Reconstitution of the 2Fe-2S Center and $g = 1.89$ Electron Paramagnetic Resonance Signal into Overproduced *Nostoc* sp. PCC 7906 Rieske Protein†

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ABSTRACT: The Rieske 2Fe-2S protein is a distinguishing subunit of the photosynthetic electron transport cytochrome b_6f complex in chloroplast and cyanobacterial thylakoid membranes. We have constructed plasmids for overproduction in *Escherichia coli* of fusion, full-length, and truncated forms of the Rieske (PetC) protein from the cyanobacterium *Nostoc* sp. PCC 7906. A glutathione *S*-transferase/Rieske fusion protein was used to prepare specific chicken egg-yolk antibodies against the Rieske protein. Expression of the nonfusion *petC* gene in a T7 RNA polymerase promoter vector produced copious quantities of the full-length Rieske protein predominantly as inclusion bodies. The highly enriched, Rieske protein from inclusion bodies has been denatured in guanidine hydrochloride and refolded and the characteristic 2Fe-2S cluster reconstituted *in vitro* by incubation with iron and sulfide under reducing conditions. Purification by chromatography on Whatman DE52 cellulose and ultrafiltration through a 30 000 molecular weight cutoff membrane yielded pure and predominantly monomeric Rieske protein. Reconstituted Rieske preparations showed intense and highly characteristic $g_x = 1.74$, $g_y = 1.89$, and $g_z = 2.03$ "Rieske-type" electron paramagnetic resonance signals at 15 K. Two methods of reconstitution yielded Rieske preparations in which 20−60% of the protein contained 2Fe-2S clusters as determined by EPR spin quantitation. The reconstituted Rieske protein was soluble and stable at 4 °C in buffers containing nonionic detergents and showed a redox midpoint potential of +321 mV at pH 7.0 as determined by optical circular dichroism (CD) spectroscopy. These data demonstrate the *in vitro* restoration of a Cys and His liganded 2Fe-2S cluster and provide the basis for mutational and structural analysis of a PetC Rieske protein of oxygenic photosynthesis.

The chloroplast/cyanobacterial cytochrome b_6f complex (plastoquinol-cytochrome c553/plastocyanin oxidoreductase) catalyzes the rate-limiting quinol-oxidation step in photosynthetic electron transport and establishes a trans-thylakoid-membrane proton gradient [reviewed by Kallas (1994) and Cramer et al. (1996)]. The complex contains four major proteins, cytochrome *f* (PetA, one c heme), cytochrome b_6 (PetB, two b hemes), the Rieske (PetC, one 2Fe-2S center), and a subunit IV (PetD). Additional low molecular weight PetG, PetL, and PetM subunits have been identified in chloroplasts (Haley & Bogorad, 1989; Pierre et al., 1995; Berthold et al., 1995). Mitochondria and many bacteria possess a cyt bc_1 complex (complex III) that shares fundamental structural and functional features with cyt b_6f complexes. "Q-cycle" models involving separate binding sites for quinol-oxidation and quinone-reduction and transmembrane electron transfer between the two b hemes explain most of the data on electron and proton transfer reactions in cyt bc_1 complexes [reviewed by Trumpower and Gennis (1994) and Gray and Daldal (1995)] but are more controversial in cyt b_6f complexes (Kramer & Crofts, 1994; Joliot & Joliot, 1994; Cramer et al., 1996, and references therein). The high-potential (ca. +320 mV) Rieske 2Fe-2S protein with its characteristic $g_y = 1.89-1.90$ electron paramagnetic resonance (EPR[1]) signal is an essential subunit of both cyt b_6f and bc_1 complexes (Rieske et al., 1964; Malkin & Aparicio, 1975). Evidence including shifts in Rieske EPR spectra caused by quinone-analog inhibitors (Malkin, 1982; McCurley et al., 1990) and mutational studies of cyt bc_1 Rieske proteins (Davidson et al., 1992a; Van Doren et al., 1993a) points to intimate involvement of the Rieske center in quinol-oxidation.

High-resolution structures based on X-ray crystallography have recently been solved for the lumenal, heme-binding domain of turnip cyt *f* (Martinez et al., 1994) and a water-

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[1] Abbreviations: Ap, ampicillin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; β-DM, dodecyl β-D-maltoside; β-ME, β-mercaptoethanol; cyt, cytochrome; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DMT, dimethoxytrityl; DTT, dithiothreitol; EPR, electron paramagnetic resonance; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton(s); LB medium, Luria−Bertani medium; MS medium, minimal salts medium; OG, octyl β-D-glucoside; NBT, nitroblue tetrazolium; M_r, relative molecular mass; MES, (2-morpholinoethanesulfonic acid); PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine.

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soluble fragment of the Rieske protein from the bovine heart mitochondrial cyt bc_1 complex (Iwata et al., 1996). Full understanding of reaction mechanisms and the molecular basis for functional differences between b_6f and bc_1 cyt complexes will require further structural characterization of these complexes. The goal of the current work was to overproduce in *Escherichia coli* the Rieske 2Fe-2S protein from the cyanobacterium *Nostoc* sp. PCC 7906 (Kallas et al., 1988) for mutational analysis and structural characterizations. *In vivo* mutations in cyanobacterial cyt b_6f complexes are limited to nonlethal ones because the complex is required for both photosynthesis and respiration [reviewed by Kallas (1994)]. A subsequent objective is *in vitro* reconstitution of an active cyt b_6f complex from overproduced Rieske protein and Rieske-depleted cyt b_6f complex to permit mutational studies of the Rieske protein in assembly and catalytic activity. Activity has previously been restored to both bovine mitochondrial bc_1 (Trumpower et al., 1980; González-Halfen et al., 1991) and spinach chloroplast b_6f (Adam & Malkin, 1987) Rieske-depleted cyt complexes by *in vitro* reconstitution with purified Rieske proteins.

Spectroscopic studies have identified His imidazole nitrogen as well as Cys sulfur ligands for Rieske clusters in *bc/bf* complexes (Britt et al., 1991; Gurbiel et al., 1991) and bacterial oxygenases (Fee et al., 1986; Gurbiel et al., 1989; Shergill et al., 1995) in contrast to the all Cys coordination of 2Fe-2S clusters in ferredoxins (Tsukihara et al., 1986; Holden et al., 1994). This is thought to account in part for the high potentials (+160 to +320 mV) of Rieske 2Fe-2S centers relative to −270 to −460 mV in plant, vertebrate, and bacterial 2Fe-2S ferredoxins (Fee et al., 1986; Riedel et al., 1995). Cys liganded 2Fe-2S (Coghlan & Vickery, 1989; Ta & Vickery, 1992), 3Fe-4S (Manodori et al., 1992), and 4Fe-4S (Zheng et al., 1992) proteins have previously been produced in *E. coli* with correctly assembled Fe-S clusters. The *Rhodobacter sphaeroides* Rieske protein has been produced in *E. coli* but not with the typical Rieske center (Van Doren et al., 1993b). Malkin and Rabinowitz (1966) showed some time ago that iron−sulfur centers could be restored to soluble ferredoxins by reconstitution *in vitro* with iron and sulfide. Variations of this procedure have been used to reconstitute Fe-S centers into overproduced 4Fe-4S PsaC protein (Li et al., 1991) and 2Fe-2S *Anabaena* sp. PCC 7120 vegetative (Cheng et al., 1994) and heterocyst (Chae et al., 1994) ferredoxins.

