Ferredoxin:NADP+ Oxidoreductase Is a Subunit of the Chloroplast Cytochrome b_6f Complex*

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Purified detergent-soluble cytochrome b_6f complex from chloroplast thylakoid membranes (spinach) and cyanobacteria (*Mastigocladus laminosus*) was highly active, transferring 300–350 electrons per *cyt f*/s. Visible absorbance spectra showed a red shift of the cytochrome *f* α-band and the Qy chlorophyll *a* band in the cyanobacterial complex and an absorbance band in the flavin 450–480-nm region of the chloroplast complex. An additional high molecular weight ($M_r \sim 35,000$) polypeptide in the chloroplast complex was seen in SDS-polyacrylamide gel electrophoresis at a stoichiometry of ~0.9 (cytochrome $f)^{-1}$. The extra polypeptide did not stain for heme and was much more accessible to protease than cytochrome *f*. Electrospray ionization mass spectrometry of CNBr fragments of the 35-kDa polypeptide was diagnostic for ferredoxin:NADP+ oxidoreductase (FNR), as were antibody reactivity to FNR and diaphorase activity. The absence of FNR in the cyanobacterial complex did not impair decyl-plastoquinol-ferricyanide activity. The activity of the FNR in the chloroplast b_6f complex was also shown by NADPH reduction, in the presence of added ferredoxin, of 0.8 heme equivalents of the cytochrome b_6 subunit. It was inferred that the b_6f complex with bound FNR, one equivalent per monomer, provides the membrane protein connection to the main electron transfer chain for ferredoxin-dependent cyclic electron transport.

The cytochrome b_6f complex provides the electronic connection between the two reaction center complexes of oxygenic photosynthesis and, by oxidizing the lipophilic plastoquinol and transferring the resulting protons to the electrochemically positive side of the membrane, also contributes significantly to the generation of the trans-membrane proton electrochemical potential (1). The complex is known to contain three redox-active polypeptide subunits, cytochrome *f*, cytochrome b_6, and the Rieske iron-sulfur protein (ISP).[1] Our understanding of the structure and function of this complex, and its relation to the

cytochrome bc_1 complex in the electron transport chain of mitochondrial respiration and photosynthetic bacteria (most recent and highest resolution structure; Ref. 2), has been extended in recent years: (i) high resolution structures have been obtained of the lumen-side soluble domain of cytochrome *f* in plant (3, 4), cyanobacterial (5), and algal (6, 7) sources and of the Rieske iron-sulfur protein from plants (8, 9); (ii) the existence of small (~4-kDa) hydrophobic polypeptides in the complex has been recognized (10); and (iii) a bound molecule of chlorophyll *a* (11–13) and of carotenoid (14), apparently not found in the bacterial bc_1 complex, has been recognized. Two-dimensional crystals of the b_6f complex from *Chlamydomonas reinhardtii* have been obtained that provide projection maps to 8–9 Å (15, 16), and three-dimensional crystals of the complex from the thermophilic cyanobacterium *Mastigocladus laminosus* diffract to somewhat lower resolution (17). As part of an effort to improve the quality of the three-dimensional crystals, the characterization of the b_6f complex isolated from spinach thylakoids and *M. laminosus* has been extended. The present study documents that a fourth redox-active subunit in addition to cytochromes *f* and b_6 and the Rieske ISP, ferredoxin:NADP+ oxidoreductase (FNR, 314 residues; mass, 35,315), is present in the cytochrome b_6f complex isolated 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 the photosystem I (PSI)- and ferredoxin-dependent cyclic electron transport pathway.

MATERIALS AND METHODS

Preparation of Cytochrome b_6f Complex

Spinach Thylakoids—Thylakoid membranes were isolated as described by Hurt and Hauska (18). Cytochrome b_6f complex was extracted in TMKNE (30 mM Tris-HCl, pH 7.5, 5 mM MgCl$_2$, 5 mM KCl, 50 mM NaCl, and 1 mM EDTA) containing 0.2% sodium cholate and 28 mM *n*-octyl-β-D-glucoside at a chlorophyll concentration of 2 mg/ml. The chloroplast suspension was stirred at room temperature for 20 min and centrifuged at $300,000 \times g$ for 40 min, the supernatant was collected, and solid ammonium sulfate was added to 35% saturation. The precipitate was removed by centrifugation at $160,000 \times g$ for 30 min, and the supernatant was loaded on a propyl-agarose column (1.5×10 cm) equilibrated with 35% saturated ammonium sulfate in TMKNE containing 0.05% *n*-undecyl-β-D-maltopyranoside (UDM). The column was washed with equilibration solution, and the cytochrome b_6f complex was eluted with 10% saturated ammonium sulfate in TMKNE with 0.05% UDM. Fractions containing cytochrome b_6f complex were pooled, concentrated in a Centriprep 10, loaded on a sucrose gradient (8–35%) in TMKNE and 0.05% UDM, and centrifuged at 35,000 rpm (16 h) in an SW-41 rotor. The brown band in the middle of the gradient was collected.

M. laminosus—Cells were harvested by centrifugation at $5000 \times g$ (10 min) and resuspended in 25 mM Hepes-KOH, pH 7.5, 10 mM CaCl$_2$, 10 mM MgCl$_2$, 0.4 M sucrose, 0.25 mM phenylmethylsulfonyl fluoride, 2

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[1] The abbreviations used are: ISP, iron-sulfur protein; FNR, ferredoxin-NADPH+ oxidoreductase; PAGE, polyacrylamide gel electrophoresis; PS, photosystem; UDM: *n*-undecyl-β-D-maltopyranoside.

mM benzamidine, and 2 mM ϵ-amino-caproic acid. The cell suspension was passed through a French pressure cell at 18,000 pounds/square inch. Unbroken cells were removed by centrifugation at $3000 \times g$ (10 min), and thylakoid membranes were collected by centrifugation at $90,000 \times g$ (45 min). The sediment was resuspended in 30 mM Tricine, pH 8.0, and washed according to the procedure described for spinach thylakoid membranes, and cytochrome b_6f complex was extracted and purified as described above for the spinach complex.

Purification of Plastocyanin

Spinach—Plastocyanin was purified according to the procedure of Morand and Krogmann (19).

