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Potential Ligands to the [2Fe-2S] Rieske Cluster of the Cytochrome bc_1 Complex of *Rhodobacter capsulatus* Probed by Site-Directed Mutagenesis†

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ABSTRACT: The Rieske protein of the ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex or b_6f complex) contains a [2Fe-2S] cluster which is thought to be bound to the protein via two nitrogen and two sulfur ligands [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; Gurbiel, R. J., Ohnishi, T., Robertson, D. E., Daldal, F., & Hoffman, B. M. (1991) *Biochemistry 30*, 11579-11584]. All available Rieske amino acid sequences have carboxyl termini featuring two conserved regions containing four cysteine (Cys) and two or three histidine (His) residues. Site-directed mutagenesis was applied to the Rieske protein of the photosynthetic bacterium *Rhodobacter capsulatus*, and the mutants obtained were studied biochemically in order to identify which of these conserved residues are the ligands of the [2Fe-2S] cluster. It was found that His159 (in the *R. capsulatus* numbering) is not a ligand and that the presence of the Rieske protein in the intracytoplasmic membrane is greatly decreased by alteration of any of the remaining six His or Cys residues. Among these mutations, only the substitution Cys155 to Ser resulted in the synthesis of Rieske protein (in a small amount) which contained a [2Fe-2S] cluster with altered biophysical properties. This finding suggested that Cys155 is not a ligand to the cluster. A comparison of the conserved regions of the Rieske proteins with bacterial aromatic dioxygenases (which contain a spectrally and electrochemically similar [2Fe-2S] cluster) indicated that Cys133, His135, Cys153, and His156 are conserved in both groups of enzymes, possibly as ligands to their [2Fe-2S] clusters. These findings led to the proposal that Cys138 and Cys155, which are not conserved in bacterial dioxygenases, may form an internal disulfide bond which is important for the structure of the Rieske protein and the conformation of the quinol oxidation (Q_o) site of the bc_1 complex.

The ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex)[1] is a key component of photosynthetic and respiratory electron-transport chains in many organisms, and is a site where energy transfer is coupled to ATP synthesis (Dutton, 1986). The subunit composition of this membrane-bound complex varies with the organism, but irrespective of the source, all have three components with the following functional prosthetic groups: a cytochrome *b* containing two *b* hemes, a cytochrome c_1 containing a *c* heme, and an iron–sulfur protein, commonly referred to as the Rieske iron–sulfur (Fe-S) protein, which

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[1] Abbreviations: bp, base pair(s); bc_1 complex, ubiquinol–cytochrome *c* oxidoreductase; cyt, cytochrome; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; E_m, redox midpoint potential; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; Q_o, quinol oxidation site; Q_i, quinone reduction site; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ps, photosynthetic growth.

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contains a [2Fe-2S] cluster. These subunits form two quinone binding sites, Q_o and Q_i, where the quinones are oxidized and reduced, respectively [for general reviews, see Cramer and Knaff (1990) and Trumpower (1990)].

The photosynthetic bacterium *Rhodobacter capsulatus* uses a bc_1 complex in both photosynthetic and respiratory electron transfer, and its isolated complex contains only the three proteins described above. The structural genes for these proteins, the *fbcFBC* (*petABC*) operon, have been cloned and sequenced (Gabellini & Sebald, 1986; Daldal et al., 1987; Davidson & Daldal, 1987). A subsequent combination of molecular genetics (Daldal et al., 1989; Atta-Asafo-Adjei & Daldal, 1991) and biophysical analyses (Robertson et al., 1990) has allowed the identification of regions, and specific amino acid residues, of cytochrome *b* which are thought to be involved in the oxidation of ubiquinol at the Q_o site. The investigation of mutations in these regions has provided a working model for the Q_o site (Robertson et al., 1990).