Here we report the production in *E. coli* of fusion, full-length, and truncated forms of the *Nostoc* sp. PCC 7906 Rieske iron−sulfur protein. A glutathione *S*-transferase (GST)/Rieske fusion protein has been used to raise a specific antibody. The full-length Rieske protein, expressed from a T7 RNA polymerase promoter vector, accumulated copiously and predominantly in the form of inclusion bodies. These have been solubilized and the Cys and His coordinated 2Fe-2S Rieske center and its characteristic $g = 1.89$ EPR signal restored by *in vitro* reconstitution. These results provide the basis for production of large quantities of the cyt b_6f Rieske protein for structural and functional studies. Preliminary aspects of this work have been reported previously (Holton et al., 1992; Wu et al., 1995).

MATERIALS AND METHODS

Bacterial Strains, Gene Manipulations, and Reagents. *E. coli* strains were grown in broth or agar LB or minimal salts (MS/glucose/thiamine) medium supplemented as needed with 150 μg of Ap/mL (Sambrook et al., 1989). Strains DH5α or DH5αMCR (Gibco-BRL, Gaithersburg, MD) were used for plasmid propagation. Expression strains are described below. DNA manipulations were performed as described in Sambrook et al. (1989). Plasmids were purified either on Qiagen-tip (Qiagen, Chatsworth, CA) or on "Wizard" (Promega, Madison, WI) columns. Oligonucleotides were synthesized on an Applied Biosystems (Perkin-Elmer, Foster City, CA) model 392 DNA synthesizer. The 5′-dimethoxytrityl (DMT) protecting group was left on and used for purification of full-length oligonucleotides by chromatography on Waters SEP-PAC columns (Millipore, Bedford, MA). Restriction endonucleases, DNA-modifying enzymes, and T4 DNA ligase were purchased from Promega or New England Biolabs (Beverly, MA). Octyl glucoside was purchased from Anatrace (Maumee, OH).

Construction of Plasmids for Production of Glutathione S-Transferase (GST)/Rieske Fusion Proteins. A DNA fragment carrying the *Nostoc* sp. PCC 7906 *petC* gene (Kallas et al., 1988) was partially degraded from its 5′ end with *Bal*31 nuclease and then ligated to *Sma*I-digested plasmid pGEX-3X (Smith & Johnson, 1988). Induction of GST gene expression in *E. coli* DH5α transformants bearing these recombinant plasmids revealed several that produced ca. 43 kDa GST/Rieske fusion proteins. Sequence analysis of two such plasmids, pGR650-1 and pGR650-32, showed that codons for N-terminal amino acids 1−13 and 1−17, respectively, had been deleted from the Rieske portions of the encoded fusion proteins.

Preparation of Chicken Antibodies against GST/Rieske Fusion Proteins. Partially purified GST/Rieske fusion proteins from *E. coli* (pGR650-1) and (pGR650-32) lysates were fractionated by SDS−PAGE. The 43 kDa GST/Rieske fusion protein bands (ca. 0.5 mg total) were excised and extracted overnight at room temperature in 1% SDS, 50 mM Tris-HCl pH 7.5, 0.1% β-mercaptoethanol. The acrylamide was washed twice in the same buffer, and the combined extracts were concentrated and dialyzed against 0.01% SDS, 20 mM Tris-HCl pH 7.5 buffer. A 0.3 mL portion of this extract (ca. 0.5 mg/mL) was mixed with 0.45 mL of 0.01 M sodium phosphate pH 7.2, 0.1 M NaCl buffer, emulsified with 0.75 mL of Freund's adjuvant, and two 0.4 mL portions were injected into the breast muscle of a White Leghorn chicken. A second set of injections without adjuvant was given 13 days later. IgY antibodies were extracted from egg yolk (starting 2−3 weeks after the second injection) by the procedure of Gassmann et al. (1990). The IgY-containing supernatant has been successfully stored at both −20 and 4 °C. Portions of the GST/Rieske IgY solutions were preadsorbed against *E. coli* lysates to minimize nonspecific cross reactivity (Sambrook et al., 1989).

Construction of Plasmids for Production of Full-Length and Truncated, Nonfusion Rieske Proteins. Plasmid p3aR contains the *Nostoc* sp. PCC 7906 *petC* gene downstream of the T7 RNA polymerase promoter and ribosome binding site of plasmid pET3a (Studier et al., 1990). Figure 1 (panel A) outlines the "5′-add-on" PCR method of Higuchi (1990) used to create the junction of plasmid pET3a with the Rieske ATG start codon. This approach minimized the possibility of base misincorporation by PCR. Four primers were used: P3af (5′-GATCGAGATCTCGATCC-3′) elongated pET3a downstream toward the ATG start; PRFe-Sr (5′-ACCAC-

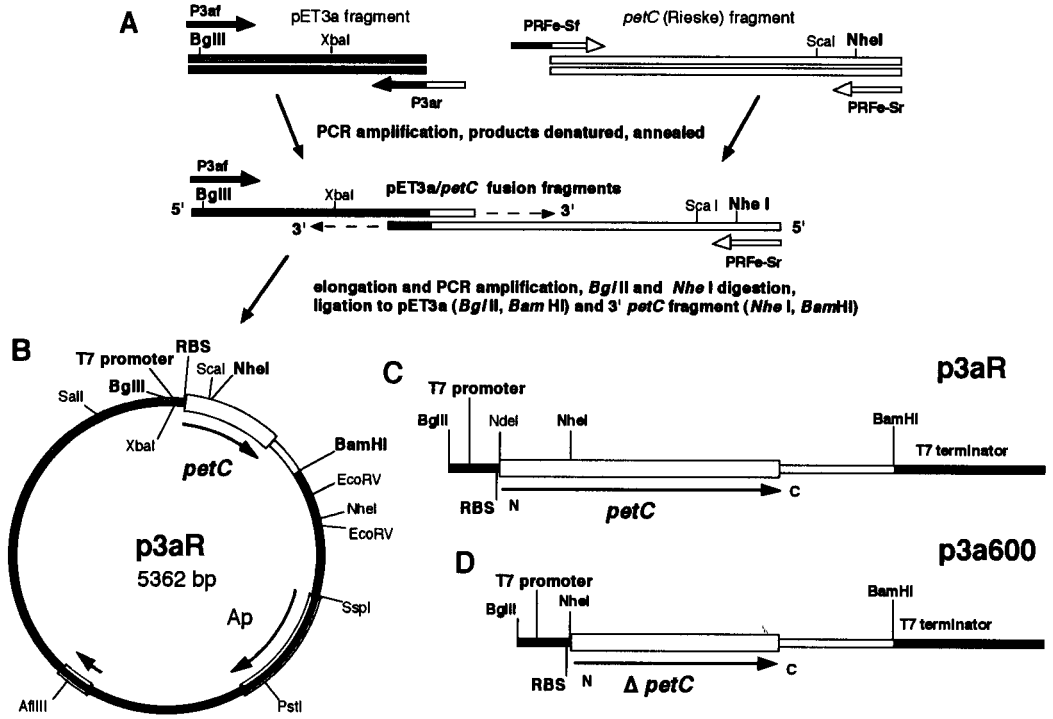

FIGURE 1: Construction of plasmids for expression of full-length and truncated *Nostoc* sp. PCC 7906 Rieske PetC proteins. Panel A: Two sets of PCR reactions were performed with the four primers described in Materials and Methods. Primers P3af and P3ar amplified the T7 promoter region of plasmid pET3a and created the pET3a/*petC* junction. Primers PRFe-Sf and PRFe-Sr amplified the 5′ region of the *petC* gene and also created the pET3a/*petC* junction. Denaturation and reannealing of the products of these PCR reactions created some overlapping molecules that annealed (as shown) via complementary base pairing of pET3a/*petC* junction sequences. Addition of the outside primers, P3af and PRFe-Sr, and PCR reagents allowed 5′ to 3′ elongation of the junction regions and subsequent, specific amplification of the entire pET3a/*petC* fusion fragment. Digestion of this PCR product with *Bgl*II/*Nhe*I and ligation to an *Nhe*I/*Bam*HI-digested 3′ *petC* fragment and to *Bgl*II/*Bam*HI-digested plasmid pET3a generated plasmid p3aR (panel B). Panel C shows the full-length *petC* gene in plasmid p3aR in relation to the T7 RNA polymerase promoter, ribosome binding site (RBS), and T7 transcription terminator. In this construct the ATG start codon of plasmid pET3a has been replaced with the ATG start codon and entire 537 base pair Rieske protein coding sequence. Panel D shows the truncated *petC* gene in plasmid p3a600 constructed as described in Materials and Methods for production of the C-terminal two-thirds of the Rieske protein.