M. laminosus—Solid ammonium sulfate was added to the supernatant of broken *M. laminosus* cells to achieve 60% saturation. The precipitate was removed by centrifugation at $35,000 \times g$ (30 min), the supernatant was collected, and ammonium sulfate was added to 90% saturation. The precipitate containing plastocyanin was sedimented by centrifugation at $36,000 \times g$ (30 min), and the pellet was resuspended in 50 mM potassium phosphate, pH 7.0, and 1 mM ferricyanide and dialyzed against 5 mM potassium phosphate$_i$, pH 7.0, overnight. This fraction was loaded onto a diethylaminoethyl-cellulose column equilibrated with 10 mM potassium phosphate$_i$, pH 7.0. The void fraction containing plastocyanin was collected and concentrated. The concentrated plastocyanin fraction was subsequently loaded on a CM-Sepharose column equilibrated with 10 mM Tris-HCl, pH 8.0. Pure plastocyanin was eluted in 10 mM Tris-HCl, pH 8.0, and 50 mM NaCl.

Absorbance Difference Spectra

Chemical difference spectra of cytochromes f and b_6 and plastocyanin were measured using a Cary 3 UV-visible spectrophotometer with a measuring beam half-bandwidth of 2 nm.

Electron Transfer Activity Plastoquinol

Plastocyanin oxidoreductase activity of cytochrome b_6f complex was assayed on a Aminco-Chance dual-beam spectrophotometer. The assay mixture contained 125 μM ferricyanide, 5 μM plastocyanin (from spinach or *M. laminosus*), 5 nM cytochrome b_6f in 30 mM 4-morpholino-ethanesulfonic acid, pH 6.0, 50 mM NaCl, and 2 mM EDTA. Reduction of plastocyanin, initiated by addition of 25 μM decyl-plastoquinol (DPQH$_2$), was monitored as the absorbance change at 600 nm relative to 500 nm based on an extinction coefficient of 4.9 mM^{-1} (20).

Diaphorase Activity

Diaphorase activity of bound FNR was measured according to the method of Avron and Jagendorf (21). The reaction mixture contained 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% UDM, 35 nM cytochrome b_6f complex, and 20 μM 2,6-dichloro-indophenol. The reduction of 2,6-dichloro-indophenol by NADPH (67 μM) was measured by the absorbance change at 620 nm relative to 500 nm and the rate of reduction of 2,6-dichloro-indophenol based on an oxidized minus reduced millimolar extinction coefficient of 20.

Electrophoresis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed on an Amersham Pharmacia Biotech Phast gel system with constant temperature control using precast gels containing 20% acrylamide. Samples were prepared in 50 mM Tris-HCl, pH 8.0, 4 M urea, 2% SDS, 5% glycerol, and 2.5% mercaptoethanol and heated for 2 min at 90 °C before loading.

Blue native gel electrophoresis was carried out by the method described by Shägger and von Jagow (22) and Schägger *et al*. (23); a 1-mm thick 8–16% gradient gel was used. Cytochrome b_6f was dissolved in 25 mM Bis-Tris, pH 7.0, 0.15% UDM, and 0.05% Serva Blue G.

Heme Stain

Heme-containing proteins on SDS-PAGE were stained using 3,3′,5,5′-tetramethyl-benzidine according to the method of Thomas et al (24).

Western Blots

Proteins were electrotransferred from gel slabs used in SDS-PAGE to polyvinylidene difluoride membranes for 1 h at 130 mA in a Hoefer TE70 semidry transfer blotting system. Immunoblotting used horseradish peroxidase-conjugated IgG at 1:2000 dilution. For the color reaction, the polyvinylidene difluoride membrane was soaked in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl containing 0.015% chloronaphthol, which was

diluted from a freshly made 0.3% solution in ethanol. After 5 min of incubation, 0.2% H$_2$O$_2$ was added. The reaction was terminated by rinsing the membrane with water.

Protease Accessibility

Cytochrome b_6f complex from spinach chloroplasts was treated with thermolysin at a ratio of 1:100 (w/w) at room temperature for 2 h. Thermolysin and proteolytically cleaved polypeptides were removed using a Centricon 100 centrifuge at $1000 \times g$. The results of the proteolysis were assayed by SDS-PAGE.

Cloning and Sequencing of the petA (cyt f) Gene from M. laminosus

M. laminosus genomic DNA was purified according to the method of Ausubel et al (25). The *pet*A gene was amplified by PCR using degenerate primer pairs based on the *pet*A gene sequence from *Nostoc*. Restriction sites (*Bam*HI and *Eco*RI) for cloning were incorporated into the PCR primers. The DNA fragments obtained from PCR were purified from an agarose gel, digested with *Bam*HI and *Eco*RI, and cloned in pBluescript II KS for sequence determination.

Electrospray Ionization Mass Spectrometry

Samples (100 μg of protein) were precipitated with chloroform/methanol and dissolved in 60% formic acid before immediate size exclusion chromatography-electrospray ionization mass spectrometry (Tosohaas Super SW2000, 4.6 × 300 mm, 40 °C) in chloroform/methanol/1% aqueous formic acid (4:4:1, v/v) as described previously (26, 27). Spectra were recorded with an API III+ quadrupole instrument equipped with an Ionspray TM source (PerkinElmer Sciex), tuned and calibrated as previously described (28). Mass spectra from the appropriate part of the chromatogram were deconvoluted to the zero-charge molecular weight spectrum (Bio Multiview, 1.3.1; Perkin Elmer Sciex) as described elsewhere.[2]

RESULTS

Spectra—Purified cytochrome b_6f complex from spinach thylakoids and the cyanobacterium *M. laminosus* showed similar absorbance spectra except for: (i) a 2-nm red shift (554–556 nm) of the reduced cytochrome f α-band (Fig. 1*A, peak 4*) caused by the residue shift Phe4→Trp4, as described by Ponamarev *et al*. (30); (ii) a 3–4-nm red shift (from 668–669 to 672 nm) in the chlorophyll a Qy band (Fig. 1*A, peak 5*) in the *M. laminosus* spectrum (14); and (iii) a small peak in the 450–480-nm region (Fig. 1*A, peak 2*) that could be characteristic of the FAD (31). When chloroplast (Fig. 1*B, solid line*) and *M. laminosus* (Fig. 1*B, dashed line*) difference spectra (dithionite minus ascorbate in the presence of glucose oxidase) are amplified and compared in the 400–500-nm spectral region, it is apparent that the spectral peak of the chloroplast b_6f complex in the 450–480-nm region is missing from the *M. laminosus* difference spectrum (Fig. 1*B, arrow*). Using an extinction coefficient at 480 nm, ϵ_{mM} = 7.4 (31), the amplitude of this absorbance band implies a FAD content of 0.8–1.0 per cytochrome *f*. The 450–480-nm difference spectra of the chloroplast b_6f complex resemble reduced minus oxidized spectra of FNR generated by flash illumination of *Chlorella* (32).