In contrast to the wealth of information concerning cytochrome *b*, relatively little is known about the Rieske protein whose function is to oxidize the QH_2 at the Q_o site and subsequently pass an electron to cytochrome c_1. The bulk of the protein, with the [2Fe-2S] cluster, is thought to the exposed in the periplasm, but the precise topology of its membrane attachment has yet to be defined. Most of our knowledge is centered on the [2Fe-2S] cluster itself [see Trumpower (1981)]. On the basis of the Mössbauer and resonance Raman spectroscopic features, the Rieske Fe-S protein has been inferred to have a binuclear-type cluster structure [see Fee et al. (1986)]. The distinct electron paramagnetic resonance (EPR) spectrum of the Rieske protein has been well characterized and exhibits a unique spectral line shape [with *g* values $g_{x,y,z}$ = 1.80, 1.90, and 2.03, respectively, with an average *g* value (g_{av} = 1.91) which is lower than that for the ferredoxin (Fd)-type [2Fe-2S] cluster (g_{av} = 1.96)]. Its redox midpoint potential (E_m), around +300 mV, is much higher (by ~700 mV) than that of typical ferredoxins [see, e.g., Fee et al. (1986)]. The g_x feature of the Rieske spectrum (g_x = 1.80 in ascorbate-reduced samples) is of particular interest since its line shape and *g* value are sensitive to the redox state of the Q pool in the membrane, presumably through interaction at the Q_o site (Matsuura et al., 1983). Q_o site inhibitors alter the E_m and EPR line shape of the Rieske cluster, strongly implying that the Rieske protein is part of the Q_o site. However, to date no Q_o inhibitor-resistant mutation has yet been mapped to the Rieske protein (di Rago et al., 1988; Howell & Gilbert, 1988; Daldal et al., 1989). One basic piece of structural information which is not known is the identity of the amino acid ligands to the cluster. The identification of the residues which bind the [2Fe-2S] cluster to the protein is the focus of this work.

The Rieske proteins share their characteristic biophysical features, i.e., relatively high E_m and EPR line shape, with several bacterial dioxygenases which are involved in degradation of aromatic compounds. These enzymes contain two different species of [2Fe-2S] cluster, one of which is frequently referred to as a "Rieske-type" cluster [e.g., see Fee et al. (1986)]. Blumberg and Peisach (1974) proposed that, for the Rieske type of cluster, at least one of the ligands was less electron-donating than sulfur. Subsequent research has confirmed this suggestion.

Studies using a number of biophysical techniques, including electron nuclear double resonance (ENDOR) and Mössbauer spectroscopy, have implied that the [2Fe-2S] cluster of dioxygenases contains two sulfur ligands and at least one nitrogen ligand (Cline et al., 1985). ENDOR and electron spin echo envelope modulation (ESEEM) studies on yeast mitochondrial bc_1 complex have suggested that the Rieske [2Fe-2S] cluster contained at least one, and probably two, nitrogen ligand (Telser et al., 1987). Extended X-ray absorption fine structure (EXAFS) studies of bovine heart mitochondrial bc_1 complex also eliminated exclusive sulfur or nitrogen ligations (Powers et al., 1989). The presence of two nitrogen ligands in the Rieske-like [2Fe-2S] cluster of *Pseudomonas cepacia* phthalate dioxygenase was firmly established by ENDOR spectroscopy (both X- and Q-band) after isolation of this dioxygenase from cells grown on ^{14}N- or ^{15}N-enriched media (Gurbiel et al., 1989). During the course of the experiments reported here, ESEEM (Britt et al., 1991) and ENDOR (Gurbiel et al., 1991) studies using purified bc_1 complexes provided evidence that two nitrogen and two sulfur atoms are ligands to the [2Fe-2S] cluster of the Rieske protein in various cytochrome bc_1 and b_6f complexes.

Amino acid sequences of the Rieske protein have been obtained from a number of sources: mitochondrial, chloroplast, and from several species of bacteria [see Hauska et al. (1988)]. The carboxyl terminus of the Rieske protein has been implicated in the binding of its [2Fe-2S] cluster on the basis of proteolytic cleavage (Li et al., 1983). Examination of the available sequence data shows that this region of the protein features two groups of six entirely conserved residues, called here box I (CTHLGC) and box II (CPCHGS). Each box contains two cysteines (Cys) and one histidine (His) (Figure 1), residues most likely to provide sulfur and nitrogen ligands to the cluster.

Comparison of available amino acid sequences has previously proven useful in assigning the His ligands to the two *b*-type hemes of cytochrome *b* of the bc_1 complex. When all cytochrome *b* sequences, including cytochrome b_6, from a range of species were compared, it was found that only four of the many histidines were universally conserved (Widger et al., 1984; Saraste et al., 1984). Unfortunately, a comparison of all available Rieske sequences shows that the number of potential ligands available as Cys and His residues exceeds the number needed (Figure 1). The four Cys and two His residues of boxes I and II are universally conserved; His159 (in the *R. capsulatus* sequence numbering) is not but is sometimes substituted by a Gln (Figure 1) which may also provide a nitrogen ligand (Gatti et al., 1989). When the first Rieske protein sequence was obtained, it was assumed that the [2Fe-2S] cluster was analogous to the [2Fe-2S] cluster of many ferredoxins in being liganded by four Cys residues (Harnisch et al., 1985). Since then, several groups [e.g., see Beckman et al. (1987) and Gatti et al. (1989)] have speculated on the identity of the ligands to the Rieske [2Fe-2S] cluster. However, it is not currently known which of these conserved Cys and His residues are the ligands of the [2Fe-2S] cluster.