CAGCACCACC-3′) elongated within *petC* upstream toward the ATG start; P3ar (5′-TGATTCTGAAAATTGAGC-CATATGTATATCTCCTTCTTA-3′) contained the Rieske/pET3a junction and elongated pET3a upstream from the ATG start; and PRFe-Sf (5′-TAAGAAGGAGATATA-CATATGGCTCAATTTTCAGAATCA-3′), essentially the inverse complement of P3ar, contained the pET3a/Rieske junction and elongated *petC* downstream from the ATG start. Denaturation and reannealing of the products of the first round of PCR amplifications allowed formation of some molecules that contained duplex pET3a/Rieske junctions as shown in Figure 1, panel A. These were elongated and further amplified from the outside, P3af and PRFe-Sr, primers. These amplification products were digested with *Bgl*II and *Nhe*I and ligated to the 3′-fragment (*Nhe*I/*Bam*HI) of *petC* and to *Bgl*II/*Bam*HI-digested pET3a to create plasmid p3aR (Figure 1, panel B). Figure 1, panel C shows the full-length Rieske, *petC* gene and flanking sequences in plasmid p3aR. Plasmid p3a600 (*petC* portion shown in Figure 1, panel D) was constructed for production of a soluble, C-terminal, iron−sulfur binding domain of the Rieske protein. Plasmid pET3a was first digested with *Eco*RV and then religated to eliminate a 200 bp fragment containing an *Nhe*I site (Figure 1, panel B). Ligation of an *Nhe*I/*Bam*HI digest of this new plasmid, pET3aNheI$^-$, to the *Nhe*I/*Bam*HI C-terminal *petC* fragment, created plasmid p3a600 in which the C-terminal portion of the Rieske gene was joined "in-frame" with the ATG start codon of pET3a (Figure 1, panel D). The Rieske polypeptide encoded by p3a600 lacks the first 45 amino acids including the N-terminal hydrophobic domain of the full-length Rieske protein.

Protein Electrophoresis and Immunoblotting. SDS−PAGE and immunoblot analyses were performed largely as described by Sambrook et al. (1989). Samples for SDS−PAGE were incubated, typically 1−2 min at 90−100 °C, with an equal volume of denaturing sample buffer. Resolving gels contained 12% acrylamide (30% acrylamide/0.8% bis-acrylamide stock). Low-range molecular weight standards were purchased from Bio-Rad (Richmond, CA). Proteins from gels were transferred electrophoretically onto Millipore (Bedford, MA) Immobilon-P, PVDF membranes. Protein standards on membranes were visualized by staining with 0.1% Amido Black (Millipore Immobilon Tech Protocol TP008). Nonfat dry milk was used as the blocking agent. The chicken anti-GST/Rieske primary antibody and the secondary rabbit anti-chicken IgG alkaline phosphatase conjugate (Sigma, St. Louis, MO) were used at 1/10000 and 1/20000 dilutions, respectively. Cross-reacting protein bands were visualized with BCIP (0.17 mg/mL) and NBT (0.33 mg/mL) purchased from Gibco-BRL (Gaithersburg, MD).

Overproduction, in Vitro Reconstitution of Iron−Sulfur Centers, and Purification of the Reconstituted Rieske Protein. Plasmids p3aR and p3a600 were transferred into *E. coli* BL21(DE3) (Studier et al., 1990). Plasmid-bearing BL21-(DE3) cultures were grown to mid-exponential phase (ca. 60 Klett Units, Klett−Summerson colorimeter, no. 66 red

filter) at 37 °C in MS/thiamine/glucose (1 g/L) medium containing 150 μg of Ap/mL. Gene expression was induced by addition of 0.4 mM IPTG. Cells were harvested after 2−4 h incubation, resuspended in 50 mM Tris-HCl pH 7.8, 10 mM EDTA, 1 mM PMSF, and broken by passage (2−3 times) through a French pressure cell at 20000 psi and 4 °C.

Two procedures were used for reconstitution. The first was based on that of Malkin and Rabinowitz (1966) for clostridial ferredoxin and that of Golbeck and co-workers (Parrett et al., 1990; Zhao et al., 1990; Mehari et al., 1991) for photosystem I Fe−S proteins. The (16000*g*) pellet fraction of the lysate (from 1−2 L of culture) was washed with distilled water and proteins solubilized by 30 min incubation in 10 mL of 5 M guanidine-hydrochloride, 25 mM MES, pH 6.8. Proteins were allowed to refold by dilution (1:10) into 50 mM MES pH 6.8, 0.5 M mannitol, 0.03% β-DM, 0.5% β-mercaptoethanol (β-ME), 1 mM PMSF, 1 mM amino caproic acid buffer and dialysis against the same buffer but with β-DM replaced by 1 mM octyl glucoside (OG) or 0.01% Triton X-100. After dialysis against 50 mM Tris-HCl pH 8.3, 1% β-ME, 1 mM OG (or 0.01% Triton X-100), the solution (1−20 mg of protein in 10 mL) was sparged with N_2 gas, anaerobic solutions of $FeCl_3$ and Na_2S (30 mM each) were added dropwise to a concentration of 100 μM, and the solution was stirred under N_2 for 1−1.5 h. Proteins were precipitated by addition of $(NH_4)_2SO_4$ to 50% of saturation, resuspended in 5−10 mL of 50 mM Tris-HCl pH 8.3, 1% β-ME (or 1 mM DTT), 1−20 mM OG (or 0.01% Triton X-100), and dialyzed against the same buffer. In the second procedure [based on Cheng et al. (1995) and Markley (John Markley, University of Wisconsin−Madison, personal communication)] inclusion bodies in 10 mL as above were solubilized by the addition of solid urea to 8 M. This solution was sparged with N_2, β-ME was added to 1% and OG to 1 mM, and $FeCl_3$ was added to 1 mM with continued sparging for 30 min. Na_2S was added to 1 mM with further sparging for 30 min. The solution was diluted 8-fold by the addition of N_2-sparged 50 mM Tris-HCl pH 8.3, 1 mM OG buffer, further diluted 2-fold by the addition of 50 mM Tris-HCl pH 8.3, 1 mM OG buffer equilibrated in air, and then dialyzed against the same buffer. Samples were concentrated on Centricon-10 (10 000 mol wt cutoff) ultrafiltration units (Amicon, Beverly, MA) to 1−3 mg of protein/mL and stored at 4 °C or for longer periods at −80 °C.