Differences in Migration on SDS-PAGE: Sequence Comparison of Cytochromes f—A comparison of SDS-PAGE profiles of the large (>15-kDa) subunits of the cytochrome b_6f complex from spinach thylakoids (Fig. 2, *lane 2*) and the thermophilic cyanobacterium *M. laminosus* (Fig. 2, *lane 3*) shows the presence of the four well known polypeptides in the b_6f complex. In order of descending molecular size, these are cytochrome *f*, cytochrome b_6, the Rieske iron-sulfur protein, and subunit IV, with M_r values of 33,000, 24,000, 21,000, and 18,000, respectively. In this gel system (20% acrylamide), there is an overlap between the b_6 and Rieske bands in the M_r 21,000–24,000

FIG. 3. **Heme-stained profile of SDS-PAGE of spinach cyto-chrome b_6f complex.** *Lane 1*, molecular weight standards; *lane 2*, spinach cytochrome b_6f complex; *lane 3*, 3,3',5,5'-tetramethyl-benzidine stain. Conditions were as described under "Materials and Methods."
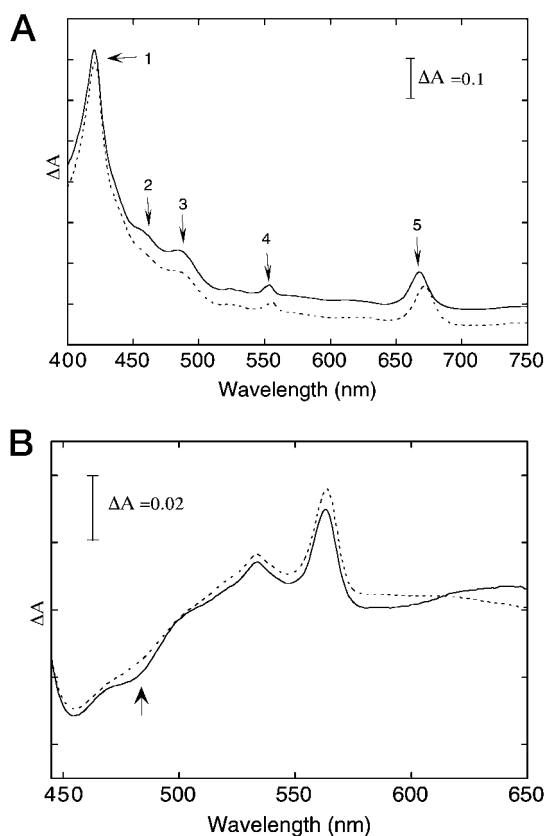

FIG. 1. *A*, visible absorbance spectra of cytochrome b_6f complex from spinach (1.0 μM *cyt f*; *solid curve*) and *M. laminosus* (1 μM *cyt f*; *dashed curve*) cytochrome b_6f complex. The complexes were reduced with Na-ascorbate (5 mM). Spectral peaks: *1*, Soret bands; *2*, FNR; *3*, FNR and carotenoid; *4*, cytochrome *f* α-band; *5*, chlorophyll *a* Qy band. B, Dithionite minus ascorbate difference spectra in the presence of glucose (5 mM)-glucose oxidase (160 units) of spinach (*solid curve*) and *M. laminosus* (*dashed curve*) b_6f complex. *Arrow*, region of reduced oxidized absorbance band of FAD in the putative FNR subunit. The positive band arises from the cytochrome b_6 subunit. The spectra of chloroplast and cyanobacterial b_6f complexes have been displaced for purposes of clarity.
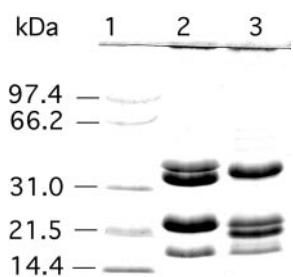

FIG. 2. **SDS-PAGE of cytochrome b_6f complexes of spinach chloroplast thylakoid (*lane 2*) and cyanobacterial (*lane 3*) membranes.** *Lane 2* contains the four components (*cyt f, cyt b_6*, ISP, and subunit IV) of the spinach thylakoid complex, with overlap between the b_6 and Rieke subunits and an additional very high M_r (~35,000) band. *Lane 3* contains the four large polypeptide subunits (*cyt f, cyt b_6*, ISP, and subunit IV) of the *M. laminosus* b_6f complex.

region of the spinach complex, as seen in the high staining density of this band (Fig. 2, *lane 2*). These subunits are separated in the *M. laminosus* complex (Fig. 2, *lane 3*). There is no doubt from the difference spectra (Fig. 1*B*), heme stain (Fig. 3), electron transfer activity, and mass spectrometry of the intact complex[2] that the spinach complex contains a full component of the cytochrome b_6 and Rieske ISP subunits.

The difference in mobility of cytochrome *f* is attributed to differences in amino acid sequences of the two cytochromes *f*
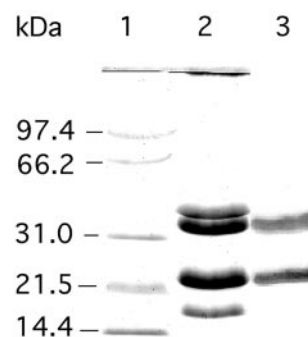
(Table I). There is 58% identity between the two cytochromes *f*, with a similar content of hydrophobic residues, 53.6 and 54.7%, respectively. The basis for the mobility difference of the Rieske protein is not known, because its nucleotide sequence in *M. laminosus* has not been determined.

Properties of the Fifth and Largest Polypeptide in the Spinach Chloroplast b_6f Complex—In addition, a fifth polypeptide with the largest M_r value, 35,000, of the subunit polypeptides is seen above the M_r ~33,000 cytochrome *f* band from chloroplasts (Fig. 2, *lane 2*) but not in cyanobacteria (Fig. 2, *lane 3*). The presence of the fifth polypeptide in the chloroplast and not the cyanobacterial complex is reflected in a smaller M_r value of the *M. laminosus* compared with the spinach complex measured on the native gel system of Schägger *et al.* (22, 23; data not shown). The M_r 35,000 polypeptide appears to be bound stoichiometrically to the other large subunits of the complex, as seen in Fig. 2. Assuming that the binding of Coomassie blue stain is proportional to molecular weight, the densitometric ratio of the subunits in the SDS-PAGE of Fig. 2, normalized relative to cytochrome *f*, is 0.95 (35-kDa subunit):1.0 (cyt *f*):1.05 (b_6 and Rieske ISP):0.85 (subunit IV) (average of two sets of scans).

