Assembly of the Rieske Iron–Sulfur Subunit of the Cytochrome bc_1 Complex in the *Escherichia coli* and *Rhodobacter sphaeroides* Membranes Independent of the Cytochrome *b* and c_1 Subunits†

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ABSTRACT: The Rieske iron–sulfur subunit of the cytochrome bc_1 complex from *Rhodobacter sphaeroides* has been expressed in *Escherichia coli* and also in a strain of *Rb. sphaeroides* lacking the other subunits of the bc_1 complex. PCR products encoding the full-length subunit were introduced into expression vectors to produce the subunit alone or the subunit fused behind the mature portion of the *E. coli* maltose binding protein (MBP), but lacking the MBP signal sequence. These proteins are both located in the cytoplasmic membrane. The unfused Rieske subunit assembles a Rieske-like iron–sulfur cluster, but with EPR characteristics which differ from the normal rhombic signal observed in the cytochrome bc_1 complex. The overproduced MBP fusion protein, on the other hand, does not contain an EPR-detectable iron–sulfur cluster. Subfragments of the Rieske subunit lacking the amino-terminal hydrophobic anchor also lack the iron–sulfur cluster were expressed in *E. coli*. When expressed in *Rb. sphaeroides* in the absence of the cytochrome *b* and c_1 subunits, the fully metalated Rieske subunit with the diagnostic $g_y = 1.90$ EPR signal is observed in the cytoplasmic membrane. The fact that the Rieske subunit has an assembled iron–sulfur cluster and is bound to either the *E. coli* or the *Rb. sphaeroides* membrane in the absence of the other subunits of the bc_1 complex demonstrates a mode of membrane attachment independent of the other components of the complex. These data are consistent with models in which the Rieske subunit is bound to the membrane via a single membrane-spanning helix located near the amino terminus. Proteolytic cleavage at a site just downstream of the putative membrane anchor results in a water-soluble form of the Rieske subunit from *Rb. sphaeroides*, providing further support for this model.

The ubiquinol:cytochrome *c* oxidoreductases, more commonly known as cytochrome bc_1 (or b_6f in chloroplasts) complexes, are protonmotive pumps central to the respiratory electron-transport chains of mitochondria and many bacteria, and to the photosynthetic electron-transport chains of chloroplasts and bacteria (Crofts, 1985; Gabellini, 1988; Hauska et al., 1988; Trumpower, 1990). The cytochrome bc_1 complexes each comprise three catalytic subunits: a two-heme cytochrome *b*, a cytochrome c_1, and a Rieske 2Fe–2S protein. The Rieske iron–sulfur subunit is unique among 2Fe–2S proteins because of its high midpoint potential [about +285 mV in *Rhodobacter sphaeroides* (Prince et al., 1975)], its low g_y value of 1.90, and its nitrogen ligation. Aromatic dioxygenases of *Pseudomonads* have 2Fe–2S clusters with electron paramagnetic resonance (EPR)[1] spectra quite similar to those of the cytochrome bc_1 complexes, but with much lower midpoint potentials of about −110 mV (Geary et al., 1984). In one such dioxygenase, ENDOR studies have indicated two histidyl nitrogen ligands to the cluster (Gurbiel et al., 1989), suggesting the bc_1 complexes may also have two nitrogen ligands to the iron–sulfur center. ENDOR studies on a bc_1 complex (Gurbiel et al., 1991) and electron spin-echo envelope modulation studies on bc_1 and b_6f complexes (DeRose et al., 1991) also implicate two distinct nitrogens in ligating the cluster. The cluster probably lies near the level of the phospholipid headgroups of the membrane bilayer (Ohnishi et al., 1989). A consensus places the bulk of the subunit on the same face of the membrane as cytochrome c_1 (Gonzalez-Halphen et al., 1988; Karlsson et al., 1983; Ohnishi et al., 1989). A recent topological model has the subunit in the mitochondrial complex peripherally associated with the mitochondrial inner membrane facing the intermembrane space, without spanning the membrane (Gonzalez-Halphen et al., 1991). Proteolysis studies have indicated, however, that a portion of the subunit is exposed to the mitochondrial matrix (Cocco et al., 1991) or, equivalently, the cytoplasmic side of the bacterial cytoplasmic membrane (Theiler & Niederman, 1991). A model with two transmembrane helices, with both amino and carboxyl termini in the intermembrane space, has been proposed (Schägger et al., 1987). An alternative model has one transmembrane helix with the amino terminus in the mitochondrial matrix (Harnisch et al., 1985). Obviously, the topology of the Rieske iron–sulfur subunit remains somewhat unclear and controversial.

Further understanding of the role of the Rieske subunit in the mechanism of the bc_1 complex awaits high-resolution structural information from X-ray crystallography, which may

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[1] Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton(s); MBP, maltose binding protein; PCR, polymerase chain reaction; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ENDOR, electron nuclear double resonance; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-benzothiazoledione; CAPS, 3-[cyclohexylamino]-1-propanesulfonic acid; MBP, maltose binding protein.

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Table I: Bacterial Strains, Genotype, and Origin

strain or plasmid	relevant characteristics	reference
Rb. sphaeroides		
Ga	carotenoid biosynthesis mutant of 2.4.1	Cohen-Bazire et al. (1957)
BC17	Δ*fbc*::Kan derivative of Ga[a]	Yun et al. (1990)
BC17C	BC17 harboring pRK plasmid with *fbc* allele	Yun et al. (1990)
CQ228stop	*fbc*Δ*C*228-263 derivative of BC17C	Konishi et al. (1991)
E. coli		
JM83	Δ(*lac-proAB*)*lacZ*	Vieira & Messing (1982)
NM522	Δ(*lac-pro*) F′ *lacI*^{q} *lacZ*ΔM15	Gough & Murray (1983)
S17-1	*pro res mod*$^+$ *recA* Tp^r Sm^r integrated plasmid RP4-Tc::Mu-Kn::Tn7	Simon et al. (1983)
SF110	Δ*degP41*::Kan *ompT*[b]	Baneyx & Georgiou (1990)
plasmids		
pRK415	*oriT lacZ* Tc^r	Keen et al. (1988)
pUC8	P_{lac} *lacZ* Amp^r	Vieira & Messing (1982)
pIN-III-ompA	$P_{lpp\text{-}lac}$ *lacI ompA* secretion signal Amp^r	Ghrayeb et al. (1984)
pKK233-2	$P_{trp\text{-}lac}$ Amp^r	Amman & Brosuis (1985)
pMON5743	P_{recA} phage T7 *g10*-L RBS Amp^r	Olins & Rangwala (1990)
pMAL-c	$P_{trp\text{-}lac}$ *lacI malE*δ2-26-fx-*lacZ* Amp^r	Maina (1988)
pRKFeS	*fbcF* Δ*fbcBC* derivative of pRK415	this study
pUC8FeS	*fbcF*Δ1-93 derivative of pUC8	this study
pOMPAFeS	*ompA-fbcF*Δ1-34 derivative of pIN-III-ompA2	this study
pKKFeS	*fbcF* derivative of pKK233-2	this study
pMONFeS	*fbcF* derivative of pMON5743	this study
pMALCFeS	*malE*Δ2-26-fx-*fbcF* pMAL-c derivative	this study