Reconstituted Rieske proteins were purified by anion exchange chromatography on DE52 resin (Whatman, Hillsboro, OR). Samples (250 μL containing 0.5−1 mg of protein) in 50 mM Tris-HCl pH 8.3, 1 mM DTT, 1 mM OG were mixed with 1.0 mL of DE52 resin equilibrated with 10 mM Tricine−NaOH pH 8.0, 1 mM DTT, 20 mM OG, loaded into an empty 1.5 cm diameter column, and washed with 5 mL of 10 mM Tricine−NaOH pH 8.0, 1 mM DTT, 1 mM OG. Following a series of washes in this buffer containing NaCl at 0.01 M (5 mL), 0.05 M (5 mL), 0.1 M (3 mL), and 0.15 M (2 mL), the brownish Rieske protein was eluted in the same buffer containing 0.2 M NaCl. Protein concentrations were estimated by the Bradford (1976) method with reagents purchased from Bio-Rad (Richmond, CA). Interfering thiol reagents and detergents were removed by ultrafiltration. Iron content in protein samples was determined relative to standard ferric nitrate solutions by atomic absorption spectrophotometry on an Instrumental Laboratory Inc. spectrometer.

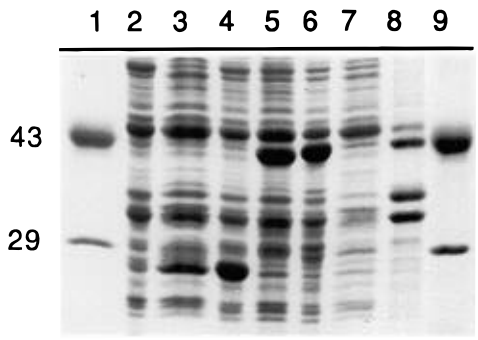

FIGURE 2: Production of GST/Rieske fusion proteins in *E. coli*. Stained SDS polyacrylamide gel showing lysates of uninduced or IPTG-induced *E. coli* carrying plasmids pGEX-3X or pGR650. Lanes 1 and 9 show size standards (kDa). Lanes 2−6 contain lysates of *E. coli* bearing uninduced pGR650-2 (an "out-of-frame" fusion, lane 2), induced pGEX-3X (lane 3), induced pGR650-2 (lane 4), induced pGR650-1 (lane 5), and induced pGR650-32 (lane 6). Lanes 7 and 8 contain supernatant and pellet fractions, respectively, from lysates of induced *E. coli* (pGR650-1).

EPR Spectroscopy. EPR spectra were obtained on a Varian E-4 X-band spectrometer at the National Biomedical ESR Center, Medical College of Wisconsin, Milwaukee, WI. Sample temperatures of 15 K were maintained by a liquid helium cryostat. Data collection from Rieske protein samples (0.3−0.4 mL at 1−3 mg/mL) reduced by β-ME or DTT usually required one scan. Typical parameters were 5 mW microwave power, 10 G modulation amplitude, 0.1 s time constant, 1000 G scan width, and 100 kHz modulation frequency. The concentration of iron−sulfur centers was estimated by double integration of the Rieske signal and comparison against a copper perchlorate standard (Aasa et al., 1976).

Circular Dichroism (CD) Spectroscopy and Redox Titration. Spectra were recorded on a Jasco 40A CD spectrometer interfaced to a PC computer. Samples contained 5 μM reconstituted Rieske Fe−S protein in 50 mM phosphate, 1 mM OG buffer pH 7.0 with 10 μM each of DAD and TMPD. E_h was measured in an anaerobic cuvette at 25 °C under flowing Ar gas by a Pt electrode versus an Ag/AgCl electrode and corrected to reflect the standard hydrogen electrode (SHE). The E_h was poised by small additions of sodium ferricyanide or sodium dithionite from anaerobic stocks of 1 mM. At least 2 min was allowed for equilibration between each addition of oxidant or reductant.

RESULTS

Production of GST/Rieske Fusion Proteins and Specific Antibodies. Induction of GST gene expression from plasmids pGR650-1 or pGR650-32 in *E. coli* resulted in overproduction of 43 kDa GST/Rieske fusion proteins (Figure 2, lanes 5 and 6) in the form of insoluble inclusion bodies (Figure 2, lane 8). Induction of plasmids pGEX-3X or pGR650-2 (an "out-of-frame" fusion) produced 27 kDa proteins of the size expected for GST alone (Figure 2, lanes 3 and 4). The 43 kDa GST/Rieske bands from partially purified pellet fractions were excised from gels, extracted, and injected into chickens for antibody production. A simple procedure (Gassmann et al., 1990) permitted large-scale isolation of egg-yolk IgY-containing anti-GST/Rieske antibodies which reacted specif-

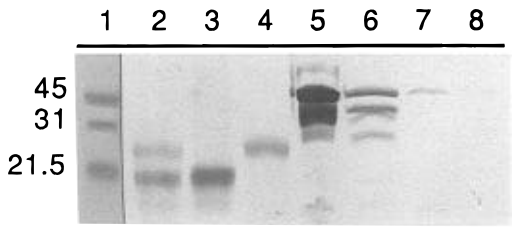

FIGURE 3: Antibody specificity for the Rieske protein. Proteins were fractionated on a 10% polyacrylamide, 8 M urea, SDS gel, blotted onto PVDF membrane (lanes 2−8), and reacted with GST/Rieske antibody as described in Materials and Methods. Lane 1 contains amido black stained molecular weight standards (kDa). Lane 2 contains combined membranes from *Nostoc* sp. PCC 7906 and *Synechococcus* sp. PCC 7002. Lanes 3 and 4 contain membranes from *Nostoc* and *Synechococcus*, respectively. Lanes 5 and 6 contain pellet and supernatant fractions, respectively, from IPTG-induced *E. coli* (pGR650-1). Lanes 7 and 8 contain pellet and supernatant fractions, respectively, from uninduced *E. coli* (pGR650-1).

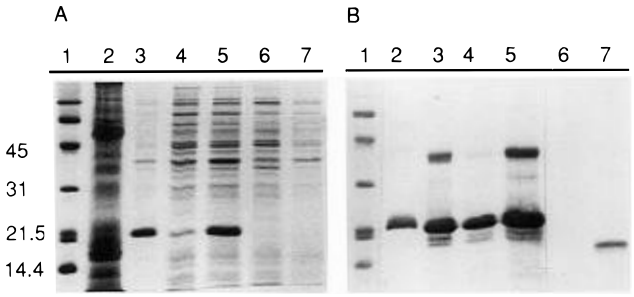

FIGURE 4: Production of full-length and truncated Rieske proteins in *E. coli*. Panel A shows a stained SDS polyacrylamide gel. Lane 1 contains molecular weight standards (kDa). Lane 2 contains an octyl glucoside extract of *Nostoc* sp. PCC 7906 membranes. Lanes 3, 4, and 5 contain the pellet, supernatant, and whole, unseparated lysate, respectively, from an induced *E. coli* BL21(DE3) culture bearing plasmid p3aR. Lanes 6 and 7 contain pellet fractions from uninduced and induced cultures, respectively, of *E. coli* BL21(DE3) bearing plasmid p3a600. Panel B shows an immunoblot of lanes 2−7 in panel A. Lane 1 shows amido black stained standards. Lanes 2−7 were reacted with the GST/Rieske antibody.

ically against Rieske proteins from *Nostoc* sp. PCC 7906 and *Synechococcus* sp. PCC 7002 membranes (lanes 2−4, Figure 3). The M_r of the cyanobacterial Rieske proteins in this gel were distinguishable and both somewhat higher than the 19.2 kDa predicted from the *Nostoc petC* sequence (Kallas et al., 1988). The GST/Rieske antibody cross reacted as expected with the GST/Rieske protein from *E. coli* (Figure 3, lanes 5 and 6) but showed little or no cross reactivity to fractions from uninduced *E. coli* (pGR650-1) cultures (Figure 3, lanes 7 and 8).