The observation of the fifth polypeptide in the complex was a major aspect of the original study by Hurt and Hauska (18) on the purification and properties of the cytochrome b_6f complex from spinach. Their study did not detect a difference in the heme-staining properties of the two bands in the M_r 33,000–34,000 cytochrome *f* region. They suggested that the two bands in this region arose from cytochromes *f* and a polypeptide containing one of the two hemes of cytochrome b_6, respectively, because it was not known at this time that the two hemes of cytochrome b_6 are bound to the same 23-kDa polypeptide. The two bands in the cytochrome *f* region were also noted by Romanowska and Albertsson (33), who did not resolve a difference in their heme-staining properties and concluded that both bands belonged to forms of cytochrome *f*. However, it was found in the present study that the fifth and largest (M_r 35,000) polypeptide in the SDS-PAGE of the spinach chloroplast complex did not stain for heme (Fig. 3). This was observed under conditions in which the second and third largest (M_r 33,000 and 24,000) 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. 3).

A different degree of integration into the spinach complex of the M_r 35,000 and 33,000 polypeptides is implied by the complete accessibility and susceptibility of the M_r 35,000 but not the M_r 33,000 polypeptide, to the protease thermolysin (Fig. 4, *lane 5 versus lanes 2–4*). Cytochrome *f* in the thylakoid membrane is known to be inaccessible to a range of proteases (34). It can be seen that the Rieske ISP and subunit IV polypeptides

FNR Subunit of the Cytochrome b_6f Complex

TABLE I
Sequence alignment of cytochrome f (pet A) from spinach and M. laminosus
Alignment was obtained using Clustal W (54). *, identical or conserved residues; :, conserved substitutions; ., semiconserved substitutions.

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M. laminosus  ------------------------------------YPFWAQQTYPETPREPTGRIVCANC
Spinach       MQTINTFSWIKEQITRSISISLILYIITRSSIANAYPIFAQQGY-ENPREATGRIVCANC
                                                  **::***  *  *.***.*********

M. laminosus  HLAAKPAEVEVPQSVLPDTVFKAVVKIPYDTKLQQVAADGSKVGLNVGAVLMLPEGFKIA
Spinach       HLANKPVDIEVPQAVLPDTVFEAVVRIPYDMQLKQVLANGKKGGLNVGAVLILPEGFELA
              *** **.:.:****:*******:***:****  :*:** *:*.* ********:*****::*

M. laminosus  PEERIPEELKKEVGDVYFQPYKEGQDNVLLVGPLPGEQYQEIVFPVLSPNPTTDKNIHFG
Spinach       PPDRISPEMKEKMGNLSFQSYRPNKQNILVIGPVPGQKYSEITFPILAPDPATKKDVHFL
              *  :**. *:*::*:: **.*:  .::*:*::**:**::*.**.**:*:*:*:*.*::**

M. laminosus  KYAIHLGANRGRGQIYPTGEKSNNNVFTASATGTITKIAKEEDEYGNVKYQVSIQTDSGK
Spinach       KYPIYVGGNRGRGQIYPDGSKSNNTVYNSTATGIVKKIVRKEK--GGYEINIADASDR-R
              **.*::*.*********** *.****.*::.:****  :.**.::*.   *. : : :::   :*   :

M. laminosus  TVVDTIPAGPELIVSEGQAVKAGEALTNNPNVGGFGQDDTEIVLQDPNRVKWMIAFICLV
Spinach       EVVDIIPRGPELLVSEGESIKLDQPLTSNPNVGGFGQGDAEVVLQDPLRIQGLLFFFASV
              *** ** ****:****:::*  .:.**.*********.*:*:***** *:: :: *:. *

M. laminosus  MLAQLMLILKKKQVEKVQAAEMNF
Spinach       ILAQIFLVLKKKQFEKVQLSEMNF
              :***::*:*****.**** :****
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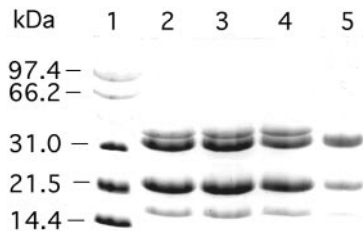

FIG. 4. **Susceptibility of subunits of spinach chloroplast b_6f complex to high ionic strength, EDTA, or exogenous protease.** *Lane 1*, Molecular weight standards; *lane 2*, control b_6f complex; *lane 3*, b_6f complex washed with 1 M NaBr; *lane 4*, b_6f complex washed with 0.25 M EDTA; *lane 5*, incubation for 2 h with thermolysin (protease/b_6f, 1:100, w/w).

FIG. 5. **Molecular weight spectrum of larger polypeptides in preparations of cytochrome b_6f complexes.** Larger polypeptides of the complex from spinach (*A*) and *M. laminosus* (*B*) were separated from lower molecular weight components by size exclusion chromatography in chloroform/methanol/1% aqueous formic acid (4:4:1, v/v). The column eluent was directed immediately to the electrospray ionization source of the mass spectrometer, which completed each successive spectral scan in 6 s, providing an ion chromatogram. The larger proteins coeluted earlier than all other components, resulting in moderately complex mass spectra, which were transformed into zero-charge molecular weight spectra by computation (BioMultiView 1.3.1; PerkinElmer Sciex). The significant peaks in the spectra are labeled with the molecular weights determined by this program, and original spectra were examined to ensure the presence of corresponding ions in the mass spectrum. The 34,624 mass peak is attributed to an artifactual dimer of subunit IV (mass, 17,314). The accuracy of masses determined by electrospray ionization mass spectroscopy according to these protocols is <0.01%.

are also sensitive to the protease (Fig. 4, *lane 5*). Although the M_r 35,000 component is accessible to protease, implying a peripheral location in the complex, it cannot be removed by washing with high concentrations of NaBr or EDTA (Fig. 4, *lanes 3 and 4 versus lane 2*).

