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Ferredoxin:NADP$^+$ Oxidoreductase, is a Subunit of the Cytochrome b_6f Complex of Spinach Chloroplasts: Implications for the Pathway of Cyclic Electron Transport

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Introduction

As part of an effort to improve the quality of 3D crystals of the cyochrome b_6f complex, the characterization of the complex isolated from spinach thylakoids (Huang *et al*., 1994) and the thermophilic cyanobacterium *M. laminosus* (Huang *et al*., 1999) has been extended. A fourth redox-active subunit in addition to cytochromes *f* and b_6 and the Rieske ISP, ferredoxin:NADP$^+$ oxidoreductase (FNR), was found in the b_6f complex from spinach thylakoids, but not in that from *M. laminosus* purified by the same protocol. The FNR bound stoichiometrically to the purified b_6f complex is enzymatically active, implying a role of the b_6f complex in a ferredoxin-dependent "cyclic" electron transport pathway (Zhang *et al*., 2001, submitted for publication).

Results and Discussion

Spectra. The visible spectra suggested the presence of a flavoprotein in the chloroplast b_6f complex. The chloroplast and cyanobacterial complexes have similar absorbance spectra except (i) the cyanobacterial complex has a 2 nm red shift (554 to 556 nm) of the reduced cytochrome *f* α-band due to the residue change, F4W (Ponamarev *et al*., 2000), and a red shift from 668-669 to 672 nm) in the Qy band of the Chl *a* molecule in the complex (Huang *et al*., 1994). In the chloroplast complex, there is an additional small band in the 450-480 nm region that could arise from the flavin FAD. Using ε_{mM} (480 nm) =7.4 (Batie & Kamin, 1981), the amplitude of this band implies an FAD content of 0.8-1.0 per cytochrome *f*. The 450-480 nm difference spectra, which could also be generated by flash illumination of *Chlorella* (Bouges-Bocquet, 1978), resemble the reduced minus oxidized spectrum of FNR.

SDS-PAGE of the large (> 15 kDa) subunits of the b_6f complex from spinach thylakoids (Fig. 1, lane 3) and *M. laminosus* (Fig. 1, lane 2), shows the presence of the four well-known polypeptides: cyt *f*, cyt b_6, the Rieske ISP, and subunit IV, with M_r 34, 24, 21, and 18 kDa. In this 20% acrylamide gel system, there is overlap between the b_6 and Rieske bands in the M_r 21-24 kDa region of the spinach complex, as seen by the high staining density of this band (Fig.1, lane 3). These subunits are separated in the *M. laminosus* complex (Fig. 1, lane 2). In addition, a 5th polypeptide with the largest $M_r \sim 35$ kDa of the subunits is seen above the $M_r \sim 33$ kDa cytochrome *f* band from spinach (lane3), but not cyanobacteria. Assuming that Coomassie stain binding is proportional to molecular weight, the subunit ratio in the SDS-PAGE, normalized to cyt *f* is: 0.95 (35 kDa subunit): 1.0 (cyt *f*): 1.05 (b_6/Rieske ISP): 0.85 (subunit IV) [avg of 2 scans].

The 5th polypeptide was seen in the b_6f complex from spinach (Hurt & Hauska, 1981), but a difference in heme staining of the 2 bands in the M_r 33–34,000 cytochrome *f* region was not detected. It was suggested that the 2 bands arose from cyt *f* and a polypeptide containing one of the two hemes of cyt b_6, since it was not known at this time that the two hemes of cyt b_6 are bound to the same 23 kDa polypeptide. The 2 bands in the cyt *f* region were also noted by Romanowska and Albertsson (1994), who inferred that both bands belonged to forms of cytochrome *f*. However, the M_r 35 kDa polypeptide of the spinach complex does not stain for heme (Fig. 1, lane 4). This was observed under conditions where the 2nd and 3rd largest (M_r 33 and 24 kDa) polypeptides in the spinach complex, those associated with cytochromes *f* and b_6 in the four component gel of *M. laminosus*, did react with the heme staining reagent (Fig. 1, lane 4).