Our experiments have therefore sought to define the liganding residues by using site-directed mutagenesis to alter all potential ligands. For this purpose, we have obtained 19 substitutions in 7 conserved positions of the Rieske protein of *R. capsulatus* and analyzed their biochemical and biophysical properties, the results of which are reported here. We have also examined the effects of these mutations on the steady-state stability and biochemical properties of the cytochrome *b* and cytochrome c_1 subunits in membranes; these studies are reported in the following paper (Davidson et al., 1992).

Materials and Methods

Media, Bacterial Strains, and Growth Conditions. The growth of *R. capsulatus* and *Escherichia coli* strains on

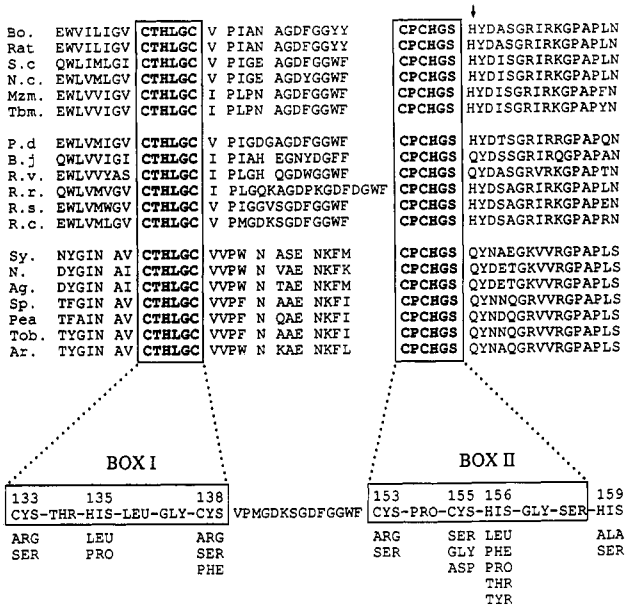

FIGURE 1: Alignment of the carboxyl termini of all available Rieske sequences showing conserved boxes I and II. The sequence identities are as follows: Bo., bovine (Schägger et al., 1987); Rat, rat (Nishikimi et al., 1989); S.c., *Saccharomyces cerevisiae* (Beckman et al., 1987); N.c., *Neurospora crassa* (Harnisch et al., 1985); Mzm., maize mitochondrion; Tbm., tobacco mitochondrion [both Huang et al. (1991)]; P.d., *Paracoccus denitrificans* (Kurowski & Ludwig, 1987); B.j., *Bradyrhizobium japonicum* (Thony-Mayer et al., 1989); R.v., *Rhodopseudomonas viridis* (Verbist et al., 1989); R.r., *Rhodospirillum rubrum* (Majewski & Trebst, 1990); R.s., *Rhodobacter sphaeroides* (Yun et al., 1990); R.c., *Rhodobacter capsulatus* (Davidson & Daldal, 1987). The lower group shows the available b_6f Rieske sequences. N., *Nostoc* (Kallas et al., 1988); Ag., *Agmenellum quadriplicatum* (Widger & Cramer, 1991); Sy., *Synechococcus* sp. PCC 7009 (Mayes & Barber, 1991); Sp., spinach (Steppuhn et al., 1987); Pea, pea; Tob., tobacco; Ar., *Arabadopsis* (all personal communication from Dr. John Gray). The putative [2Fe-2S] cluster binding region of the *R. capsulatus* Rieske protein is shown with the mutants made in boxes I and II underneath the sequence at the appropriate position. The position of the nonconserved His adjacent to box I is indicated by an arrow.

MPYE, RCV, and LB media, respectively, was described previously (Daldal et al., 1989). The strains used are listed in Table I, and in the text we refer to each merodiploid by the nature of the amino acid replacement; e.g., the strain C133SER (pFC133S/MT-RBC1) contains the composite plasmid pFC133S (pRK404-pPET1 derivative) on which the codon corresponding to cysteine at position 133 of *fbcF* (*fbcF*:*C133S*) has been altered to encode a serine, in MT-RBC1.