The M_r 35,000 Polypeptide in the Spinach Cytochrome b_6f Complex Is FNR—The absence of heme staining of the M_r 35,000 polypeptide in the spinach complex and the similarity of its M_r value to that of the "true" M_r 33,000 spinach cytochrome f suggested that the M_r 35,000 polypeptide might be preapocytochrome, *i.e.* cytochrome f with its 35-residue leader peptide (35) but without the heme, the incorporation of which requires a free N terminus of the mature cytochrome f (3). Electrospray mass ionization mass spectroscopy displayed a component in the mass spectrum of the spinach b_6f complex with a mass of 35,314, greater than the mass component of 31,972 attributed to mature holocytochrome f (Fig. 5A). The 34,624 mass component is an artifact with a mass that corresponds to that of a dimer of subunit IV. The 35-kDa component was absent from the mass spectrum of the b_6f complex from *M. laminosus* (Fig. 5B). The 35,314 mass of the high molecular weight component in the spinach b_6f complex was indeed almost the same as that
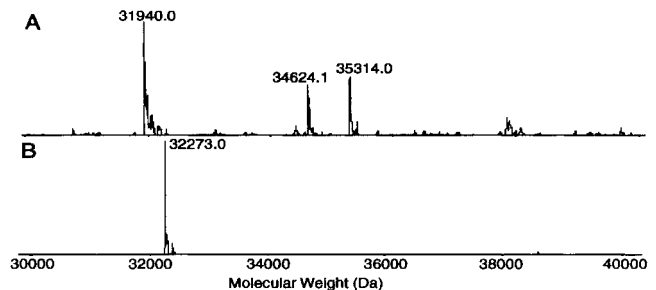
(M_r 35,328) calculated for acetylated preapocytochrome f but as well was very similar to that of FNR (M_r 35,315). Western blots of the M_r 35,000 polypeptide in the spinach complex showed that it reacted with an antibody to spinach FNR (Fig. 6, *lane 4 versus lane 2*), whereas the M_r 33,000 polypeptide reacted with an antibody to cytochrome f (Fig. 6, *lane 3 versus lane 2*). Furthermore, electrospray ionization mass spectroscopy of CNBr fragments of the M_r 35,314 polypeptide, isolated chromatographically from the chloroplast complex, ranging from 3020 to 6538 in molecular weight, were similar to those ex-
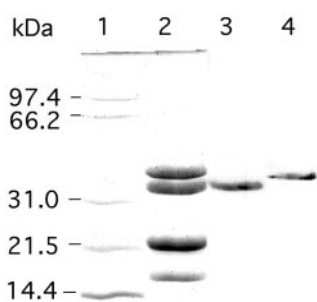
FIG. 6. **Western blot with antibody to cytochrome f (*lane 3*) and FNR (*lane 4*) of spinach cytochrome b_6f complex.** *Lane 1*, molecular weight standards; *lane 2*, spinach cytochrome b_6f complex; *lanes 3* and *4*, immune blots with antibody to cytochrome f and FNR, respectively.

TABLE II

Comparison of intact spinach 35-kDa polypeptide and its CNB fragments with calculated masses of FNR and cytochrome f by electrospray ionization mass spectroscopy

Measured mass of 35-kDa polypeptide[a] and CNBr fragments	Calculated mass of FNR[b,c] and fragments[b]	Calculated mass of preapocyt f[b,c] and fragments[b]
Da	*Da*	
$35,320.3 \pm 5.9$	$35,314\ (1{-}313)^{d,e}$	$35,328.7\ (2{-}318)$
CNBr		
$1,145.4 \pm 0.2$		$486.6\ (129{-}132)$
$3,020.2 \pm 0.7$	$3,020.3\ (220{-}245)$	
$4,097.7 \pm 0.7$	$4,099.6\ (24{-}60)$	$3,952.6\ (91{-}128)$
$4,357.9 \pm 0.5$	$4,355.9\ (278{-}314)^{f}$	$4,485.3\ (91{-}132)^{f}$
$5,657.2 \pm 0.7$		
$6,538.4 \pm 1.2$	$6,539.5\ (1{-}60)^{d,f}$	$10,018.6\ (2{-}90)$
		$20,471.5\ (133{-}318)$

[a] Intact masses were measured on API III (Sciex) and computed from raw spectra (Hypermass; Sciex); fragments on LCQ-DECA (Thermoquest) with Turbosequest software (Thermoquest). The intact mass reported here is 6 mass units higher than the value obtained with the software (BioMultiView 1.3.1, Sciex). See Fig. 5.

[b] Masses were calculated from SwissProt entries P00455 and P16013 with modifications using PeptideMass (www.expasy.org) set to generate the average mass for the uncharged species based on natural isotopic abundance. Only FNR fragments with sizes similar to the observed masses are shown.

[c] FNR with Q1 of mature form; pre apo *cyt f* with initiating Met removed and N-acetylated in calculation.

[d] Subtracted 17 for N-terminal pyroGlu.

[e] Subtracted 2 for single disulfide; assumed F268V (29).

[f] Added 16 because of single internal MetO, which resulted in a missed cleavage.
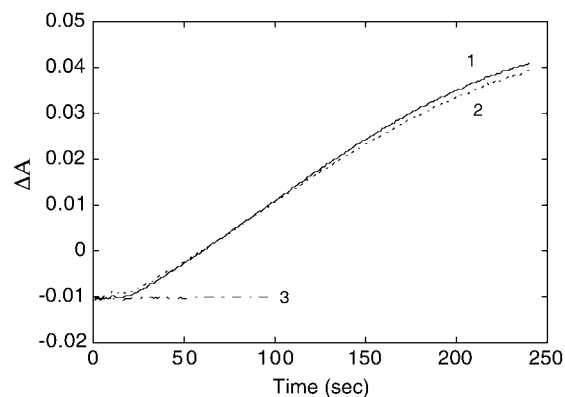
pected from FNR but not from cytochrome f (Table II). The M_r 1145 and 5657 CNBr fragments (Table II, first column) are thought to result from nonspecific cleavage.

Diaphorase activity (5–10 electrons per *cyt f*/s), arising from the presence of FNR, was readily detected in the spinach b_6f complex (Fig. 7, *trace 1*), identical to that in an EDTA-washed complex (Fig. 7, *dashed line, trace 2*), but not in the b_6f complex from *M. laminosus* (Fig. 7, *trace 3*). It was concluded that the M_r 35,000 polypeptide in the spinach b_6f complex is FNR.