M_r 35,000 polypeptide in the spinach b_6f complex is FNR. (A) Electrospray mass ionization mass spectroscopy displayed a component in the mass spectrum of the spinach b_6f complex with a mass of 35,362, greater than the mass component of 31,972 attributed to mature holo-cytochrome *f*. The 35 kDa component was absent from the mass spectrum of the b_6f complex from *M. laminosus*. The 35,316 mass of the high molecular component in the spinach b_6f complex was indeed almost the same as that (MW = 35,328) calculated for acetylated pre-apocytochrome *f* (Alt & Herrmann, 1984), but as well very similar to that of ferredoxin: $NADP^+$ reductase (FNR) (MW = 35,362). (B) Western blots of the M_r 35,000 polypeptide in the spinach complex showed that it reacted with antibody to spinach FNR (Fig. 1, lane 6 vs 3), whereas the M_r 32,000 polypeptide reacted with antibody to cytochrome *f* (Fig. 1, lane 5 vs 3). Furthermore, ESI-MS of CNBr fragments of the MW 35,316 polypeptide, isolated chromatographically from the chloroplast complex, ranging from 3,020 to 6,538 in molecular weight, were similar to those expected from FNR, but not from cytochrome *f* (Table 1). The 1,145 and 5,657 CNBr fragments (Table 1, column 1) result from non-specific cleavage. (C) Diaphorase activity (5-10 electrons/cyt *f*/sec), arising from the presence of FNR, was readily detected in the spinach b_6f complex, but not in b_6f complex from *M. laminosus* (data not shown). It was concluded that the M_r 35,000 polypeptide in the spinach b_6f complex is FNR.

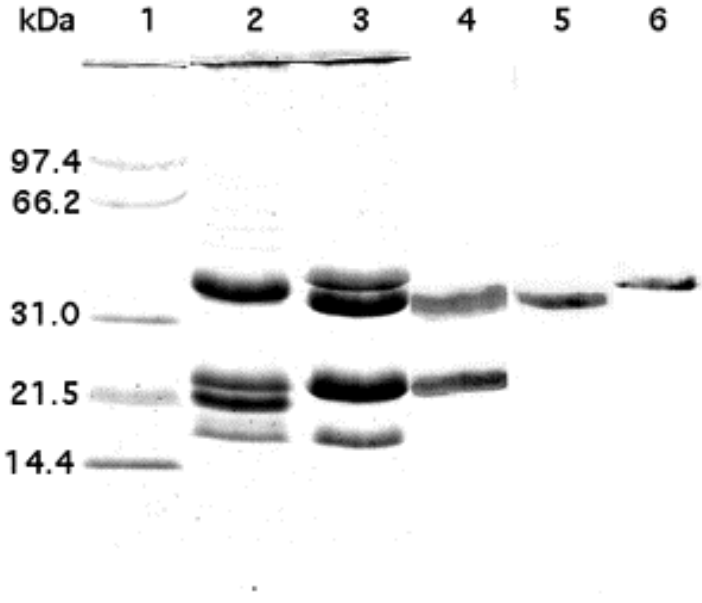

Fig. 1. Cytochrome b_6f complex was purified from spinach thylakoids by procedures similar to those in (Hurt & Hauska, 1981, Huang *et al*., 1994) and from cells of *M. laminosus* (Huang *et al*., 1999). SDS-PAGE of cyt b_6f complexes of cyanobacterial (lane 2) and spinach chloroplast thylakoid (lane 3) membranes; Heme stain profile of spinach cyt b_6f complex (lane 4), and Western blot with antibody to cyt *f* (lane 5) and FNR (lane 6) of spinach cyt b_6f complex. Lane 2: four large polypeptide subunits (cyt *f*, cyt b_6, ISP, and subunit IV) of the *M. laminosus* b_6f complex. Lane 3: four components (cyt *f*, cyt b_6, ISP, and subunit IV) of the spinach complex, with overlap between the b_6 and Rieske subunits, and an additional "very high M_r" (~ 35,000) band.

Absence of FNR does not affect electron transfer activity. High levels of DBMIB-inhibitable electron transfer activity were measured through the high potential chain of the spinach b_6f complex, 290 ± 60 electrons (cyt *f*)$^{-1}$(sec)$^{-1}$at 22° C measured from decylplastoquinol to ferricyanide via spinach plastocyanin. A similar level of activity, 340 ± 50 electrons (cyt *f*)$^{-1}$(sec)$^{-1}$, was measured in b_6f complex isolated from *M. laminosus*, in which the FNR complex is not present (data not shown). Thus, the presence of bound FNR in the spinach chloroplast b_6f complex does not affect the activity of the complex associated with non-cyclic electron transfer.