Site-Directed Mutagenesis and DNA Sequencing. This was performed as described previously (Atta-Asafo-Adjei & Daldal, 1991) using the uracilated phage M13-73R2BC1 (containing an appropriate fragment of the *fbc* operon) as a template. The following mixed mutagenic oligonucleotides were used: petA-C133, 5′-AGGTGGGTGC(T/G/C)-CACGCCA-3′; petA-H135, 5′-CAGCCGAG(G/C)-NNGGTGCACAC-3′; petA-C138, 5′-ATCGGCAC(G/C)(A/C/T)(N)GCCGAGGTG-3′; petA-C153, 5′-TGGCAGGGGC(T/G/C)GAACCAG-3′; petA-C155, 5′-GAGCCGTG(G/C)(A/C/T)(N)GCAGGGGCA-3′; petA-H156, 5′-TGCGAGCC(G/C)NNGCAGGGGCA-3′; petA-H159, 5′-AATCGTA(G/C)NNCGAGCCGTGG-3′.

The merodiploid *R. capsulatus* strains containing plasmid-borne mutations were constructed essentially as shown in Figure 2 of Atta-Asafo-Adjei and Daldal (1991), except that the 600 bp *Bst*XI–*Eco*RI (instead of the 450 bp *Eco*RI–*Sma*I) fragment carrying the Rieske mutations was shuttled from the replicative form of phage M13-73R2BC1 to the plasmid pPET1. The resulting pPET1 derivatives were ligated into the *Hin*dIII site of the broad host-range plasmid pRK404 (Ditta et al., 1985) for conjugation into the *R. capsulatus* strain MT-RBC1, from which the *fbc* operon has been deleted and replaced by a spectinomycin resistance cartridge (Atta-Asafo-Adjei & Daldal, 1991).

Table I: Characteristics of *R. capsulatus* Rieske FeS Protein Mutants

box	strains	mutation	Ps[a]	QH_2–cyt *c*[b]	reversion[c]
I	MT0-404	wild type	+	100	
	C133ARG	TGC→CGC	–	0.0	10^{-5}
	C133SER	TGC→AGC	–	0.2	10^{-9}
	H135LEU	CAC→CTC	–	ND	10^{-8}
	H135PRO	CAC→CCC	–	0.1	
	C138ARG	TGC→CGC	–	ND	3×10^{-8}
	C138SER	TGC→AGC	–	ND	6×10^{-6}
	C138PHE	TGC→TTC	–	ND	
II	C153ARG	TGC→CGC	–	ND	2×10^{-8}
	C153SER	TGC→AGC	–	0.0	
	C155ASP	TGC→GAC	–	ND	$<10^{-10}$
	C155GLY	TGC→GGC	–	ND	
	C155SER	TGC→AGC	–	0.0	6×10^{-7}
	H156LEU	CCC→CTC	–	0.2	
	H156PRO	CCC→CCC	–	0.0	3×10^{-6}
	H156PHE	CCC→TTC	–	0.0	$<10^{-10}$
	H156THR	CCC→ACC	–	0.0	$<10^{-10}$
	H156TYR	CCC→TAC	–	0.0	10^{-6}
	H159ALA	CAG→GCG	+	100	
	H159SER	CAG→TCC	+	100	
	MT-RBC1	deleted	–	0.0	

[a] Ps indicates the ability to grow photosynthetically on MPYE plates at 35 °C. [b] Ubiquinol–cyt *c* reductase activities were assayed as described under Materials and Methods and expressed as a percentage of the wild-type overproducer MT0-404. ND, not determined. [c] Approximate reversion frequency of the mutants measured on MPYE by the ratio of Ps^+ to Ps^- colonies observed after plating a culture grown by respiration.