Absence of FNR Does Not Affect Electron Transfer Activity in the High Potential Chain—The oxidation of quinol by the b_6f complex uses a bifurcated electron transfer pathway though a high (redox) potential chain and a low potential chain, in which the high potential chain contains the Rieske iron-sulfur protein, cytochrome f, plastocyanin, or cytochrome c_6 and P700. High levels of electron transfer activity were measured through the high potential chain of the spinach b_6f complex, 290 ± 60 electrons per *cyt f*/s at 22 °C measured from decylplastoquinol to ferricyanide via spinach plastocyanin. A similar level of activity, 340 ± 50 electrons per *cyt f*/s, was measured in b_6f complex isolated from *M. laminosus*, in which the FNR complex was not present (data not shown). This activity for both complexes is a measure of "noncyclic" electron transfer. The activity of the spinach and cyanobacterial complexes was inhibited by 1–2 μM 2,5-dibromo-6-isopropyl-1,4-benzoquinone. Stigmatellin (1 μM) was an effective inhibitor in chloroplast membranes but not of the complex from *M. laminosus* or spinach. An important conclusion from the activity measurements is that the presence of bound FNR in the spinach chloroplast b_6f complex does not affect the activity of the complex associated with noncyclic electron transfer.

Electron Transfer from NADPH to Cytochrome b_6—The presence of FNR bound to the b_6f complex suggests the possibility that NADPH could reduce the intramembrane redox centers of the complex, the two hemes of cytochrome b_6, heme b_n and heme b_p on the electronegative (stroma) and electropositive (lumen) sides of the membrane. Addition of NADPH alone causes no reduction of the cytochrome b hemes (Fig. 8, *spectrum 1*). However, in the presence of ferredoxin, NADPH reduces 0.4 of the 2 heme cytochrome b complement, a total of 0.8 heme (Fig. 8, *spectrum 2*), as seen in the comparison with the complete reduction of both hemes by dithionite (Fig. 8, *spectrum 3*). In this experiment, cytochrome f is initially reduced by ascorbate. Ferredoxin-dependent reduction by NADPH of half of the cytochrome b heme complement by NADPH was demonstrated previously with spinach thylakoid membranes, in which the half-complement was inferred from other data to be the stromal-side heme b_n. In any case, the ferredoxin-dependent b_6 reduction by NADPH, *in situ* (36) and *in vitro*, as shown in the present studies, implies that the b_6f complex with bound

FIG. 7. **FNR-dependent diaphorase activity in control (*1*) and EDTA-treated (*2*) spinach b_6f complex and *M. laminosus* cytochrome b_6f complex (*3*).**

FNR provides the membrane protein connection between the binding site of ferredoxin on the *psa*D subunit of the photosystem I reaction center complex and the plastoquinone pool. This presumably defines the pathway of cyclic electron transport (Fig. 9).

DISCUSSION

FNR in the Spinach Chloroplast Cytochrome b_6f Complex: Reality or Artifact?—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 (37). Although the preparation of Clark and Hind (38) was less well characterized with respect to purity, polypeptide composition, and activity than that utilized in the present study, some of the data (*e.g.* immunoreactivity and cyanogen bromide cleavage pattern) implying the presence of FNR in the complex are similar to those used in the present work. However, in subsequent studies by the same group, it was concluded that the presence of bound FNR in the spinach
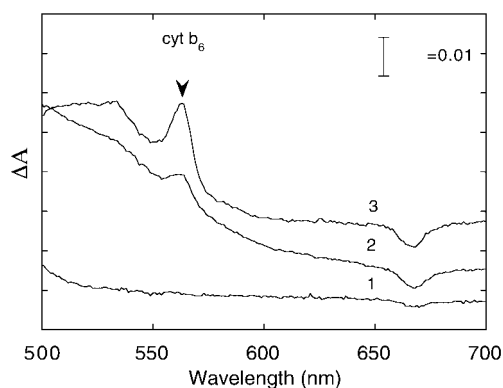
FIG. 8. **Cytochrome b_6 reduction by NADPH in the presence of ferredoxin.** Cytochrome b_6f complex was dissolved to a final concentration of 0.5 μM in 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.05% UDM, 5 mM glucose, 160 units of glucose oxidase, and 5 mM Na-ascorbate. NADPH (0.7 mM, *spectrum 1*), ferredoxin (10 μM, *spectrum 2*), and dithionite (*spectrum 3*) were added sequentially.
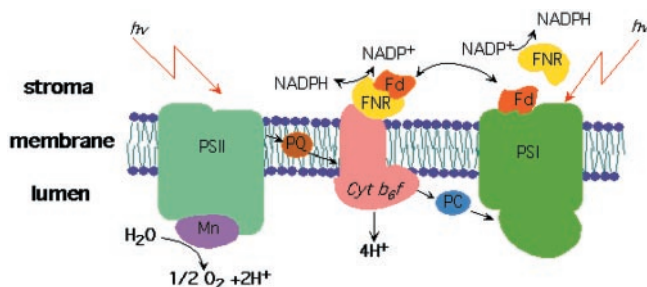

FIG. 9. **Schematic of electron transport complexes of oxygenic photosynthesis in thylakoid membrane showing the photosynthetic reaction center complexes, PSI and PSII and the cytochrome b_6f complex.** The location of FNR bound to the cytochrome b_6f complex and a site of ferredoxin binding on the FNR, as concluded from the ferredoxin-dependent partial reduction of cytochrome b_6 (Fig. 8), 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 that is proposed to be the stromal side pathway for ferredoxin-mediated cyclic electron transport. The soluble pool of FNR described by Matthijs *et al*. (44), which could also function as a ferredoxin-diffusive pathway for NADP$^+$ reduction, is shown.

b_6f complex was artifactual, based on the findings that: (i) the FNR could be cross-linked to a 17-kDa polypeptide of the lumen-side oxygen-evolving complex (39, 40); and (ii) there was a different pH dependence of diaphorase activity in pure FNR and in thylakoids (39). On the other hand, in the present study, biochemically meaningful binding in the spinach complex is implied by the stoichiometric and relatively tight binding of FNR to the complex after exposure to high ionic strength hydrophobic extraction and chromatography, sucrose gradient separation, and the diaphorase activity of the purified b_6f complex. A structural basis for the binding interaction between the FNR and b_6f complex can be proposed, as discussed below, but will not be definitive until there is a high resolution structure.

On the other hand, the absence of FNR from the cyanobacterial complex may well be an artifact of preparation, resulting in the loss of FNR during preparation arising from weaker binding to the b_6f complex, or a consequence of a different distribution of FNR in the cyanobacterial photosynthetic apparatus. Regarding the latter possibility, the deduced amino acid sequences of FNR from the cyanobacteria, *Synechococcus* 7002, *Anabaena*, and *Synechocystis* PCC 6803 have longer N-terminal extensions than those of *Spirulina* and chloroplasts. These N-terminal extensions have high sequence similarity to a phycocyanin-associated linker protein. It was hypothesized that the N-terminal domain of FNR localizes it to the extremities of

the peripheral rods of the phycobilisomes on the cyanobacterial membrane (41).