Production of Nonfusion, Full-Length, and Truncated Rieske Proteins. To permit the solubilization and refolding of a functional Rieske protein from inclusion bodies, we assembled plasmid p3aR for production of a nonfusion, full-length, Rieske protein as illustrated in Figure 1. Induction of plasmid p3aR in *E. coli* BL21(DE3) led to overproduction of a protein that comigrated with authentic *Nostoc* sp. Rieske protein (Figure 4, panel B, lane 2) and cross reacted with the GST/Rieske antibody (Figure 4, panels A and B, lanes 3−5). Most of the Rieske protein was produced as inclusion bodies which pelleted at 12000*g*. Washes of these pellets in buffer yielded highly enriched Rieske protein preparations (Figure 4, lanes 3). We deleted the 5′ end of the *petC* gene to create plasmid p3a600 (Figure 1, panel D) for production of a truncated, water-soluble, Rieske polypeptide. Expression of plasmid p3a600 in *E. coli* BL21(DE3) produced the expected, soluble Rieske fragment but only at a low level requiring immunological detection (Figure 4, panel B, lane 7).

Reconstitution of the 2Fe-2S Center, Purification, and Characterization of the Rieske Protein. We were unable to detect the characteristic $g = 1.89$ EPR signal directly from Rieske samples overproduced in *E. coli*. Copropagation and induction in *E. coli* BL21(DE3) of plasmids p3aR and pGroESL encoding the GroEL and GroES molecular chaperones (Goloubinoff et al., 1989) did result in incorporation of iron−sulfur but not a Rieske cluster into Rieske protein inclusion bodies (data not shown). We therefore pursued solubilization of inclusion bodies, refolding, and *in vitro* reconstitution with reduced iron and sulfide as described in Materials and Methods. This approach allowed assembly of the characteristic Rieske 2Fe-2S cluster into the overproduced *Nostoc* Rieske protein as demonstrated by intense $g_x = 1.74$, $g_y = 1.89$, and $g_z = 2.03$ Rieske EPR signals observed at 15 K (data not shown but similar to those in Figure 5C, below). One liter of *E. coli* BL21 (DE3, p3aR) typically yielded several milligrams of reconstituted protein.

Further purification of the reconstituted Rieske protein was accomplished by anion exchange chromatography on Whatman DE52 cellulose. Rieske protein loaded directly onto DE52 resin in a column bound irreversibly to the matrix, possibly the result of aggregation caused by high local protein concentration. When mixed with DE52 resin in a more dilute bulk solution prior to loading into the column as described in Materials and Methods, the Rieske protein eluted in buffer containing 0.2 M NaCl, 10 mM Tricine−NaOH pH 8.0, 1 mM DTT, and 1 mM octyl glucoside. SDS−PAGE analysis of overloaded samples from such fractions showed a protein band with M_r ca. 20 000 (Figure 5A,B, lanes 1 and 2). The Rieske protein in the eluate was predominantly monomeric as judged by passage through a 30 000 mol wt cutoff ultrafilter (Figure 5A,B, lanes 1). Higher and lower molecular weight bands that sometimes appeared in purified preparations represented aggregation or degradation products of the Rieske protein as demonstrated by immunoblotting against the GST/Rieske antibody (Figure 5B). Panel C in Figure 5 shows an EPR spectrum of the overproduced, reconstituted, and purified *Nostoc* sp. PCC 7906 Rieske protein displayed in Figure 5A,B, lanes 1. The $g_x = 1.74$, $g_y = 1.89$, and $g_z = 2.03$ resonances are characteristic of the Rieske 2Fe-2S cluster. The concentration of 2Fe-2S centers estimated from this spectrum was 50 μM. Based on a protein concentration of 150 μM, approximately one-third of the reconstituted Rieske protein in Figure 5 contained iron−sulfur centers. Atomic absorption determination showed a total Fe content of 258 μM in this sample, suggesting either the presence of nonspecifically bound "free" iron or underestimation of Fe-S clusters by EPR spin quantification. Other samples showed 20−66% reconstitution with Rieske 2Fe-2S clusters based on EPR. Figure 6 displays a reduced-minus-oxidized optical CD spectrum and redox titration of reconstituted *Nostoc* sp. Rieske protein. These data indicate a midpoint potential of +321 mV at pH 7.0 and 25 °C.

DISCUSSION

We have reported here the overproduction of a full-length Rieske, PetC protein from the cyanobacterium *Nostoc* sp.

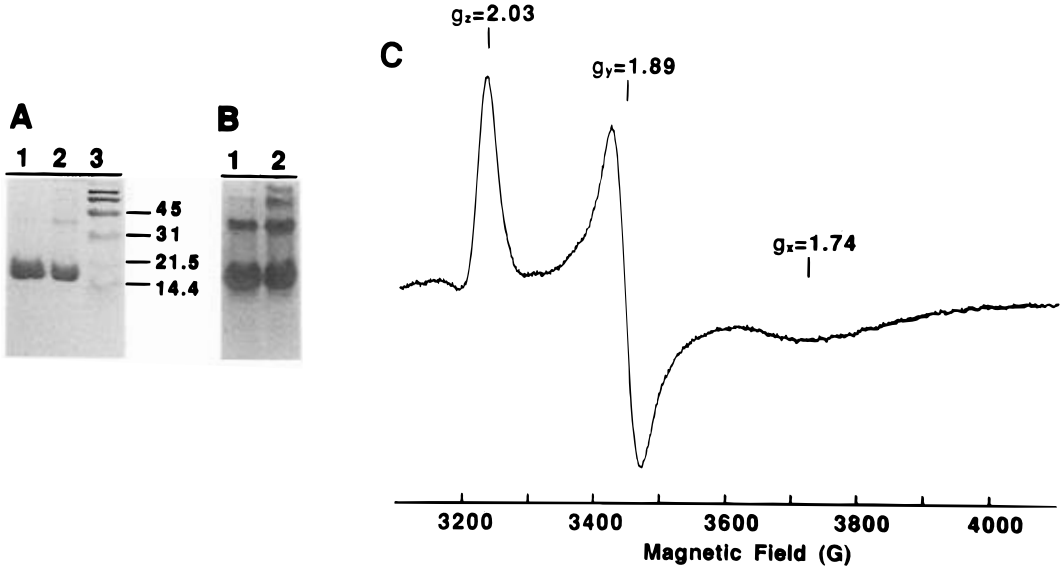

FIGURE 5: Reconstituted and purified, full-length, nonfusion *Nostoc* sp. PCC 7906 Rieske protein and EPR spectrum. Panels A and B show a stained SDS polyacrylamide gel and an immunoblot of the gel reacted against the GST/Rieske antibody, respectively. Lanes 1 show 10 μL from a Rieske protein preparation that had been passed through a 30 000 mol wt cutoff ultrafilter after elution in 0.2 M NaCl, 10 mM Tricine−NaOH pH 8.0, 1 mM DTT, 1 mM octyl glucoside buffer from a Whatman DE52 column. Lanes 2 show the same sample before the filtration step. Lane 3 shows molecular weight standards (kDa). Panel C shows an EPR spectrum of the Rieske protein shown in panels A and B, lanes 1. Data were collected at 15 K from a 0.3 mL (ca. 150 μM) sample in 0.2 M NaCl, 10 mM Tricine−NaOH pH 8.0, 1 mM DTT, 1 mM octyl glucoside buffer. The characteristic $g_x = 1.74$, $g_y = 1.89$, and $g_z = 2.03$ Rieske EPR features are indicated. EPR parameters were as described in Materials and Methods.