Electron transfer from NADPH to cytochrome b_6 . Addition of NADPH caused no reduction of cytochrome *b* (data not shown). However, with ferredoxin, NADPH reduced 0.4 of the 2 hemes *b* (0.8 heme). In this experiment, cyt *f* is initially reduced in the presence of ascorbate. In previous experiments with thylakoid membranes, the half-complement of reduced cyt b_6 was inferred to be the heme b_n (Furbacher *et al*., 1989). The ferredoxin-dependent b_6 reduction by NADPH *in situ*, and *in vitro* as shown in the present studies, implies that the b_6f complex with bound FNR provides the membrane protein connection between the binding site of ferredoxin on the *psa*D subunit of the PS I reaction center complex and the plastoquinone pool, defining the pathway of cyclic electron transport (Fig. 2).

Table.1. Comparison of Masses of CNBr Fragments of 35 kDa Polypeptide Determined by ESI-MS with Calculated Masses of Putative Fragments of FNR

Measured mass of 35 kDa polypeptide and CNBr fragments (Da)	**Calculated mass of FNR and fragments (Da)**	**Amino acid number**
35320.3 ± 5.9	35313.7	1-313
CNBr		
1145.4 ± 0.2	—	—
3020.2 ± 0.7	3020.3	220-245
4097.7 ± 0.7	4099.6	24-60
4357.9 ± 0.5	4355.9	27-314
5657.2 ± 0.7	—	—
6538.4 ± 1.2	6538.5	1-60

FNR Subunit: Artifact or Reality. It is possible that the presence of FNR in the spinach cytochrome b_6f complex, or its absence in the cyanobacterial complex, is an artifact of preparation with respect to FNR binding. The presence of FNR in the spinach complex has been noted previously (Clark *et al*., 1984). However, it was subsequently concluded that the presence of bound FNR in the spinach b_6f complex was artifactual (Coughlan *et al*., 1985, Soncini and Vallejos, 1989). In the present study, meaningful binding in the spinach complex is implied by stoichiometric binding of FNR to the complex after exposure to high ionic strength, hydrophobic extraction and chromatography, sucrose gradient separation, and as well the diaphorase and cytochrome *b* reductase activities of the purified b_6f complex. On the other hand, the absence of FNR from the cyanobacterial complex may be an artifact of preparation, arising from weaker binding to the b_6f complex.

FNR and ferredoxin-mediated cyclic electron transport. The presence of active FNR bound stoichiometrically to the b_6f complex, with a requirement for ferredoxin for its activity, implies that the cytochrome b_6f complex is the membrane protein complex responsible for coupling electron transfer from the reducing side of PS I to the main electron transport chain in ferredoxin-dependent cyclic electron transport. It has previously been argued on the basis of antimycin insensitivity of flash-induced heme b_6 oxido-reduction and dye-mediated cyclic phosphorylation, with 40 % inhibition of ferredoxin-mediated phosphorylation by antimycin A, that the pathway of ferredoxin-mediated cyclic electron transport and phosphorylation bypasses the b_6f complex (Moss & Bendall, 1984). The existence of a unique "ferredoxin-plastoquinone oxidoreductase" integral membrane protein was proposed as a membrane protein interface alternative to the b_6f complex, but it has not been possible subsequently to identify or purify the latter complex. Kinetic competence of electron transfer to FNR was indicated by the finding of a half-time < 10 µsec in *Chlorella* for reduction by a light flash of a spectral component whose difference spectrum is similar to that of FNR (Bouges-Bocquet, 1978).

