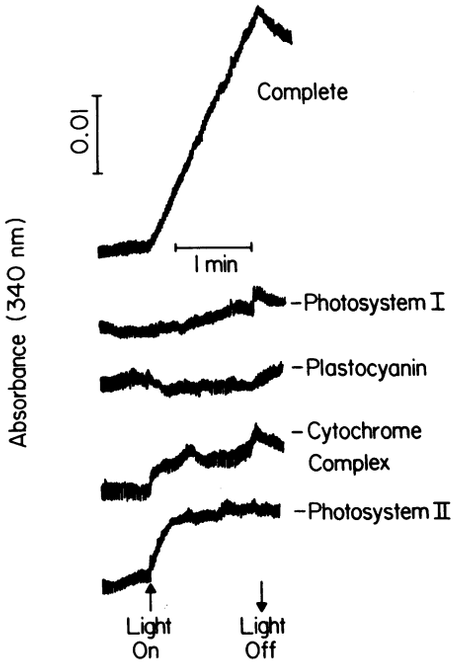


FIG. 2. Reconstruction of NADP photoreduction with chloroplast protein complexes and plastocyanin. The reaction mixture contained in 1 ml: 50 mM Mes buffer (pH 6.0), 5 mM $MgCl_2$, 15 mM NaCl, 2 mM NADP, 8 μM ferredoxin, 0.2 μM ferredoxin-NADP reductase and, where present, 1.2 μM plastocyanin, cytochrome $b_6$–*f* complex (0.8 μM cytochrome *f*), photosystem II fragments (100 μg of chlorophyll), and photosystem I fragments (70 μg of chlorophyll). NADP photoreduction was measured at 340 nm.

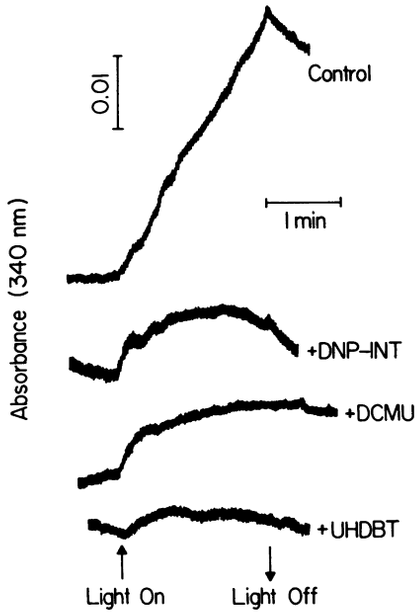


FIG. 3. Effect of inhibitors on NADP photoreduction activity. The complete reaction mixture was as described for Fig. 2. Where present, inhibitors were added at the following concentrations: DCMU, 2 μM; DNP-INT, 4 μM; UHDBT, 5.6 μM. NADP photoreduction was measured at 340 nm.

electron carrier by $O_2$ is responsible for the net uptake of $O_2$.

Shown in Fig. 3 are results indicating that the reconstructed noncyclic pathway is sensitive to known inhibitors of noncyclic electron transport. DCMU, UHDBT, and DNP-INT all cause complete inhibition of NADP reduction at inhibitor concentrations comparable to those commonly used in unfractionated spinach thylakoid membranes.

A preparation of a cytochrome $b_6$–*f* complex that is devoid of the Rieske iron–sulfur center has recently been described (21). We have found that this depleted preparation is lacking a tightly bound plastoquinone molecule that is present in the cytochrome complex (unpublished observations). The effect of subsitution of the depleted cytochrome complex for the intact cytochrome complex in NADP photoreduction is shown in Fig.

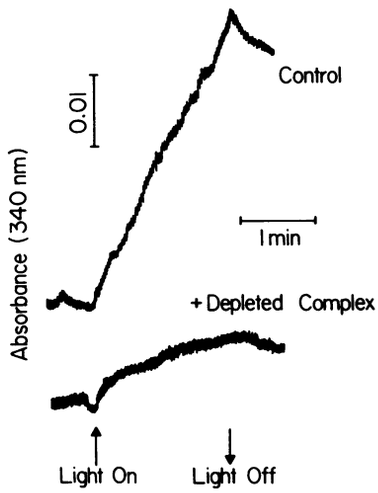


FIG. 4. Effect of depleted cytochrome complex on NADP photoreduction activity. The complete reaction mixture was as described for Fig. 2. Where indicated, the depleted cytochrome complex (0.8 μM cytochrome *f*) was substituted for the intact complex. NADP photoreduction was measured at 340 nm.