Membrane Binding Site on FNR—FNR is a charged water-soluble protein, suggesting that it might bind to the membrane electrostatically. However, FNR is not released from the membrane by exposure to high ionic strength or EDTA (Fig. 4, *lanes 3* and *4*), suggesting the possibility of a nonpolar binding mode. A large (200 Å^3, ~30–40 Å^2) hydrophobic cavity or channel in the NADP$^+$ binding domain of FNR was suggested as a possible membrane binding region of the protein (42). The structure of the spinach FNR:ferredoxin adduct (43) shows that the bound ferredoxin would block the NADP$^+$-proximal side of this cavity, and only the NADP$^+$-distal face of the cavity would be sufficiently exposed to allow binding to the membrane surface. Furthermore, only the latter arrangement would allow ferredoxin and the NADP$^+$ binding site to be exposed to the solvent, as they must be. Inspection of the NADP$^+$-distal face of the cavity in spinach FNR shows it to be made of three α-helices, 8–15 residues in length extending from residues 262 to 305, which have an amphipathic character. It is proposed that this amphipathic face of the hydrophobic cavity is the membrane binding face of FNR.

Soluble and Membrane-bound FNR—Approximately half of the FNR content of intact spinach chloroplasts could be extracted by osmotic shock (44), implying that this fraction is not membrane-bound. The membrane-bound fraction could be removed by a zwitterionic detergent (44). The only known membrane binding site for FNR is that on the cytochrome b_6f complex demonstrated in the present study. The finding that FNR bound to cytochrome b_6f complex (44) or that chloroplast thylakoid membranes (40) could be cross-linked to a 17-kDa polypeptide, which is not subunit IV of the b_6f complex but rather a lumen-side polypeptide of the oxygen-evolving complex, on the side of the membrane opposite to that at which NADP$^+$ reduction occurs, added a confusing element to the previous studies (40). At present, one can say that at least one membrane-bound component of FNR is tightly bound to the chloroplast cytochrome b_6f complex at a level of one polypeptide per cytochrome f or per monomer of the complex. Then, two FNR polypeptides would be bound per dimeric unit. The question then arises of the role of this component in NADP(H)-linked reactions.

Site of NADP$^+$ Reduction—As inferred from cross-linking experiments, the reduction of ferredoxin occurs on the stromal side of the photosystem I reaction center (45), where its extrinsic *psa*D subunit, to which ferredoxin can be cross-linked (46), is located. The reduction of ferredoxin is the last known membrane-bound electron transfer step in the pathway of NADP$^+$ reduction. Cross-linking of FNR to the PSI reaction center has not been reported (47), although cross-linking of flavodoxin, which can substitute for ferredoxin in the reduction of NADP$^+$, has been demonstrated (48). NADP$^+$ reduction can use membrane-bound or soluble FNR (44), ferredoxin either diffusing to the latter from its *psa*D binding site (Fig. 9) or binding FNR transiently at the *psa*D site. Alternatively, NADP$^+$ reduction could occur via membrane-bound FNR, for which the only defined site at present is the cytochrome b_6f complex defined in the present study. Electron transfer from ferredoxin bound at the PSI *psa*D site to the cytochrome b_6f complex would be mediated by either ferredoxin diffusion (Fig. 9) or a close interaction between the PSI and b_6f complexes. The rationale for and properties of such a "supercomplex" between the bacterial cytochrome bc_1 and reaction center complexes has been discussed and debated (49, 50).

FNR and Ferredoxin-mediated Cyclic Electron Transport—The presence of active FNR (Figs. 7 and 8) bound stoichiomet-

rically to the b_6f complex, with a requirement for ferredoxin for its activity (Fig. 8), implies that the cytochrome b_6f complex is the membrane protein complex responsible for coupling electron transfer from the reducing side of photosystem I to the main electron transport chain in ferredoxin-dependent cyclic electron transport. It has previously been argued on the basis of anti-mycin insensitivity of flash-induced heme b_6 oxidoreduction 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 (51). 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. The identification in the present study of FNR as an integral, stoichiometric, active polypeptide subunit of the b_6f complex suggests that the previous conclusions based on partial *versus* complete inhibition of cyclic phosphorylation by anti-mycin A may not have been a sufficient basis to exclude a role of the cytochrome b_6f complex in this pathway (51). Kinetic competence of electron transfer to FNR was indicated by the studies of Bouges-Bocquet (32), who measured a half-time of <10 μs in *Chlorella* for reduction by a light flash of a spectral component with a difference spectrum that was very similar to FNR.

In light of the recent structural data on the bc_1 complex (*e.g.* Ref. 2), the stromal-side heme *b* seems an obvious candidate for a redox group at the stromal-side membrane interface to mediate electron transfer from stromal side peripheral and soluble electron donor proteins such as FNR and ferredoxin. Reduction of half of the heme *b* complement by NADPH, the same result as obtained previously with thylakoid membranes (36), suggests that only the stromal side heme is reduced via NADPH and ferredoxin. The two-electron reduction of plastoquinone would then require either transfer of two electrons through this heme, or cooperative one-electron reductions by both stromal and lumen side hemes as proposed by Furbacher *et al.* (36).

Differences between Cytochrome bc_1 and b_6f Complexes—The cytochrome bc_1 and b_6f complexes are thought to be fundamentally similar in their core structure and some functions. However, it is now known that, except for the core cytochrome *b* (52) and the cluster binding domain of the iron-sulfur protein (9), there are major differences in the polypeptide subunits of the complex, the greatest being between cytochromes *f* and c_1 (3). Additional differences in function are expected because of the different membrane and cellular environments. The 15.2-kDa polypeptide with kinase function (53) in the b_6f complex of *C. reinhardtii* and the 35-kDa FNR subunit in the plant chloroplast cytochrome b_6f allow unique functions to be carried out by the b_6f complex that are not expressed in the cytochrome bc_1 complex from mitochondria or photosynthetic bacteria.