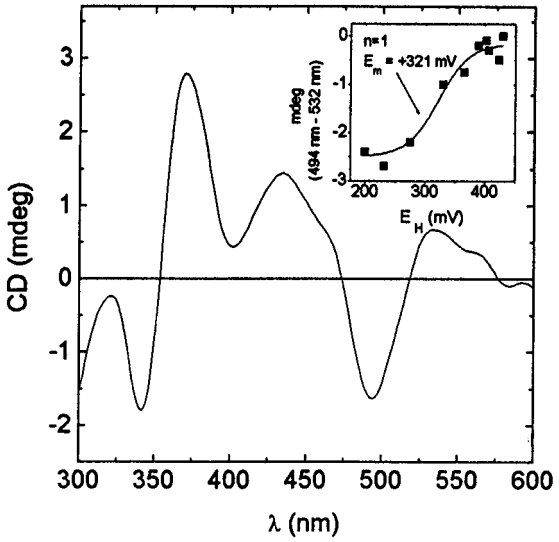

FIGURE 6: Dithionite-reduced minus ferricyanide-oxidized optical circular dichroism (CD) difference spectrum of reconstituted *Nostoc* sp. PCC 7906 Rieske protein and redox titration. Data were recorded from 5 μM samples of reconstituted Rieske protein in 50 mM phosphate, 1 mM octyl glucoside buffer pH 7.0 with 10 μM each of DAD and TMPD at 25 °C as described in Materials and Methods. The oxidant was sodium ferricyanide, and the reductant was sodium dithionite. Spectra were the average of 10 traces with spectral resolution of 3 nm. The inset shows titration of the signal at 494 nm (minus 532 nm) as a function of redox potential.

PCC 7906 as well as fusion and deletion forms of this protein. Denaturation, refolding, and incubation of the full-length PetC protein with reduced iron and sulfide allowed reconstitution of the "Rieske-type" 2Fe-2S center *in vitro* as demonstrated by the $g_x = 1.74$, $g_y = 1.89$, and $g_z = 2.03$ EPR spectrum detected at 15 K (Figure 5C). This spectrum is typical of the highly characteristic, rhombic EPR spectra displayed by Rieske iron−sulfur clusters (Rieske et al., 1964; Malkin & Aparicio, 1975; Riedel et al., 1995). "Plant-type" 2Fe-2S ferredoxins such as those found in cyanobacteria (Cheng et al., 1994) and the ferredoxin centers of some bacterial dioxygenases (Riedel et al., 1995) also display rhombic EPR spectra, but with g values at about $g_x = 1.89$, $g_y = 1.96$, and $g_z = 2.05$. The g_x feature is noteworthy because it has seldom been detected from cyt b_6f Rieske centers and then only in the isolated cyt b_6f complex at high protein concentration (Riedel et al., 1991). Our results confirm that the g_x resonance is an intrinsic feature of the *Nostoc* sp. Rieske protein not necessarily dependent on its association with the cyt b_6f complex. The intensity and position of the g_x resonance in cyt bc_1 Rieske proteins are sensitive to occupancy of the quinol-oxidation site by quinones and inhibitors (Siedow et al., 1978; De Vries et al., 1979; Meinhardt et al., 1987) and have been used in elegant characterizations of mutations at this site (Robertson et al., 1990; Ding et al., 1995).

The full-length *Nostoc* sp. Rieske protein appeared predominantly in the 12000*g* pellet fraction of *E. coli* lysates as insoluble inclusion bodies (Mitrake & King, 1989). Inclusion body formation in *E. coli* as well as aggregation of the purified protein *in vitro* appeared to result primarily from hydrophobic interactions involving the N-terminus. Expression of the p3a600 construct, from which the N-terminal hydrophobic domain was deleted, allowed the production of a soluble, truncated Rieske protein, although only at low levels. The transcription/translation sequences upstream of the *petC* genes in plasmids p3aR and p3a600 are identical, suggesting that low expression of the P3a600 Rieske protein may have resulted from proteolysis to which the full-length Rieske aggregates were resistant. Alternatively, hydrophobic or suboptimal amino acids near the N-terminus of the truncated Rieske protein may have resulted in low translation, as shown for bacteriorhodopsin (Karnik et al., 1987).

One to five percent of the full-length Rieske protein (based on scanning densitometry, not shown) in *E. coli* lysates

appeared in 25000*g* and 150000*g* pellet fractions which represent membrane fragments and vesicles (e.g., Van Doren et al., 1993b). The N-terminal, hydrophobic domain of the mature pea chloroplast Rieske protein contains a thylakoid-membrane targeting sequence for translocation into the chloroplast lumen (Madueño et al., 1994). It is not known whether the Rieske protein targeting to membranes requires specific receptors. Expression of the full-length *R. sphaeroides* Rieske protein both in *R. sphaeroides* and in *E. coli* resulted predominantly in protein localization within membrane fractions (Van Doren et al., 1993b), but protein production was very low. In contrast, our full-length *Nostoc* sp. Rieske protein was produced in quantities as high as 50% of the total *E. coli* protein. The *Nostoc* protein too may have initially integrated into or associated with membranes until available "sites" or assembly chaperones became saturated. The subsequently produced Rieske protein may then have aggregated to form the inclusion bodies that we predominantly observed. The pea chloroplast Rieske protein associates with chaperones Cpn60 and Hsp70 in the chloroplast stroma (Madueño et al., 1993) and mitochondrial Rieske protein with Hsp10 and Hsp60 en route to the intermembrane space (Höhfeld & Hartl, 1994). The actual topology of Rieske proteins in membranes remains controversial. We believe that our results, and those of Van Doren et al. (1993b), showing membrane association of a fraction of the Rieske proteins produced in *E. coli*, are compatible with either a single membrane-spanning helix as suggested by Van Doren et al. (1993b) and others (Harnisch et al., 1985; Steppuhn et al., 1987), integration into one leaflet of the bilayer as in prostaglandin H2 synthase-1 (Picot et al., 1994) as suggested by Cramer et al. (1996), or tight peripheral association with the membrane. Several lines of evidence now support extrinsic association of the Rieske protein with cyt complexes (Breyton et al., 1994; Szczepaniak et al., 1995). Preliminary results from the crystal structure of the intact beef heart mitochondrial cyt bc_1 complex are compatible with this interpretation (Chang-An Yu, Oklahoma State University, personal communication).

We were unable to detect a Rieske 2Fe-2S cluster in the full-length *Nostoc* sp. Rieske protein produced in *E. coli*. These results and those of Van Doren et al. (1993b) with the *R. sphaeroides* Rieske protein suggested that *E. coli* lacks the molecular machinery to assemble Cys/His liganded "Rieske-type" 2Fe-2S clusters. Indeed, proteins with such clusters have not been identified in *E. coli*. However, we have recently overproduced a Rieske fragment as a fusion to thioredoxin and found that *E. coli* inserts the characteristic Rieske 2Fe-2S cluster into this soluble fusion protein *in vivo* (Cho and Kallas, unpublished material). The inability of *E. coli* to assemble the Rieske center into the full *Nostoc* protein thus appears to be a function of protein insolubility and misfolding rather than a lack of specific cofactors. Consistent with this is the production in *E. coli* of active toluene dioxygenase subunits which require Rieske Fe-S centers (Zylstra & Gibson, 1989), monooxygenase *tmoC* Rieske protein from *Pseudomonas mendocina* (Markley et al., 1996), and the ISP_{BPH} and FER_{BPH} Rieske-containing subunits of *Comamonas testosteroni* biphenyl dioxygenase (Hurtubise et al., 1995). These findings suggest that the pathway for Rieske cluster assembly may be rather nonspecific or that alternative mechanisms can substitute. For Fe-S clusters in nitrogenase, the *nifS* and *nifU* gene products have roles in supplying inorganic sulfide and iron, respectively, for cluster formation (reviewed by Dean et al., 1993). NifS catalyzes the desulfurization of L-cysteine, and *nifS* gene homologs have been found in non-nitrogen-fixing organisms, suggesting a broad role for NifS-like proteins as sulfur donors in Fe-S cluster assembly (Dean et al., 1993; Zheng & Dean, 1994).