Biochemical Analyses. Intracytoplasmic membranes, as chromatophores, were isolated after French pressure cell treatment according to Atta-Asafo-Adjei and Daldal (1991). Protein concentrations were determined by the method of Lowry et al. (1951). SDS–PAGE and Western blot analysis were performed as described previously using monoclonal (Daldal, 1988) or polyclonal (Davidson et al., 1987) antibodies and horseradish peroxidase conjugated secondary antibodies from Cappel (Malvern, PA) or Bio-Rad (Richmond, CA), respectively. Samples for protein gels were prepared by 2-fold dilution with the loading buffer to a final concentration of 0.1 M dithiothreitol and heated at 37 °C for 10–15 min. Under these conditions, the *R. capsulatus* Rieske protein runs as a single band, and the cytochrome *b* is not aggregated. The enzymatic activities of the bc_1 complexes in membranes were assayed by a DBH oxidation assay, measuring the reduction of horse heart cytochrome *c* as previously described (Atta-Asafo-Adjei & Daldal, 1991).

EPR spectra were recorded using a Varian E-109 X-band spectrometer, and the potentiometric titrations were performed as in Dutton (1978). The Rieske titrations were carried out in the presence of 40 μM aliquots of the redox-mediating dyes 1,2-naphthoquinone, 1,4-naphthoquinone, 1,2-naphthoquinone-4-sulfonate, duroquinone, quinhydrone, indigo disulfonate, indigo trisulfonate, and indigo tetrasulfonate. EPR conditions for each measurement are given in the appropriate figure legend.

RESULTS

The box I (CTHLGC) and box II (CPCHGS) sequences

thought to be involved in liganding the [2Fe-2S] cluster in the carboxyl terminus of the Rieske proteins contain four Cys (133, 138, 153, and 155 in *R. capsulatus*) and two His residues (135 and 156) which are universally conserved (Figure 1). There is also an additional His (159 in *R. capsulatus*, indicated by an arrow in Figure 1) which is adjacent to box II. This residue is replaced by a Gln in *Rhodopseudomonas viridis* (Verbist et al., 1989), in *Bradyrhizobium japonicum* (Thony-Meyer et al., 1989), and in all known cyanobacterial and chloroplast sequences (see Figure 1). We cannot exclude His159 on the basis of the Gln substitution since Gln may have the potential to provide a nitrogen ligand to the [2Fe-2S] cluster (Gatti et al., 1989). We have therefore considered His159 to be a potential ligand.

Our strategy for investigating which four of these seven residues were the ligands of the [2Fe-2S] cluster was based on the rationale that a nonligand residue could be altered by site-directed mutagenesis without impairing the incorporation of a cluster. Thus, a number of site-specific substitutions were obtained at seven distinct positions of the box I and II regions (Figure 1) as described under Materials and Methods, and the corresponding nucleotide and amino acid changes are listed in Table I.

Photosynthesis-Competent Mutants. Among the 19 *R. capsulatus* Rieske mutants obtained (Figure 1), only the H159ALA and H159SER mutants yielded photosynthesis-competent (Ps^+) strains (Table I). These mutants were similar to a wild-type strain overproducing the bc_1 complex, MT0-404, in respect to their photosynthetic and respiratory growth, Q_o inhibitor sensitivity in vivo (data not shown), and DBH–cyt *c* reductase activities (Table I). Since residue 159 is adjacent to the presumed cluster binding site, the effects of these substitutions on the physicochemical properties of the [2Fe-2S] cluster were tested. The H159 mutant strains were found to be very similar to wild type (Figure 2A,B). The characteristic *g* value shifts and broadening of the g_x feature of the Rieske protein in response to the reduction of the membrane Q pool (Matsuura et al., 1983) are unaffected by these substitutions (Figure 2A), suggesting that the Q_o site itself is not perturbed. The E_m of each cluster was also determined by titration of the g_y signals in these mutants at pH 7.0 (Figure 2B). The E_m's of the wild-type overproducer strain MT0-404 and H159SER were approximately +290 and +287 mV, respectively, and that of H159ALA titrated a little higher at approximately +317 mV. We do not consider this last deviation to be meaningful, and conclude that the elimination of H159 has very little effect on the redox midpoint of the [2Fe-2S] cluster of *R. capsulatus* Rieske protein. Therefore, His 159 can be conclusively discounted as a ligand to the [2Fe-2S] cluster despite its high degree of conservation (Figure 1).