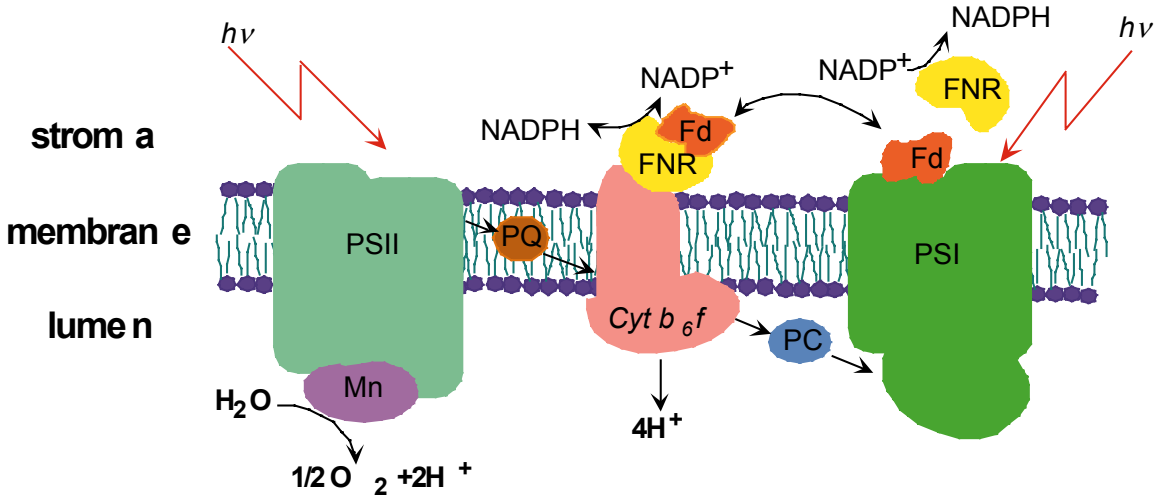

Fig. 2. Schematic of electron transport complexes of thylakoid membrane showing the reaction center complexes, PSI and PSII, and the cytochrome b_6f complex. The sites of FNR bound to the cytochrome b_6f complex and of ferredoxin binding on FNR, as concluded from the ferredoxin-dependent reduction of cyt b_6 are shown. A diffusive connection is shown between the ferredoxin binding site on the *psa*D subunit of the PSI complex and FNR on the b_6f complex. A soluble pool of FNR (Matthijs *et al.*, 1986), which could also function in $NADP^+$ reduction, is shown.

Thus, it is concluded that the cytochrome b_6f-FNR complex provides the protein connection to the main electron transport chain for Fd-dependent cyclic electron transport. Some remaining questions are:

(1) Is the diffusive connection between PSI and the b_6f complex long- or short-(supercomplex) range?

(2) Can $NADPH^+$ reduction occur on the b_6f complex?

(3) Is only heme b_n reduced by NADPH-ferredoxin?

(4) Is cyclic electron transport then controlled or regulated by the redox state of the heme b_n?

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References

Alt, J., and Herrmann, R. G. (1984) *Current Genetics* **8,** 551-557
Batie, C. J., and Kamin, H. (1981) *J. Biol. Chem.* **256,** 7756-7763
Bouges-Bocquet, B. (1978) *FEBS Lett.* **85,** 340-344
Clark, R. D., *et al*. (1984) *FEBS Lett.* **174,** 137-142
Coughlan, S., Matthijs, H. C. P., and Hind, G. (1985) *J. Biol. Chem.* **260,** 14891-148940.
Furbacher, P. N., Girvin, M. E., and Cramer, W. A. (1989) *Biochemistry* **28,** 8990-8998
Huang, D., Zhang, H., Soriano, G. M., Dahms, T. E. S., Krahn, J. M., Smith, J. L., and Cramer, W. A. (1999) in *Photosynthesis: Mechanisms and Effects* (Garab, G., ed), Vol. 3, pp. 1577-1580, Kluwer Academic Publishers, Dordrecht
Huang, D., Everly, R. M., Cheng, R. H., Heymann, J. B., Schägger, H., Sled, V., Ohnishi, T., Baker, T. S., and Cramer, W. A. (1994) *Biochemistry* **33,** 4401-4409
Hurt, E. C., and Hauska, G. (1981) *Eur. J. Biochem.* **117,** 591-599
Matthijs, H. C. P., Coughlan, S. J., and Hind, G. (1986) *J. Biol. Chem.* **261,** 12154-12158
Moss, D. A., and Bendall, D. S. (1984) *Biochim. Biophys. Acta* **767,** 389-395
Ponamarev, M. V., Schlarb, B. G., Howe, C. J., Carrell, C. J., Smith, J. L., Bendall, D. S., and Cramer, W. A. (2000) *Biochemistry* **39,** 5971-5976
Romanowska, E., and Albertsson, P.-A. (1994) *Plant Cell Physiol.* **35,** 557-568
Soncini, F. C., and Vallejos, R. H. (1989) *J. Biol. Chem.* **264,** 21112-21115
Zhang, H., Whitelegge, J. P., and Cramer, W. A., 2001, submitted for publication