[a] The *fbc* operon encodes the Rieske iron–sulfur, cytochrome *b*, and cytochrome c_1 subunits of the *Rb. sphaeroides* cytochrome bc_1 complex. [b] *degP41* and *ompT* encode periplasmic proteases.

come from studies of crystals recently reported (Yue et al., 1991). Previously, crystals have been reported of water-soluble domains of both the Rieske iron–sulfur and cytochrome c_1 subunits with their respective membrane anchors cleaved by chymotrypsin (Römisch et al., 1987). Unfortunately, these crystals do not diffract well. One goal of the work described in this paper was to prepare a water-soluble domain of the Rieske subunit via a bacterial expression system, in the hope of obtaining samples suitable for structural studies. The water-soluble heme binding domain of cytochrome c_1 has been expressed in and purified from *Rb. sphaeroides* (Konishi et al., 1991). *E. coli* contains an endogenous 2Fe–2S ferredoxin which is located in the cytoplasm (Ta & Vickery, 1992). This ferredoxin can be overproduced in *E. coli* with a properly assembled 2Fe–2S cluster (Ta & Vickery, 1992). A report (Coghlan & Vickery, 1989) of a foreign 2Fe–2S ferredoxin successfully assembling in the *E. coli* cytoplasm and a report (Zylstra & Gibson, 1989) of expression of active toluene dioxygenase, perhaps with its Rieske-type cluster inserted in the *E. coli* cytoplasm, suggest that other foreign 2Fe–2S proteins might also assemble in *E. coli*. However, successful expression in *E. coli* of foreign ferredoxins containing their iron–sulfur clusters is not universal, as exemplified by the expression of the *psa*C gene product from *Synechococcus* (Li et al., 1991). In this case, in vitro reconstitution of the 4Fe–4S clusters in the polypeptide has been successful (Li et al., 1991).

This paper reports construction and characterization of *E. coli* and *Rb. sphaeroides* strains expressing the full-length Rieske iron–sulfur subunit of the *Rb. sphaeroides* cytochrome bc_1 complex, and of *E. coli* strains expressing subdomains of the subunit. The full-length Rieske polypeptides are assembled in the *E. coli* or *Rb. sphaeroides* cytoplasmic membranes with iron–sulfur clusters.

Previous studies on the bc_1 complex from *Rhodobacter capsulatus* have shown that the Rieske subunit is not necessary for the assembly of a subcomplex containing the cytochrome *b* and cytochrome c_1 subunits which retains partial activity (Davidson et al., 1992). It has also been demonstrated that the Rieske subunit can be removed from the purified bovine bc_1 complex to yield an otherwise intact subcomplex (Brandt et al., 1991). The data in the present work indicate that the cytochrome *b* or c_1 subunits are not essential either for membrane binding or for assembly of the 2Fe–2S cluster, although the stability of the subunit is clearly affected.

Assembly of the Rieske subunit in the bacterial cytoplasmic membranes in the absence of the cytochrome subunits, predicted structural features, and proteolytic release of a soluble domain of the subunit all suggest that the subunit spans the membrane once with a hydrophobic amino-terminal helix.

MATERIALS AND METHODS

Recombinant DNA Techniques. Enzymes were obtained from Bethesda Research Laboratories, Inc., or New England Biolabs, Inc. Cloning techniques were as described by Sambrook et al., (1989). Extraction of DNA fragments prior to ligation was performed using the Gene Clean Kit from BIO 101.

Synthetic oligodeoxynucleotides for priming PCR amplifications were obtained from the Biotechnology Center of the University of Illinois where they were synthesized on an Applied Biosystem Model 380A DNA synthesizer. PCR reactions were performed with a COY temperature cycler over 20 or 22 cycles with 2 min for denaturation at 92 °C, 2 min for annealing at 48 °C, and 3 min for elongation at 72 °C. Concentrations were 0.4 mM in each deoxynucleotide and 2.5 mM in Mg^{2+}.

Bacterial Strains, Growth, and Induction. Strains and plasmids used in this study are summarized in Table I. *E. coli* strains NM522 or JM83 were used for screening for insertional inactivation of *lacZ* when subcloning. The strain SF110 with the *ompT* and *degP* periplasmic proteases deleted (Baneyx & Georgiou, 1990; Strauch & Beckwith, 1988; Strauch et al., 1989) was a gift of G. Georgiou, used with the permission of J. Beckwith (Harvard University). Plasmid pMON5743 was a gift of S. Rangwala of Monsanto Chemical Co. (St. Louis, MO). Plasmid pIN-III-ompA2 was obtained from M. Inouye (Robert Wood Johnson Medical School, Piscataway, NJ). Plasmid pKK233-2 was purchased from Pharmacia LKB Biotechnology. Plasmid pMAL-c was

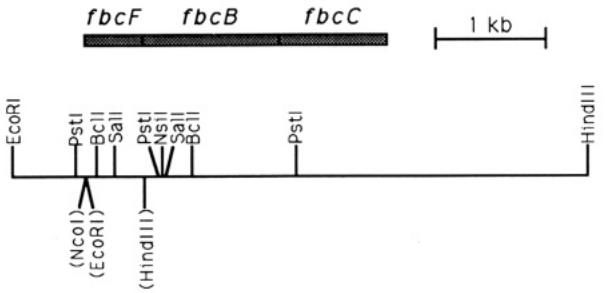

FIGURE 1: Restriction map of the *fbc* operon of *Rb. spaheroides*. The three cistrons of the operon encoding the Rieske iron–sulfur, cytochrome *b*, and cytochrome c_1 subunits of the cytochrome bc_1 complex are shown above. Restriction sites used in subcloning domains of the iron–sulfur subunit for expression are shown above the bottom line. Restriction sites introduced into PCR products are shown below the bottom line in parentheses.

purchased from New England Biolabs. Plasmid pUC8 was purchased from Bethesda Research Laboratories. Expression of portions of the Rieske subunit in strains harboring plasmid pUCFeS, pOMPAFeS, or pKKFeS was induced in early log phase with 1 mM IPTG. In strains harboring pMALCFeS, expression was induced with 0.01–0.3 mM IPTG. Expression in strains with pMONFeS was induced at mid-log phase with 50 μg/mL nalidixic acid as described by Olins and Rangwala (1990). Cultures were typically induced 3 h and then harvested. *E. coli* strains were grown at 37 °C in Luria broth (Sambrook et al., 1989) supplemented with 0.5 mg of $FeSO_4 \cdot 7H_2O$/L. SF110 derivatives were grown in the presence of 50 μg/mL kanamycin. Strains harboring expression plasmids were grown in the presence of 150 μg/mL ampicillin.

