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 $f$ $a$-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 $\varepsilon_{\text{M}}$ (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 $M_r$ ~ 35 kDa of the subunits is seen above the $M_r$ ~ 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 Mr 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 Mr 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 (Mr 33
and 24 kDa) polypeptides in the spinach complex, those associated with cytochromes $f$ and $b_6$
in the four component gel of $M. \text{laminosus}$, 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 $M. \text{laminosus}$. 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 Mr 35,000 polypeptide in the spinach
complex showed that it reacted with antibody to spinach FNR (Fig. 1, lane 6 vs 3), whereas the
Mr 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 $M. \text{laminosus}$ (data not shown). It was concluded that the
Mr 35,000 polypeptide in the spinach $b_{6f}$ complex is FNR.

![Fig. 1](image_url)

**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 $M. \text{laminosus}$ (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
cytochrome $b_{6f}$ complex. Lane 2: four large polypeptide subunits (cyt $f$, cyt $b_6$, ISP, and subunit IV) of the $M. \text{laminosus}$
$b_{6f}$ 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 Mr" (~ 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$_6$f 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$_6$f 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$_6$f complex does not affect the activity of the complex associated with non-cyclic electron transfer.

Electron transfer from NADPH to cytochrome b. 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$_6$f complex with bound FNR provides the membrane protein connection between the binding site of ferredoxin on the _psaD_ subunit of the PS I reaction center complex and the plastoquinone pool, defining the pathway of cyclic electron transport (Fig. 2).

<table>
<thead>
<tr>
<th>Measured mass of 35 kDa polypeptide and CNBr fragments (Da)</th>
<th>Calculated mass of FNR and fragments (Da)</th>
<th>Amino acid number</th>
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<tr>
<td>35320.3 ± 5.9</td>
<td>35313.7</td>
<td>1-313</td>
</tr>
<tr>
<td><strong>CNBr</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1145.4 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3020.2 ± 0.7</td>
<td>3020.3</td>
<td>220-245</td>
</tr>
<tr>
<td>4097.7 ± 0.7</td>
<td>4099.6</td>
<td>24-60</td>
</tr>
<tr>
<td>4357.9 ± 0.5</td>
<td>4355.9</td>
<td>27-314</td>
</tr>
<tr>
<td>5657.2 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6538.4 ± 1.2</td>
<td>6538.5</td>
<td>1-60</td>
</tr>
</tbody>
</table>

FNR Subunit: Artifact or Reality. It is possible that the presence of FNR in the spinach cytochrome b$_6$f 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$_6$f 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$_6$f 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$_6$f 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).

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 $b_6$ reduced by NADPH-ferredoxin?
4. Is cyclic electron transport then controlled or regulated by the redox state of the heme $b_6$?
Acknowledgment

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References