The Rieske clusters of several organisms display p*K*'s on their oxidized forms [e.g., see Prince and Dutton (1976)]. In *Rhodobacter sphaeroides*, the E_m of the Rieske cluster is constant in a pH range of approximately 5–8 (Prince et al., 1975), but decreases by 60 mV per pH unit above pH 8 (Prince & Dutton, 1976). The situation is similar in *R. capsulatus*; the E_m of the Rieske cluster is constant in the same pH range (Prince et al., 1975), and is 100 mV lower at pH 9.6 (Robertson et al., 1986). Histidines are good candidates for residues responsible for a p*K* effect; we therefore determined what effect the substitution of His159 would have on the p*K* of the *R. capsulatus* Rieske E_m. We observed that the E_m's of the Rieske clusters in H159ALA and MT0-404 were decreased by almost identical amounts when redox titrations were carried out at pH 9.7 (data not shown), suggesting that

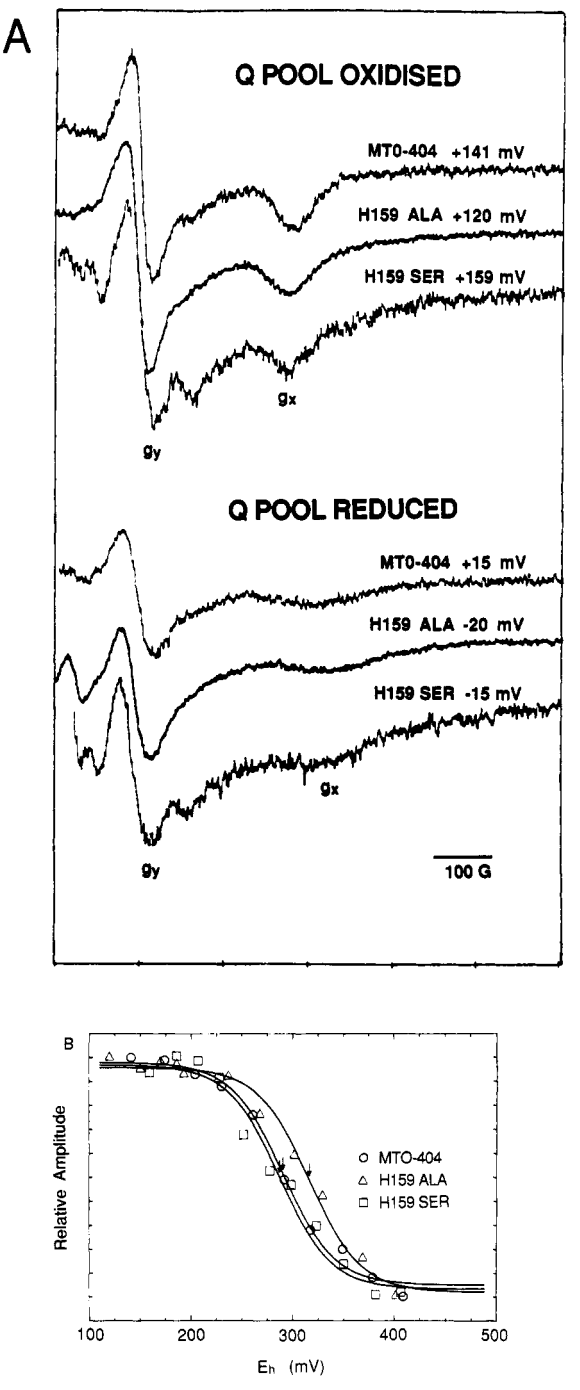

FIGURE 2: (A) EPR spectra showing the effect of quinone (Q) pool oxidation on the line shape of Rieske spectra in wild-type (MT0-404), H159ALA, and H159SER chromatophores. EPR conditions: T = 20 K; microwave power = 1 mW. (B) Redox titration of the g_y signal of wild-type (MT0-404), H159ALA, and H159SER chromatophores. Redox-mediating dyes were as described under Materials and Methods. EPR conditions: T = 20 K; power = 1 mW. The data points are fit to a Nernst curve, n = 1. The E_m's of the curves are indicated by arrows.

His159 is not responsible for the p*K* on the Rieske cluster.

Photosynthesis-Incompetent Mutants. All of the mutants obtained by substitutions in the remaining six positions of the Rieske protein (Figure 1) were photosynthetically incompetent (Ps^-) although most of them could revert at detectable frequencies to Ps^+ (Table I). Their very low DBH–cyt *c* reductase activity indicated that their bc_1 complexes were defective (Table I). Membranes isolated from these mutants were first examined by SDS–PAGE to determine if they contained any Rieske apoprotein; typical examples are shown