Rb. sphaeroides strains were grown chemoheterotropically at 30 °C in Sistrom's minimal medium A (Leuking et al., 1978) in the presence of 25 μg/mL kanamycin. Tetracycline was added to 1 μg/mL if a pRK415-derivative plasmid carrying a wild type or mutant *fbc* operon was present.

Construction of Expression Vectors. The expression vectors constructed in this study are listed in Table I. The restriction sites in the *fbc* operon used for subcloning are displayed in Figure 1. The resulting amino termini of the full-length and fused subfragments of the Rieske polypeptide expressed are summarized in Table II.

The vector pUC8FeS, encoding the carboxy-terminal half of the Rieske protein containing the ligands to the iron–sulfur cluster, was constructed by ligating a *SalI*/*PstI* fragment of *fbcF* into plasmid pUC8 cut with *SalI* and *PstI*. This fused the first eight residues of β-galactosidase in-frame with the carboxy-terminal half of the Rieske subunit (residues 94–187). The vector pOMPAFeS was constructed by ligating the *BclI* fragment of *fbc*, encoding the carboxy-terminal 80% of the Rieske subunit, with the secretion expression vector pIN-III-ompA2 which had been cut with *Bam*HI. This fused the cleavable *ompA* signal sequence with a soluble domain of the Rieske subunit beginning at Ile35. The site at which this soluble domain begins is identical to the amino terminus of the soluble domain of the subunit generated by chymotrypsin cleavage in this study.

The pKKFeS and pMONFeS expression vectors each encode the full-length, unfused *Rb. sphaeroides* Rieske sequence. PCR amplification of *fbcF* from plasmid pBC1 (Yun et al., 1990) was employed to generate a fragment encoding the entire subunit and to introduce both a codon for fMet with an *NcoI* site and a *Hin*dIII site downstream of the stop codon. See Figure 1 for the sites introduced. These upstream and downstream primer sequences are respectively 5′-GAA GTT CCC ATG GCC AAC GC-3′ and 5′-GCG CAA GCT TCC TTA CCC GAG CTG GATG-3′. The PCR product was cut with *NcoI* and *Hin*dIII and ligated into both of the vectors pKK233-2 and pMON5743, each cut with *NcoI* and *Hin*dIII. The plasmid pMALCFeS was also generated from a PCR product encoding the full-length Rieske subunit lacking the first two amino acid residues. The upstream primer generating an *Eco*RI site before residue 3 is 5′-A GAA GTT CTC GAA TTC AAC GCA GAA G-3′. The downstream primer generating a *Hin*dIII site beyond the stop codon is 5′-ACA TGG CGT GAA GCT TAC CCG AGC T-3′. Both the resulting PCR product and the vector pMAL-c were cut with *Eco*RI and *Hin*dIII and ligated to generate fusion of the mature portion of maltose binding protein with its signal deleted with the Rieske subunit (Table II and Figure 2). The factor Xa cut site, IEGR, lies at the junction of the two domains.

The vector pRKFeS was used for expressing the full-length Rieske iron–sulfur subunit in the absence of the cytochrome subunits in *Rb. sphaeroides* BC17, from which the genes encoding the bc_1 complex have been deleted. This vector was constructed by ligating the *Eco*RI/*Nsi*I fragment containing *fbcF* with plasmid pRK415 cut with *Eco*RI and *Pst*I (Figure 1). This fragment also encodes the first 67 amino acids of cytochrome *b*, but the remainder of the operon downstream is absent. This vector was transferred into *Rb. sphaeroides* by diparental mating of donor *E. coli* S17-1 with recipient *Rb. sphaeroides* BC17 by the method of Donohue et al. (1988).

NaDodSO₄–Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out using the Tricine-buffered 10%T, 3%C gels described by Schägger and von Jagow (1987). Samples were incubated 10 min at 45 °C in loading buffer before being loaded. Prestained low-range molecular weight standards were from Bio-Rad.

Western Blotting. Proteins were electrophoretically transferred to 0.2-μm nitrocellulose (Schleicher & Schuell) using 20% isopropyl alcohol, 25 mM Tris, and 140 mM glycine. Membranes were blocked for 30 min with 1% BSA in 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% (v/v) Tween 20, incubated for 30 min with anti-*Rb. sphaeroides* Rieske iron–sulfur protein antiserum (a generous gift from R. Niederman, Rutgers University), and then incubated for 30 min with goat anti-rabbit IgG (Bio-Rad) conjugated to alkaline phosphatase. Following antibody incubations, membranes were washed 3 times with 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% (v/v) Tween 20. Alkaline phosphatase activity was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega). Scanning densitometry was performed on a Pharmacia LKB UltroScan XL densitometer. Nitrocellulose from Western blots was made opaque for scanning by dipping in microscope immersion oil. Dilution series were scanned for quantitation.

Cell Fractionation. To prepare membranes from *E. coli* or *Rb. sphaeroides*, celi pellets (about 1 g) were resuspended in 25 mM Tris and 10 mM $MgCl_2$, pH 7.5, with about 2 mg of DNase (Sigma) per gram of cell paste, and disrupted by twice passing through a French pressure cell (SLM—Aminco). Cell debris was pelleted by centrifugation for 10 min at 12000*g*. Large membrane fragments were pelleted at 25000*g* for 20 min. The small membrane vesicles were pelleted at 150000*g* or more for at least 1.5 h. Cytoplasmic membranes were purified by spinning at 100000*g* on a 44% sucrose (w/w) cushion overnight. Spheroplasts of *E. coli* were formed by the method of Witholt et al. (1976). Spheroplast-derived vesicles of *Rb. sphaeroides*, enriched in right-side-out vesicles, were prepared by the method of Picorel et al. (1990) with the