REFERENCES

1. Cramer, W. A., Soriano, G. M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S. E., and Smith, J. L. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **47,** 477–508
2. Hunte, C., Koepke, J., Lange, C., Rossmanith, T., and Michel, H. (2000) *Structure* **8,** 669–684
3. Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A., and Smith, J. L. (1994) *Structure* **2,** 95–105
4. Martinez, S., Huang, D., Ponomarev, M., Cramer, W. A., and Smith, J. L. (1996) *Protein Sci.* **5,** 1081–1092
5. Carrell, C. J., Schlarb, B. G., Bendall, D. S., Howe, C. J., Cramer, W. A., and Smith, J. L. (1999) *Biochemistry* **38,** 9590–9599
6. Sainz, G., Carrell, C. J., Ponamarev, M. V., Soriano, G. M., Cramer, W. A., and Smith, J. L. (2000) *Biochemistry* **39,** 9164–9173
7. Chi, Y. I., Huang, L. S., Zhang, Z., Fernandez-Velasco, J. G., and Berry, E. A. (2000) *Biochemistry* **39,** 7689–7701
8. Zhang, H., Carrell, C. J., Huang, H., Sled, V., Ohnishi, T., Smith, J. L., and Cramer, W. A. (1996) *J. Biol. Chem.* **271,** 31360–31336
9. Carrell, C. J., Zhang, H., Cramer, W. A., and Smith, J. L. (1997) *Structure* **5,** 1613–1625
10. Haley, J., and Bogorad, L. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1534–1538
11. Bald, D., Kruip, J., Boekema, E. J., and Roegner, M. (1992) in *Research in Photosynthesis: Proceedings of the Ninth International Congress on Photosynthesis* (Murata, N., ed), pp. 629–632, Kluwer Academic Publishers, Dordrecht
12. 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
13. Pierre, Y., Breyton, C., Kramer, D., and Popot, J. L. (1995) *J. Biol. Chem.* **270,** 29342–29349
14. Zhang, H., Huang, D., and Cramer, W. A. (1999) *J. Biol. Chem.* **274,** 1581–1587
15. Mosser, G., Breyton, C., Olofsson, A., Popot, J.-L., and Rigoud, J.-L. (1997) *J. Biol. Chem.* **272,** 20263–20268
16. Breyton, C. (2000) *J. Biol. Chem.* **275,** 13195–13201
17. 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
18. Hurt, E. C., and Hauska, G. (1981) *Eur. J. Biochem.* **117,** 591–599
19. Morand, L. Z., and Krogmann, D. W. (1993) *Biochim. Biophys. Acta* **1141,** 105–106
20. Katoh, S., Shiratori, I., and Takamiya, A. (1962) *J. Biochem. Tokyo* **51,** 32–40
21. Avron, D. I., and Jagendorf, A. T. (1956) *Arch. Biochem. Biophys.* **65,** 475–483
22. Schägger, H., and von Jagow, G. (1991) *Anal. Biochem.* **199,** 223–231
23. Schägger, H., Cramer, W. A., and von Jagow, G. (1994) *Anal. Biochem.* **217,** 220–230
24. Thomas, P. E., Ryan, D., and Levin, W. (1976) *Anal. Biochem.* **75,** 168–176
25. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J., and Struhl, K. (eds) (2000) *Current Protocols in Molecular Biology, Preparation and Analysis of DNA*, Vol. 1, John Wiley and Sons, New York
26. Whitelegge, J. P., le Coutre, J., Lee, J. C., Engel, C. K., Prive, G. G., Faull, K. F., and Kaback, H. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10695–10698
27. le Coutre, J., Whitelegge, J. P., Gross, A., Turk, E., Wright, E. M., Kaback, H. R., and Faull, K. F. (2000) *Biochemistry* **39,** 4237–4242
28. Whitelegge, J. P., Gundersen, C., and Faull, K. F. (1998) *Protein Sci.* **7,** 1423–1430
29. Karplus, A. P., Walsh, K. A., and Herriott, J. R. (1984) *Biochemistry* **23,** 6576–6583
30. 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
31. Batie, C. J., and Kamin, H. (1981) *J. Biol. Chem.* **256,** 7756–7763
32. Bouges-Bocquet, B. (1978) *FEBS Lett.* **85,** 340–344
33. Romanowska, E., and Albertsson, P.-A. (1994) *Plant Cell Physiol.* **35,** 557–568
34. Szczepaniak, A., Black, M. T., and Cramer, W. A. (1989) *Z. Naturforsch.* **44c,** 453–461
35. Alt, J., and Herrmann, R. G. (1984) *Curr. Genet.* **8,** 551–557
36. Furbacher, P. N., Girvin, M. E., and Cramer, W. A. (1989) *Biochemistry* **28,** 8990–8998
37. Clark, R. D., Hawkesford, M. J., Coughlan, S. J., Nennett, J., and Hind, G. (1984) *FEBS Lett.* **174,** 137–142
38. Clark, R. D., and Hind, G. (1983) *J. Biol. Chem.* **258,** 10348–10354
39. Coughlan, S., Matthijs, H. C. P., and Hind, G. (1985) *J. Biol. Chem.* **260,** 14891–14894.0
40. Soncini, F. C., and Vallejos, R. H. (1989) *J. Biol. Chem.* **264,** 21112–21115
41. Schluchter, W. M., and Bryant, D. A. (1992) *Biochemistry* **31,** 3092–3102
42. Bruns, C. M., and Karplus, A. P. (1995) *J. Mol. Biol.* **247,** 125–145
43. Kurisu, G., Kusunoki, M., Katoh, E., Yamazaki, T., Teshima, K., Onda, Y., Kimata-Ariga, Y., and Hase, T. (2001) *Nat. Struct. Biol.* **8,** 117–121
44. Matthijs, H. C. P., Coughlan, S. J., and Hind, G. (1986) *J. Biol. Chem.* **261,** 12154–12158
45. Krauss, N., Schubert, W.-D., Klukas, O., Fromme, P., Witt, H. T., and Sänger, W. (1996) *Nat. Struct. Biol.* **3,** 965–973
46. Wynn, R. M., Omaha, J., and Malkin, R. (1989) *Biochemistry* **28,** 5554–5560
47. Golbeck, J. H. (1994) in *The Molecular Biology of Cyanobacteria* (Bryant, D. A., ed), pp. 319–360, Kluwer Academic Publishers, Dordrecht
48. Muehlenhoff, U., Zhao, J., and Bryant, D. A. (1996) *Eur. J. Biochem.* **235,** 324–331
49. Vermeglio, A., and Joliot, P. (1999) *Trends Microbiol.* **8,** 106–107
50. Crofts, A. R. (2000) *Trends Microbiol.* **8,** 107–108
51. Moss, D. A., and Bendall, D. S. (1984) *Biochim. Biophys. Acta* **767,** 389–395
52. Widger, W. R., Cramer, W. A., Herrmann, R. G., and Trebst, A. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 674–678
53. Hamel, P., Olive, J., Pierre, Y., Wollman, F.-A., and de Vitry, C. (2000) *J. Biol. Chem.* **275,** 17072–17079
54. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680