Malkin and Rabinowitz (1966) first demonstrated reconstitution of iron−sulfur centers into soluble ferredoxins by *in vitro* incubations with reduced iron and sulfide. Variations of this procedure have been used to reconstitute Fe-S clusters into overproduced 4Fe−4S PsaC protein (Zhao et al., 1990; Li et al., 1991) and 2Fe-2S *Anabaena* sp. PCC 7120 vegetative (Cheng et al., 1994, 1995) and heterocyst (Chae et al., 1994) ferredoxins. Data presented here extend iron−sulfur cluster reconstitution to proteins with "Rieske-type" Cys and His cluster ligands. Reconstituted *Nostoc* sp. Rieske proteins appeared to be in a native conformation as judged by EPR and optical CD spectra and by CD spectroscopic determination of a redox midpoint potential of +321 mV at pH 7.0. This value is comparable to the +320 mV potential determined by EPR titration at 15 K for the Rieske cluster in isolated spinach cyt b_6f complex (Nitschke et al. 1992). The reduced-minus-oxidized CD spectrum of the *Nostoc* sp. PetC Rieske protein (Figure 6) is similar to those reported for the soluble fragment of the bovine mitochondrial and the *Pseudomonas putida* FD_{BED} Rieske proteins (Link et al., 1996). A negative CD feature near 500 nm appears to be highly characteristic of reduced Rieske clusters.

Reconstituted *Nostoc* sp. Rieske proteins contained approximately 20−60% Fe-S clusters as determined by EPR spin quantitation. A sample reconstituted to 66% was obtained by the Cheng et al. (1995) and Markley method described in Materials and Methods and showed 80 μM Fe by atomic absorption relative to 30 μM protein. An excess of Fe in this and other samples relative to Fe detected in Rieske clusters may reflect the recognized imprecision of EPR spin quantitation, nonspecifically bound "free" iron, or the presence of some aberrant "EPR silent" clusters. The latter are unlikely in 2Fe-2S proteins unless the cluster environment and geometry have become altered. We also detected a $g = 4.3$ EPR signal in reconstituted samples (not shown). This signal is attributed to free Fe(III) (Wertz & Bolton, 1972) and could account for the extra Fe in our preparations. Such $g = 4.3$ EPR signals are commonly observed in reconstituted iron−sulfur proteins as well as in purified cyt complexes (McCurley et al., 1990). Step gradient elution from Whatman DE52 resin did not resolve fully reconstituted *Nostoc* sp. Rieske 2Fe-2S holoprotein from apoproteins lacking clusters, but altered chromatographic conditions should allow such separation because of expected differences in folding and surface charge between these forms. Reconstitution by the method of Cheng et al. (1995) and Markley yielded the highest percentage of proteins with Rieske 2Fe-2S clusters. In this approach, proteins were allowed to refold in the presence of reduced iron and sulfide and finally exposed to aerobic buffer in the absence of reducing agents. Exposure to oxidizing conditions may stabilize the Rieske Fe-S cluster by allowing formation of the disulfide predicted by Graham and Trumpower (1991) and Davidson et al. (1992b) and shown in the Iwata et al. (1996) structure.

Overproduction and reconstitution of the full-length Rieske iron−sulfur protein from the cyanobacterium *Nostoc* sp. PCC 7906 opens the way to further characterization of this key protein involved in the quinol-oxidation step of oxygenic photosynthesis. Because the *Nostoc* sp. Rieske protein is expressed in *E. coli*, it is now readily amenable to site-directed mutagenesis and selective isotopic labeling approaches. The emerging structure of the soluble bovine heart mitochondrial Rieske fragment (Iwata et al. 1996) provides a firm basis for such studies.

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REFERENCES

Aasa, R., Albracht, S. P. J., Falk, E.-E., Laane, B., & Vänngård, T. (1976) *Biochim. Biophys. Acta 422*, 260−272.

Adam, Z., & Malkin, R. (1987) *FEBS Lett. 225*, 67−71.

Berthold, D. A., Schmidt, C. L., & Malkin, R. (1995) *J. Biol. Chem. 270*, 29293−29298.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.

Breyton, C., de Vitry, C., & Popot, J.-L. (1994) *J. Biol. Chem. 269*, 7597−7602.

Britt, R. D., Sauer, K., Klein, M. P., Knaff, D. B., Kriauciunas, A., Yu, C.-A., Yu, L., & Malkin, R. (1991) *Biochemistry 30*, 1892−1901.

Chae, Y. K., Abildgaard, F., Mooberry, E. S., & Markley, J. L. (1994) *Biochemistry 33*, 3287−3295.

Cheng, H., Xia, B., Reed, G. H., & Markley, J. L. (1994) *Biochemistry 33*, 3155−3164.

Cheng, H., Westler, W. M., Xia, B., Oh, B.-H., & Markley, J. L. (1995) *Arch. Biochem. Biophys. 316*, 619−634.

Coghlan, V. M., & Vickery, L. E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 835−839.

Cramer, W. A., Soriano, G. M., Ponomarev, M., Huang, D., Zhang, H., Martinez, S. E., & Smith, J. L. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 47*, 477−508.

Davidson, E., Ohnishi, T., Tokito, M., & Daldal, F. (1992a) *Biochemistry 31*, 3351−3358.

Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E., & Daldal, F. (1992b) *Biochemistry 31*, 3342−3351.

Dean, D. R., Bolin, J. T., & Zheng, L. (1993) *J. Bacteriol. 175*, 6737−6744.

De Vries, S., Albracht, S. P. J., & Leeuwerik, P. (1979) *Biochim. Biophys. Acta 546*, 316−333.

Ding, H., Moser, C. C., Robertson, D. E., Tokito, M. K., Daldal, F., & Dutton, P. L. (1995) *Biochemistry 34*, 15979−15996.

Fee, J. A., Kuila, D., Mather, M. W., & Yoshida, T. (1986) *Biochim. Biophys. Acta 853*, 153−185.

Gassmann, M., Thommes, P., Weiser, T., & Hubscher, U. (1990) *FASEB J. 4*, 2528−2532.

Goloubinoff, P., Gatenby, A. A., & Lorimer, G. H. (1989) *Nature 337*, 44−47.

González-Halphen, D., Vázquez-Acevedo, M., & García-Ponce, B. (1991) *J. Biol. Chem. 266*, 3870−3876.

Graham, L. A., & Trumpower, B. L. (1991) *J. Biol. Chem. 266*, 22485−22492.

Gray, K. A., & Daldal, F. (1995) in *Anoxygenic Photosynthetic Bacteria* (Blankenship, R. E., Madigan, M. T., & Bauer, C. E., Eds.) pp 746−774, Kluwer Academic Publishers, The Netherlands, Dordrecht.

Gurbiel, R. J., Batie, C. J., Sivaraja, M., True, A. E., Fee, J. A., Hoffman, B. M., & Ballou, D. P. (1989) *Biochemistry 28*, 4861−4871.

Gurbiel, R., Ohnishi, T., Robertson, D. E., Daldal, F., & Hoffman, B. M. (1991) *Biochemistry 30*, 11579−11584.

Haley, J., & Bogorad, L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1534−1538.

Harnisch, U., Weiss, H., & Sebald, W. (1985) *Eur. J. Biochem. 149*, 95−99.

Higuchi, R. (1990) in *PCR Protocols* (Innis, M. A., Gelfand, D. H., Sninsky, J. L., & White, T. J., Eds.) pp 177−183, Academic Press, San Diego.