Table II: Products Expressed from Vectors

expression vector[a]	amino-terminal sequence[b]	localization	EPR-detectable iron–sulfur cluster[c]
pRKFeS	M[1]SNAEDHAGTRRDFLYYATAGAGAVATGAAVWPLINQMNPS	membrane	+
pUC8FeS	MTITNSRGSVDTNARNANIDAGAEA[94]TDQNRTLDEAGEWLVM...	cytoplasm	–
pOMPAFeS	MKKTAIAIAVALAGFATVAQA-AEFQAWINQMNP[35]SADVQAL...	membrane	–
pKKFeS	M[1]SNAEDHAGTRRDFLYYATAGAGAVATGAAVWPLINQMNPS...	membrane	+
pMONFeS	M[1]SNAEDHAGTRRDFLYYATAGAGAVATGAAVWPLINQMNPS...	membrane	+
pMALCFeS	*mal*EΔ2-26SSVPGRGSIEGR-PEFN[3]AEDHAGTRRDFLYYA...	membrane	–

[a] These vectors are expressed in *E. coli* except for pRKFeS whose host is *Rb. sphaeroides* BC17, from which the *fbc* operon is deleted. [b] Amino acid residues of the Rieske subunit are underlined. The position of the first residue in the Rieske subunit sequence, counted from fMet, is numbered. Protease recognition sites engineered into fusion proteins are denoted by (-). [c] See Figures 4 and 5 for EPR spectra of membranes containing iron–sulfur clusters.

modifications that spheroplasts were formed by the method of Arata et al. (1987) and the linear sucrose gradient for purifying these vesicles was replaced by a two-step (0.75 and 0.25 M) sucrose cushion. Purified vesicles were collected from the interface. Osmotic shock of *E. coli* to release the periplasmic contents was performed according to Neu and Heppel (1965). Solubilization of membranes at a protein concentration of 10 mg/mL was performed in 1.0% *n*-dodecyl β-D-maltoside (CalBiochem), or 1.0% Deriphat (a gift from the Henkel Corp.), or 0.5% Tween 20 (Sigma). Amylose affinity purification of the maltose binding protein fused to the Rieske domain was performed according to Maina et al. (1988) with amylose resin supplied by New England Biolabs. Factor Xa (New England Biolabs) was incubated with the MBP–Rieske fusion protein according to Nagai and Thøgersen (1984).

EPR Spectroscopy. EPR spectra were recorded on a Bruker ESP-300 X-band spectrometer in the University of Illinois Molecular Spectroscopy Lab. Cryogenic temperatures were maintained with an Oxford Instruments liquid helium cryostat and temperature control system. Membranes were suspended with 25 mM Tris-HCl–10 mM EDTA, pH 7.5, reduced with 10 mM ascorbate in the presence of 20 μM *N*-methylphenazonium methosulfate, and frozen using a methylcyclohexane–isopentane mixture (1:5, v/v) at liquid nitrogen temperature.

Preparation of a Soluble Chymotryptic Fragment of the Rieske Subunit. Spheroplast-derived vesicles of an *Rb. sphaeroides* mutant which lacks cytochrome b_L and produces a soluble cytochrome c_1 (Konishi et al., 1991) were used for proteolysis experiments on the Rieske subunit. These membranes were solubilized at a protein concentration of 10 mg/mL in 1.0% octyl β-D-glucoside (Boehringer Mannheim) for 30 min at 4 °C in 10 mM Tris-HCl, pH 7.5. Chymotrypsin (Sigma) was added to 0.1 mg/mL (ratio of 1:100 w/w) and the solution was incubated for 3 h at 32 °C. The suspension was diluted 5-fold with 10 mM Tris-HCl, pH 7.5, and was applied to a column of DEAE-Sephacel (Pharmacia) equilibrated with the same buffer. The column was washed with 10 mM Tris-HCl, pH 7.5, and eluted with a 0–600 mM NaCl gradient in the absence of detergent. Most of the applied protein did not elute, as the octyl glucoside concentration was diluted to far below is critical micelle concentration (0.73%). The soluble Rieske fragment did elute, and was monitored by the absorbance at 420 nm and by dot-blotting with rabbit antiserum against the Rieske iron–sulfur protein of *Rb. sphaeroides*. Peak fractions containing the Rieske fragment were concentrated by ultrafiltration with an Amicon PM10 membrane, applied to a column of Sephacryl S-300 (Pharmacia), and eluted with 10 mM Tris-HCl–200 mM NaCl, pH 7.5, in the absence of detergent. Analysis of fractions from the gel filtration column by SDS–PAGE suggests the Rieske fragment does not aggregate and that the purity is greater than 80%. The peak fractions were pooled and concentrated by ultrafiltration with an Amicon PM10 membrane.

Amino-Terminal Protein Sequencing. The purified chymotryptic fragment of the Rieske iron–sulfur subunit was electroblotted from a 10% polyacrylamide–3% cross-linker Tricine gel (Schägger & von Jagow, 1987) onto ProBlott PVDF membranes (Applied Biosystems) using 10 mM CAPS, pH 11.0, and 20% methanol as described previously (Matsudaira, 1987; Ploug et al., 1989). Automated Edman degradation of the Coomassie-stained band was performed on an Applied Biosystems 477A pulsed-liquid-phase sequencer equipped with a 120A high-performance liquid chromatograph system in the University of Illinois Biotechnology Center.

Secondary Structure Prediction. Calculations of hydropathy, amphipathy (hydrophobic moment), and mutability (moment of conservation) were performed with the AMPHI and INFORMAT (Crofts et al., 1989) programs of the SEQANAL (version 2.0) software package of Antony R. Crofts (copyright, 1987, University of Illinois).

RESULTS

Expression of the Rieske Iron–Sulfur Subunit in a Strain of Rb. sphaeroides from Which the fbc Operon Has Been Deleted. Rb. sphaeroides strain BC17(pRKFeS) expresses the assembled, full-length Rieske subunit in the absence of genes encoding the *b* and *c* cytochrome subunits, schematically illustrated in Figure 2C. The full-length subunit is located in the cytoplasmic membrane as revealed by Western blots of membranes purified on sucrose cushions (Figure 3, lane E). The membranes used for the Western blot shown in Figure 3 were prepared from spheroplasts and were enriched in right-side-out vesicles (periplasmic or positive-side-out). A 1 M KCl wash of these vesicles removes water-soluble proteolytic fragments of the Rieske subunit which form upon cell disruption. Figure 3 shows that the Rieske subunit, when expressed independently of the complex [strain BC17-(pRKFeS)], is much more susceptible to endogenous proteolysis than when it is part of the intact bc_1 complex (strain BC17C).