Table 1. Effect of preincubation conditions on NADP photoreduction

| Conditions | $\Delta A_{340}$/min |
|---|---|
| Control, no preincubation | 0.0110 |
| Preincubation mixture | |
| + Photosystem II + cytochrome complex | 0.0090 |
| + Photosystem II + photosystem I | 0.0063 |
| + Photosystem I + cytochrome complex | 0.0100 |
| + Photosystem II + photosystem I + cytochrome complex | 0.0220 |

In the control sample, components were mixed under dilute conditions, as described for Fig. 2. During preincubations, the designated components were incubated at 25°C for 5 min in concentrated form and the remaining reaction mixture components were then added just prior to illumination. NADP photoreduction was monitored at 340 nm.

4. NADP photoreduction is abolished when the depleted complex is used, and this finding would argue for a specific requirement for the Rieske iron–sulfur center and the bound plastoquinone of the cytochrome complex in the chloroplast noncyclic electron transport chain.

**Properties of the Reconstructed NADP-Reducing System.** Evidence for an interaction of the integral protein complexes required for NADP reduction has been obtained in studies of preincubation of various complexes. As shown in Table 1, approximately a 2-fold stimulation of the rate of NADP reduction was observed when the three complexes were mixed in concentrated forms and diluted just prior to assay as compared with a control sample in which the complexes were mixed under more dilute conditions. It is interesting to note that this stimulation was observed only when all three complexes were present, and the absence of any one prevented the stimulatory effect.

No exogenous lipids were required for the reconstruction, and the addition of sonicated phospholipids (1–5 mg per reaction mixture) had no effect on the rate of NADP reduction. The endogenous lipid content of the complexes is not known, and this may be a factor in our failure to observe any lipid requirement for activity.

## DISCUSSION

Our results indicate that it is possible to reconstruct the noncyclic electron transport chain from water to NADP by combining three thylakoid integral protein complexes in the presence of soluble protein cofactors. Arntzen *et al.* (22) and Ke and Shaw (23) reported the reconstitution of NADP photoreduction with photosystem I and II preparations, but the photosystem II preparations used could not use water as an electron donor and nonphysiological electron donors were required. In addition, no specific requirement for the chloroplast cytochrome complex was reported at that time, presumably because the carriers of the cytochrome complex were present as contaminants of either the photosystem I or the photosystem II preparation used. Our resolved complexes allow a clear demonstration of the requirement for photosystem I, photosystem II, and the cytochrome complex in NADP photoreduction with water as the electron donor.

Inhibitor sensitivity and the requirement for specific electron carriers of the cytochrome complex (the Rieske iron–sulfur center and a bound plastoquinone molecule) indicate that the reconstructed pathway is specific and probably does not involve bypasses or shuttles that do not exist under physiological conditions. Because of the stimulatory effect observed during preincubation of the three complexes, it is probable that these three complexes associate with each other in a manner that facilitates the overall electron transfer reaction.

These results bear on two recent hypotheses concerning the mechanism of chloroplast noncyclic electron transport (24) and the organization of the thylakoid integral protein complexes (11). It has been proposed by Arnon and co-workers (24) that chloroplast NADP photoreduction is mediated solely by photosystem II with no requirement for either photosystem I or the cytochrome complex. Our results, which indicate a mandatory requirement for both photosystems as well as the cytochrome complex in the restoration of NADP photoreduction, argue against this model. No photoreduction of NADP could be observed by photosystem II alone, even after the addition of plastocyanin to this preparation. Other studies of the reconstitution of NADP photoreduction also have demonstrated a photosystem I requirement (22, 23).

Anderson has recently discussed a model for the organization of integral protein complexes in the thylakoid membrane (11). According to this model, photosystem I is spatially separated from photosystem II, because the former is proposed to be located in the stromal membranes whereas the latter is localized in the granal regions. Studies of the localization of the cytochrome complex by Cox and Andersson (25) as well as Anderson (26) have reported an equal distribution of this complex between granal and stromal membranes. Although our results cannot directly be related to this model, it is apparent from the present work that photosystem II can interact with the cytochrome complex in the absence of any added soluble factors. This conclusion has also been reached on the basis of our unpublished studies of the reconstruction of cytochrome *f* photoreduction with electrons from water catalyzed by the photosystem II and cytochrome complex. Whether this system can be related to *in vivo* functioning of the complexes remains to be determined.

We thank Dr. A. Melis for his aid in measuring $P_{700}$ and the fluorescence induction curves and Drs. A. Trebst and B. Trumpower for inhibitors used in this study. This investigation was supported in part by grants from the National Institutes of Health and the National Science Foundation. E.L. was supported by National Institutes of Health Training Grant T32 GM07379.

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