Höhfeld, J., & Hartl, F. U. (1994) *J. Cell Biol. 126*, 305−315.

Holden, H. M., Jacobson, B. L., Hurley, J. K., Tollin, G., Oh, B.-H., Skjeldal, L., Chae, Y. K., Cheng, H., Xia, B., & Markley, J. L. (1994) *J. Bioenerg. Biomembr. 26*, 67−88.

Holton, B., Middleton, K., Zarka, D., Alsaadi, R., & Kallas, T. (1992) in *Research in Photosynthesis* (Murata, N., Ed.) pp 567−570, Kluwer Academic Publishers, Dordrecht.

Hurtubise, Y., Barriault, D., Powlowski, J., & Sylvestre, M. (1995) *J. Bacteriol. 177*, 6610−6618.

Iwata, S., Saynovits, M., Link, T. A., & Michel, H. (1996) *Structure 4*, 567−579.

Joliot, P., & Joliot, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1034−1038.

Kallas, T. (1994) in *The Molecular Biology of the Cyanobacteria* (Bryant, D. A., Ed.) pp 259−317, Kluwer Academic Publishers, The Netherlands, Dordrecht.

Kallas, T., Spiller, S., & Malkin, R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5794−5798.

Karnik, S. S., Nassal, M., Doi, T., Jay, E., Sgaramella, V., & Khorana, H. G. (1987) *J. Biol. Chem. 262*, 9255−9263.

Kramer, D. M., & Crofts, A. R. (1994) *Biochim. Biophys. Acta 1184*, 193−201.

Li, N., Zhao, J., Warren, P. V., Warden, J., Bryant, D. A., & Golbeck, J. H. (1991) *Biochemistry 30*, 7863−7872.

Link, T. A., Hatzfield, O. M., Unalkat, P., Shergill, J. K., Cammack, R., & Mason, J. R. (1996) *Biochemistry 35*, 7456−7552.

Madueño, F., Napier, J. A., & Gray, J. C. (1993) *Plant Cell 5*, 1865−1876.

Madueño, F., Bradshaw, S. A., & Gray, J. C. (1994) *J. Biol. Chem. 269*, 17458−17463.

Malkin, R. (1982) *Biochemistry 21*, 2945−2950.

Malkin, R., & Rabinowitz, J. C. (1966) *Biochem. Biophys. Res. Commun. 23*, 822.

Malkin, R., & Aparicio, P. J. (1975) *Biochem. Biophys. Res. Commun. 63*, 1157−1160.

Manodori, A., Cecchini, G., Schröder, I., Gunsalus, R. P., Werth, M. T., & Johnson, M. K. (1992) *Biochemistry 31*, 2703−2712.

Markley, J. L., Xia, B., Chae, Y. K., Cheng, H., Westler, W. M., Pikus, J. D., & Fox, B. G. (1996) in *Protein Structure Function Relationship* (Zaidi, Z., & Smith, D., Eds.), Plenum Press, London (in press).

Martinez, S. E., Huang, D., Szczepaniak, A., Cramer, W. A., & Smith, J. L. (1994) *Structure 2*, 95−105.

McCurley, J. P., Miki, T., Yu, L., & C.-A., Y. (1990) *Biochim. Biophys. Acta 1020*, 176−186.

Mehari, T., Parrett, K. G., Warren, P. V., & Golbeck, J. H. (1991) *Biochim. Biophys. Acta 1056*, 139−148.

Meinhardt, S. W., Yang, X., Trumpower, B. L., & Ohnishi, T. (1987) *J. Biol. Chem. 262*, 8702−8706.

Mitrake, A., & King, J. (1989) *Bio/Technology 7*, 690−697.

Nitschke, W., Joliot, P., Liebl, U., Rutherford, A. W., Hauska, G., Muller, A., & Riedel, A. (1992) *Biochim. Biophys. Acta 1102*, 266−268.

Parrett, K. G., Mehari, T., & Golbeck, J. H. (1990) *Biochim. Biophys. Acta 1015*, 341−352.

Phillips, J. D., Graham, L. A., & Trumpower, B. L. (1993) *J. Biol. Chem. 268*, 11727−11736.

Picot, D., Loll, P. J., & Garavito, R. M. (1994) *Nature 367*, 243−249.

Pierre, Y., Breyton, C., Kramer, D., & Popot, J.-L. (1995) *J. Biol. Chem. 270*, 29342−29349.

Riedel, A., Rutherford, A. W., Hauska, G., Muller, A., & Nitschke, W. (1991) *J. Biol. Chem. 266*, 17838−17844.

Riedel, A., Fetzner, S., Rampp, M., Lingens, F., Liebl, U., Zimmermann, J.-L., & Nitschke, W. (1995) *J. Biol. Chem. 270*, 30869−30873.

Rieske, J. S., Zaugg, W. S., & Hansen, R. E. (1964) *J. Biol. Chem. 239*, 3017−3022.

Robertson, D. E., Daldal, F., & Dutton, P. L. (1990) *Biochemistry 29*, 11249−11260.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.

Shergill, J. K., Joannou, C. L., Mason, J. R., & Cammack, R. (1995) *Biochemistry 34*, 16533−16542.

Siedow, J. N., Power, S., de la Rosa, F. F., & Palmer, G. (1978) *J. Biol. Chem. 253*, 2392−2399.

Smith, J. S., & Johnson, K. S. (1988) *Gene 67*, 31−40.

Steppuhn, J., Rother, C., Hermans, J., Hanson, T., Salnikow, J., Hauska, G., & Herrmann, R. G. (1987) *Mol. Gen. Genet. 210*, 171−177.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−88.

Szczepaniak, A., Frank, K., & Rybka, J. (1995) *Z. Naturforsch. 50C*, 535−542.

Ta, D. T., & Vickery, L. E. (1992) *J. Biol. Chem. 267*, 11120−11125.

Trumpower, B. L., & Gennis, R. B. (1994) *Annu. Rev. Biochem. 63*, 675−716.

Trumpower, B. L., Edwards, C. A., & Ohnishi, T. (1980) *J. Biol. Chem. 255*, 7487−7493.

Tsukihara, T., Fukuyama, K., & Katsube, Y. (1986) in *Iron−Sulfur Protein Research* (Matsubara, H., Ed.) pp 59−68, Japan Sci. Soc. Press: Tokyo and Springer Verlag: Berlin.

Van Doren, S. R., Gennis, R. B., Barquera, B., & Crofts, A. R. (1993a) *Biochemistry 32*, 8083−8091.

Van Doren, S. R., Yun, C.-H., Crofts, A. R., & Gennis, R. B. (1993b) *Biochemistry 32*, 628−636.

Wertz, J. E., & Bolton, J. R. (1972) in *Electron Spin Resonance*, McGraw Hill, New York.

Wu, X., Tsapin, A., Cho, Y. S., Lee, T.-X., Metzger, S., Genzlinger, L., Holton, B., Whitmarsh, J., & Kallas, T. (1995) in *Photosynthesis: from Light to Biosphere* (Mathis, P., Ed.) pp 769−772, Kluwer Academic Publishers, Dordrecht.

Zhao, J., Warren, P., Li, N., Bryant, D. A., & Golbeck, J. R. (1990) *FEBS Lett. 276*, 175−180.

Zheng, L., & Dean, D. R. (1994) *J. Biol. Chem. 269*, 18723−18726.

Zheng, L., Kennedy, M. C., Beinert, H., & Zalkin, H. (1992) *J. Biol. Chem. 267*, 7895−7903.

Zylstra, G. J., & Gibson, D. T. (1989) *J. Biol. Chem. 264*, 14940−14946.

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