The Rieske subunit in the native bc_1 complex displays a diagnostic EPR transition at $g_y = 1.90$ (Figure 4, spectrum A). The position and breadth of the upfield g_x transition reflect the redox state of the quinone pool (Meinhardt et al., 1987). When the quinone pool is reduced, the EPR spectrum of the native complex shows a broad transition of $g_x = 1.76$. When the pool is oxidized, the spectrum reveals a narrow transition of $g_x = 1.80$. The change from one state to the other titrates with an $E_{m,7}$ of 90 mV, the midpoint of the quinone pool. The conditions used here have the quinone pool reduced, as illustrated for the intact complex with $g_x =$

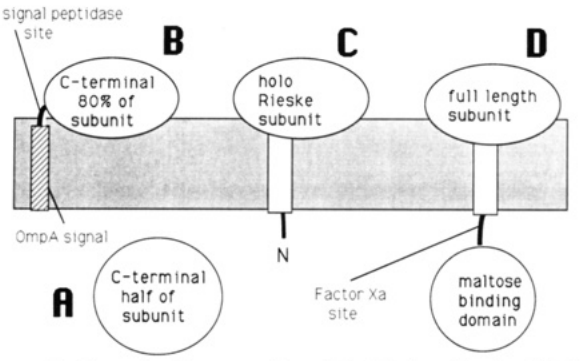

FIGURE 2: Strategies for expression of the Rieske subunit. (A) The carboxyl-terminal half, containing the conserved cysteine and histidine residues expected to ligate a 2Fe–2S cluster, expressed by the vector pUC8FeS in the cytoplasm of *E. coli*. (B) The carboxyl-terminal 80%, equivalent to the water-soluble chymotryptic fragment (Figure 6A,D and Figure 7A), directed to the periplasm of *E. coli* by the secretion expression vector pOMPAFeS. (C) The full-length, unfused polypeptide expressed by the vectors pKKFeS and pMONFeS in *E. coli* and by pRKFeS in *Rb. sphaeroides*. (D) The mature domain of the maltose binding protein fused to the amino terminus of the full-length Rieske polypeptide produced by the vector pMALCFeS in *E. coli*. The foreign domains are stipled. Expected protease recognition sites in the fusion proteins are indicated.

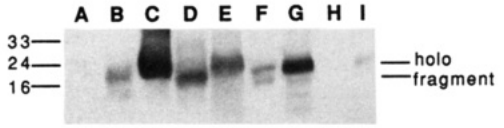

FIGURE 3: Western blot showing the localization of the full-length Rieske subunit and proteolytic fragments. Fractionations were performed as described under Materials and Methods. The membrane fractions loaded in lanes A, C, E, G, and I are inner membranes purified on sucrose cushions. The lanes were loaded as follows: (A) 25 μg of French-pressed *Rb. sphaeroides* BC17 membranes; (B) 25 μg of the soluble fraction of *Rb. sphaeroides* BC17C; (C) 25 μg of spheroplast-derived vesicles from *Rb. sphaeroides* BC17C, washed with 1 M KCl; (D) 100 μg of the soluble fraction of *Rb. sphaeroides* BC17(pRKFeS); (E) 100 μg of spheroplast-derived vesicles from *Rb. sphaeroides* BC17(pRKFeS), washed with 1 M KCl; (F) 75 μg of the soluble fraction of *E. coli* SF110(pKKFeS); (G) 75 μg of French-pressed membranes of *E. coli* SF110(pKKFeS); (H) 75 μg of the soluble fraction of *E. coli* SF110; (I) 75 μg of French-pressed membranes of *E. coli* SF110.

1.76 (Figure 4A). EPR spectra of membranes of the *Rb. sphaeroides* mutant strain which contains only the Rieske subunit, BC17(pRKFeS), reveal the ascorbate-reducible Rieske center with the diagnostic transition at $g = 1.90$ (Figure 4, spectrum B). A shallow upfield feature at $g = 1.745$ is scarcely discerned above the noise. Additionally, though the inhibitor stigmatellin induces shifts in the EPR spectrum of the iron–sulfur center in the intact cytochrome bc_1 complex (Andrews et al., 1990; Ding et al., 1992; von Jagow & Ohnishi, 1985), it does not shift the spectrum of the Rieske cluster in this mutant (not shown). This suggests the cytochrome subunits are necessary for stigmatellin binding.

The amount of Rieske subunit present in the membranes of the strain lacking the cytochrome *b* and c_1 subunits is only 1–2% of the amount (per milligram of protein) in the membrane of the wild-type control, BC17C. This is determined both from quantitative Western blotting and by EPR spectroscopy which suggests that the subunit is fully metalated although it is present at a very low level. This probably reflects instability due to enhanced susceptibility to proteolysis.

Expression of Fragments of the Rieske Polypeptide in E. coli. Fragments of the iron–sulfur subunit expected to be soluble were expressed in *E. coli* (see Figure 2A,B). Vector pUC8FeS should express the carboxyl-terminal portion of

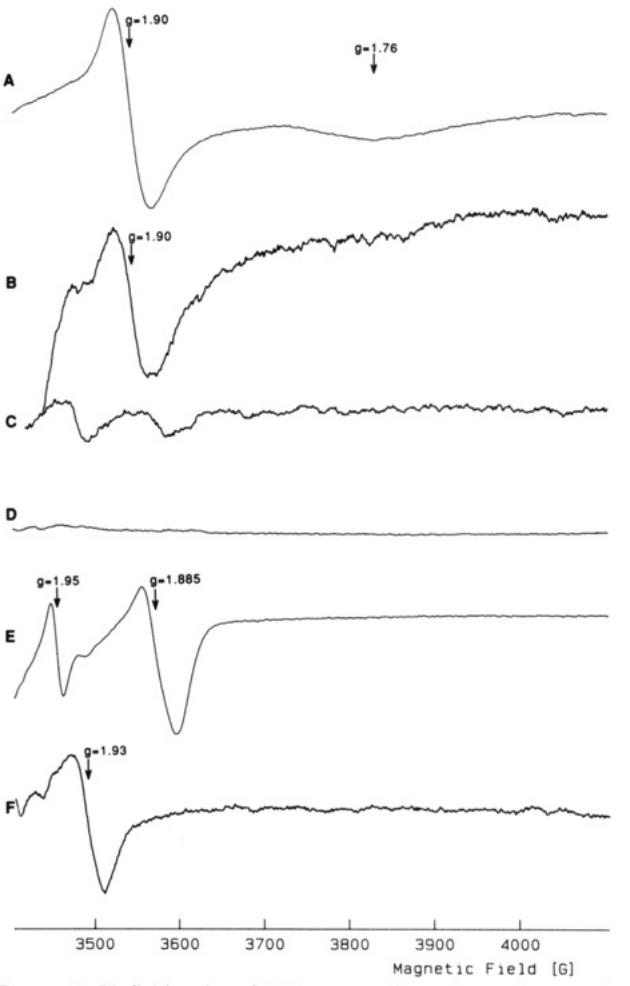

FIGURE 4: Upfield region of EPR spectra of membranes from strains containing the Rieske subunit. See Materials and Methods for details. *Spectrum A* is a single scan of *Rb. sphaeroides* BC17C membranes which contain wild-type cytochrome bc_1 complex. *Spectrum B* is a spectrum of *Rb. sphaeroides* BC17(pRKFeS) membrane from which cytochromes *b* and c_1 have been genetically deleted. Spectrum C is a spectrum of *Rb. sphaeroides* BC17 membranes from which all of the catalytic subunits of the cytochrome bc_1 complex have been genetically deleted. *Spectra B and C* are on a scale 10-fold more sensitive than that of spectrum A, and are averages of 12 scans with the cavity background spectrum subtracted out of each. *Spectrum D* is a single scan of *E. coli* SF110 membranes not expressing foreign protein (negative control). *Spectrum E* is a single scan of *E. coli* SF110(pKKFeS) high-speed-pellatable (170000*g*) membranes. Spectrum F is an average of two scans of *E. coli* SF110(pKKFeS) low-speed-pellatable (25000*g*) membrane fragments, on a scale about 5 times more sensitive than that of spectra A, D, and E. EPR spectra were recorded under the following conditions: temperature, 25 K; microwave power, 20 mW; modulation amplitude, 16 G; frequency, 9.44 GHz; modulation frequency, 100 kHz; sweep time, 170 s; time constant, 330 ms.

the Rieske subunit fused to the first few residues of β-galactosidase. As expected, the protein produced is located in the cytoplasm (Table II). The product of pOMPAFeS fuses the *ompA* signal sequence with the equivalent of the water-soluble domain generated by chymotrypsin cleavage (Table II). Although the fusion protein should be processed at the leader peptidase cleavage site and released into the *E. coli* periplasm, osmotic shock treatment to release the periplasmic contents yielded little of the Rieske domain. Rather, the bulk of the fusion protein appears to be uncleaved and is located in the cytoplasmic membrane. EPR signals characteristic of the Rieske iron–sulfur cluster are absent from whole cells, soluble fractions, or membrane fractions of strains harboring either pUC8FeS or pOMPAFeS (not shown).

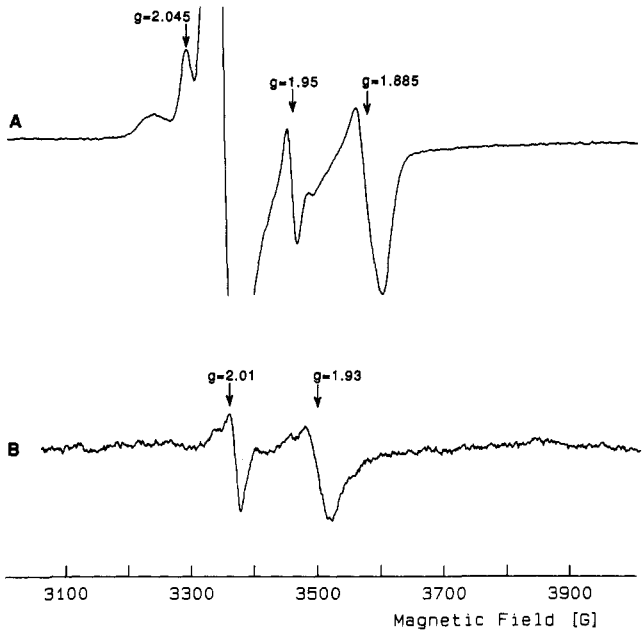

FIGURE 5: Full EPR scans of *E. coli* membranes expressing the full-length, metalated Rieske subunit of the cytochrome bc_1 complex. Conditions were those used in Figure 4, but the sweep width was 1000 G. *Spectrum A* is of *E. coli* SF110(pKKFeS) high-speed-pelleted membranes as in Figure 4E. *Spectrum B* is of *E. coli* SF110-(pMONFeS) low-speed-pelleted membranes as in Figure 4F.

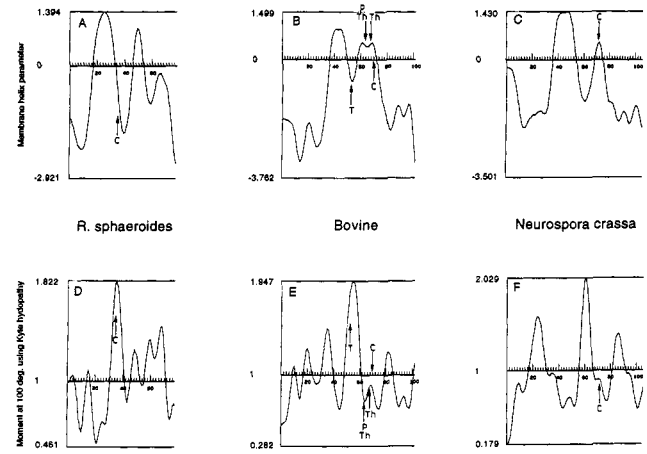

FIGURE 6: Hydropathy and amphipathy profiles of the amino-terminal portions of the mature Rieske iron–sulfur subunits from the *Rb. sphaeroides*, bovine, and *Neurospora crassa* cytochrome bc_1 complexes. The upper panels A–C are calculated with the Rao–Argos hydropathy scale (Rao, 1986) using a window of seven residues, with two cycles of smoothing using a window of five residues. Peaks represent hydrophobic regions. The lower panels D–F are calculated using the Kyte–Doolittle hydropathy scale (Kyte & Doolittle, 1982) at an α-helical repeat of 100°, using a sliding window of seven residues with two cycles of smoothing using a window of five residues. Peaks represent helical amphipathy. The sites of proteolysis yielding soluble, carboxyl-terminal domains of the Rieske subunits from *Rb. sphaeroides*, bovine (Gonzalez-Halphen, 1988; Cocco, 1991), and *N. crassa* (Weiss, personal communication) are marked with arrows. Abbreviations of proteases used are as follows: C, chymotrypsin; T, trypsin; P, papain; Th, thermolysin.

Expression of the Full-Length Rieske Subunit in E. coli. *E. coli* strains hosting plasmid pKKFeS or pMONFeS both express the full-length, unfused Rieske iron–sulfur polypeptide (Table II). The full-length polypeptide is located primarily in the cytoplasmic membrane, though very small proportions of the polypeptide and proteolytic fragment appear in the soluble phase (Figure 3, lanes F and G).

The strains expressing the full-length Rieske polypeptide do assemble ascorbate-reducible iron–sulfur clusters but with unusual EPR signals for Rieske iron–sulfur centers (Figures 4 and 5). The EPR signals associated with the Rieske subunit expressed in *E. coli* are heterogeneous. The spectrum is different depending on the sample preparation. In all cases, the broad upfield feature at about $g = 1.76$ is absent (Figure 4E,F). Membranes obtained from low-speed centrifugation have a prominant peak at $g = 1.93$, whereas membranes pelleted at higher centrifugation speeds exhibit two transitions at $g = 1.95$ and $g = 1.885$. When the "high speed" membranes were solubilized in 1% dodecyl maltoside or 1% Deriphat, the spectrum of the iron–sulfur cluster changed substantially to that of Figure 4F. The cluster is labile in other detergents. As observed with the *Rb. sphaeroides* mutant lacking the cytochrome subunits, stigmatellin does not shift the spectrum of the iron–sulfur subunit expressed in *E. coli*. Membranes from SF110(pMONFeS) give signals identical to those of SF110(pKKFeS) shown in Figure 4.

Wider EPR scans of samples prepared as in Figure 4E,F are shown in Figure 5A,B respectively. The spectrum of the Rieske iron–sulfur center of the bc_1 complex is rhombic, with $g_z = 2.03$, $g_y = 1.90$, and $g_x = 1.76$ (when the quinone pool is reduced). The signals from the *E. coli* expression systems, however, are less anisotropic and probably axial. The spectrum from the "low speed" membranes has $g_\parallel = 2.01$ and $g_\perp = 1.93$ (Figure 5B). Two overlapping axial signals appear to be present in the spectrum of the "high speed" membranes. One species has the sharp $g_\perp = 1.95$, and the other has $g_\perp = 1.885$. One of these species has $g_\parallel = 2.045$ while the $g_\parallel$ feature of the other species is likely buried in the free radical region of the spectrum. All of these species have power saturation properties similar to each other and to the *Rb. sphaeroides* wild-type complex. Altogether, three different forms of high-potential iron–sulfur centers are associated with the Rieske subunit expressed in *E. coli*.

Expression in E. coli of the Maltose Binding Protein Fused to the Rieske Polypeptide. Strains hosting the vector pMALCFeS produce fusion of the mature portion of the maltose binding protein, lacking its signal sequence, with the full-length Rieske polypeptide (see schematic in Figure 2D). This fusion protein is located primarily in the membrane fraction (Table II). As expression levels are increased by increasing the IPTG concentration in the growth medium to 0.3 mM, both the growth rate and cell yield decrease. The fusion product is readily purified on an amylose affinity resin. Factor Xa, however, does not cleave at the proteolysis site engineered into the fusion protein, but does cleave the fusion protein elsewhere. Regardless of IPTG induction level or growth temperature, EPR signals indicative of an ascorbate-reducible iron–sulfur cluster are not observed in spectra of membranes containing this fusion protein.

Amino-Terminal Sequence of a Chymotrypsin-Cleaved, Water-Soluble Fragment of the Rieske Subunit. A water-soluble fragment of the Rieske protein obtained by chymotrypsin cleavage was purified and sequenced. The chymotrypsin cleavage product from solubilized *Rb. sphaeroides* membranes initially retains its iron–sulfur cluster, as determined by EPR spectroscopy, but the cluster is labile. The amino-terminal sequence determined for the product is INQMNPSADVQA (Figures 6 and 7).

Predicted Structural Features and Pattern of Conservation in the Amino-Terminal Region. Alignment of amino-terminal sequences of Rieske subunits reveals a pair of conserved positive charges, not present in the fungal sequences, followed by a hydrophobic stretch of 18 or 19 residues (Figures 6 and 7). This is a common motif for signal and start-transfer sequences

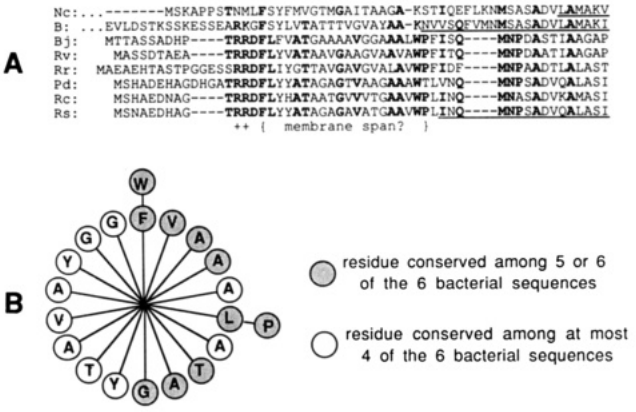

FIGURE 7: Conservation of amino-terminal resdiues among bacterial Rieske subunits. Part A shows an alignment of the amino-terminal portions of six bacterial and two mitochondrial Rieske subunits. Species are designated as follows: Nc, *Neurospora crassa* (Harnisch et al., 1986); B, bovine (Schägger et al., 1987); Bj, *Bradyrhizobium japonicum* (Thöny-Meyer et al., 1989); Rv, *Rhodopseudomonas viridis* (Verbist et al., 1989); Rr, *Rhodospirillum rubrum* (Majewski & Trebst, 1990); Pd, *Paracoccus denitrificans* (Kurowski & Ludwig, 1987); Rc, *Rhodobacter capsulatus* (Davidson et al., 1987); Rs, *Rhodobacter sphaeroides* (Yun et al., 1990). The extreme amino termini of the eukaryotic sequences are omitted. Boldfaced residues are conserved among at least five of the bacterial sequences. Underlined residues represent the amino termini of soluble proteolytic fragments of the *Rb. sphaeroides*, *N. crassa*, and bovine subunits, also indicated in Figure 6. The pair of conserved positive charges and the hydrophobic stretch, suggestive of a membrane-spanning signal/anchor, are labeled below the aligned sequences. Part B shows a helical wheel representing the putative membrane span of the *Rb. sphaeroides* sequence. Shaded circles represent residues conserved among at least five of the six bacterial sequences.

(Sjostrom et al., 1987). In the hydrophobic region, the aligned bacterial sequences display a pattern of amino acid conservation that suggests a helical structure. One side of the proposed hydrophobic helix is nearly fully conserved, and the opposite side is less so, as illustrated by the helical wheel of Figure 7B. At the end of this putative helix is a helix-breaking residue (Figure 7A), followed by a short segment of highly amphipathic character with a helical repeat (see Figure 6).

DISCUSSION

This paper has explored various expression systems for the production of either the full-length Rieske iron–sulfur protein from *Rb. sphaeroides* or the metal binding hydrophilic domain. Although the work was not successful in defining a protocol to produce large quantities of a water-soluble version of this unusual 2Fe–2S protein, two conclusions can be deduced from the data obtained.

(1) The Rieske polypeptide with its iron–sulfur cluster is assembled in the cytoplasmic membrane of aerobically grown *Rb. sphaeroides* in the absence of the cytochrome *b* and c_1 subunits of the bc_1 complex. This suggests a mode of membrane binding via direct interaction with the lipid bilayer, e.g., a transmembrane helix, as previously suggested (Harnisch et al., 1986). The Rieske subunit is subject to endogenous proteolysis in *Rb. sphaeroides*, and this susceptibility is substantially enhanced in the absence of the cytochrome *b* and c_1 subunits. This is probably why the residual fully metalated Rieske subunit in the membrane is 1–2% of the control values when the subunit is produced in *Rb. sphaeroides* without the other components of the bc_1 complex.

(2) The full-length Rieske subunit with an iron–sulfur cluster can be produced in *E. coli*. The cluster has a high midpoint potential and has EPR characteristics which are Rieske-like. However, the EPR spectra indicate at least three distinct species. The spectra all differ from that of the native complex and have shifted *g* values, similar to the spectroscopic perturbations caused by bc_1 inhibitors such as stigmatellin, UHDBT (Andrews et al., 1990; von Jagow & Ohnishi, 1985), and DBMIB (Degli Esposti et al., 1984; Malkin, 1981, 1982). In the native complex, DBMIB causes a large shift of the g_y feature from 1.90 to 1.95, but this shift is not accompanied by significant changes in the coordination of the cluster as determined by electron spin-echo spectroscopic studies (Britt et al., 1991). Hence, subtle conformational differences may be responsible for the different EPR spectra observed for the Rieske subunit in *E. coli*. Such subtle changes may be a sufficient and simpler explanation than rearrangement to different ligands, the latter of which has been observed for a 4Fe–4S cluster of a mutant ferredoxin (Martin et al., 1990). Since *E. coli* does not contain an endogenous Rieske protein or bc_1 complex, these data strongly imply that a bc_1-specific system is not required for insertion of the 2Fe–2S cluster in the Rieske subunit.

Stigmatellin does not affect the EPR spectrum of the Rieske center when the cytochromes are absent. Furthermore, in a *Rb. sphaeroides* mutant with both the cytochrome *b* and the Rieske proteins present, but with cytochrome c_1 absent from the membrane, stigmatellin does shift the EPR spectrum (Van Doren et al., unpublished results). Thus, both cytochrome *b* and the Rieske subunit together appear to make up the binding site for stigmatellin.

Previous studies have clearly implicated the amino-terminal portion of the Rieske subunit as required for binding to the bc_1 complex (Cocco et al., 1991; Gonzalez-Halphen et al., 1988, 1991; Li et al., 1981). Water-soluble fragments of the Rieske subunit with the intact iron–sulfur cluster have been prepared by proteolytic cleavage of the bc_1 complex from bovine (Cocco et al., 1991; Gonzalez-Halphen et al., 1988) and *Neurospora crassa* (Li et al., 1981). These soluble domains do not reconstitute with Rieske-depleted bc_1 complex (Gonzalez-Halphen et al., 1991). The cleavage sites are shown in Figure 6, along with the comparable cleavage site in the *Rb. sphaeroides* subunit. In all cases, a hydrophobic stretch at the amino terminus of the subunit is removed by proteolytic treatment. These data also show that the putative amphiphilic helix, which invariably follows the hydrophobic domain (Figure 6), is not sufficient for binding to the complex, although it may be functionally important (Cocco et al., 1991). Unfortunately, the soluble Rieske domain from *Rb. sphaeroides* is not sufficiently stable to permit isolation and characterization on a large scale.

The importance of the hydrophobic domain in mediating the binding of the bovine Rieske subunit to the bc_1 complex was recently directly demonstrated (Gonzalez-Halphen et al., 1991). A 20-residue synthetic peptide corresponding to this region of the bovine subunit (Lys33–Lys52) was shown to compete with the Rieske subunit in reconstitution experiments with Rieske-depleted bc_1 complex.

Comparison of the amino acid sequences of six bacterial Rieske subunits (Figure 7) reveals a pattern of residue conversion that strongly suggests that this region is an α-helix. The hydropathy and amphipathy plots of Figure 6 suggest that the probable hydrophobic α-helix is, furthermore, uniformly hydrophobic on all sides, making it unlikely to be a surface-seeking helix. The fact that the Rieske subunit can be assembled in the membrane in the absence of the other subunits suggests that this is most likely a transmembrane helix which anchors the subunit to the membrane. It should be noted that the bacterial Rieske subunit is synthesized in

vivo without a cleaved signal sequence at the amino terminus (Andrews et al., 1990). Examples of bacterial periplasmic proteins without a cleaved signal sequence are rare (Friedrich et al., 1986; Van Hove et al., 1990), whereas there are numerous examples of proteins with uncleaved "signal/anchors" (Sjostrom et al., 1987) as suggested here for the Rieske subunit. It does not seem likely that the entire Rieske subunit is exported entirely across the *Rb. sphaeroides* cytoplasmic membrane and binds primarily to the cytochrome *b* subunit as a peripheral membrane protein. The pattern of conserved residues (Figure 7) is similar to that in several transmembrane helices of the photosynthetic reaction center (Komiya et al., 1988), where the more conserved side of the helices is involved in protein interactions and the less conserved side faces the lipid bilayer. Perhaps the more conserved side of the putative transmembrane helix in the Rieske subunit binds to the other subunits of the bc_1 complex.

The strongest evidence in support of a peripheral location of the entire Rieske subunit, e.g., without a transmembrane domain, is that of Hartl et al. (1986), who reported that an alkaline wash (100 mM sodium carbonate) removes 80% of the Rieske subunit from the membranes of *N. crassa*. In a similar experiment with *Rb. sphaeroides*, 100 mM sodium carbonate (pH 12) removed one-third or less of the Rieske subunit either from spheroplast-derived vesicles or from chromatophores (unpublished results). However, this criterion for peripheral localization must be considered suspect, since Szczepaniak and Cramer (1991) have demonstrated that under similar conditions the very hydrophobic, transmembrane cytochrome b_6 is easily removed from chloroplast thylakoids. The fact that the Rieske subunit is most easily removed from the isolated bc_1 complex by adding salt or chaotropic agents (Hovmoller et al., 1981; Trumpower & Edwards, 1979) is also not pertinent, since similar conditions remove the H subunit from the purified photosynthetic reaction center (Feher & Okamura, 1978), even though it has a transmembrane anchor (Deisenhofer et al., 1985).

Overall, the evidence presented here for a single transmembrane helix supports conclusions based on previous labeling (D'Souza & Wilson, 1982) and proteolysis (Oritz & Malkin, 1985) studies that have indicated that the Rieske subunit is transmembranous.

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