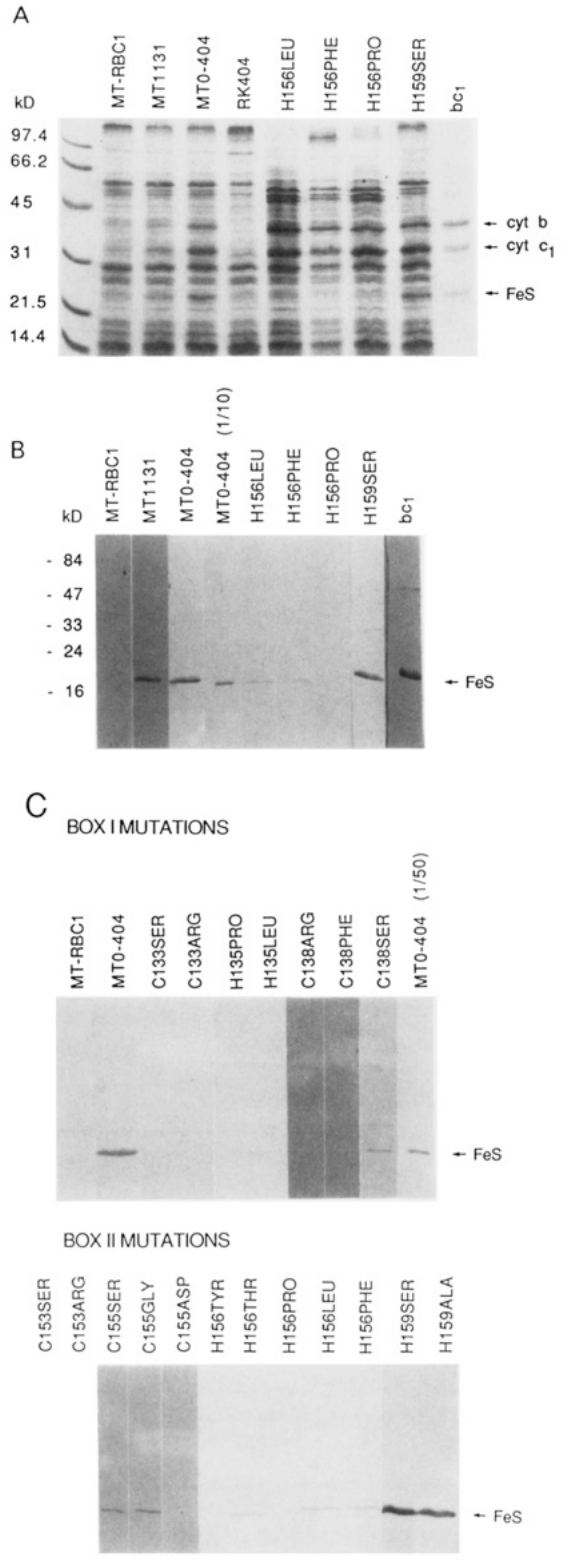

FIGURE 3: (A) SDS–PAGE of chromatophore membranes, stained for protein with Coomassie blue R250; 15% acrylamide; 40 μg of protein per lane. (B) Western blot of chromatophores. Gels similar to that shown in panel A were run, blotted to nitrocellulose, and subsequently probed with polyclonal antibodies raised against the Rieske protein. (C) Western blots, performed as for panel B (except that each lane contained 120 μg of protein), showing the presence or absence of the Rieske protein in various mutants. bc_1 corresponds to purified bc_1 complex; cyt *b*, cyt c_1, and FeS indicate the three subunits of the complex.

in Figure 3A. While the Rieske apoprotein was clearly seen in the wild-type overproducer MT0-404 and in H159ALA and H159SER mutants, it could not be readily detected in the other mutants after Coomassie staining (Figure 3A). We therefore used immunoblotting as a more sensitive detection method. The monoclonal antibody D23, which recognizes the wild-type Rieske apoprotein (Daldal, 1988), did not detect any antigen in the mutants (data not shown), but the results shown in Figure 3B,C were obtained with polyclonal antibodies (Davidson et al., 1987) raised against Rieske protein from purified *R. capsulatus* bc_1 complex. We estimate the amount of the Rieske apoprotein in the mutant strains to be less than 3% of that seen in the wild-type overproducer strain MT0-404 after comparison with a 10-fold-diluted wild-type sample (Figure 3B). The effect of overproduction can be seen in a comparison of MT0-404 with MT1131, a wild-type strain with only a chromosomal copy of the *fbc* operon (Davidson et al., 1987). The Rieske apoprotein was detected only in membranes of C138SER and C138PHE (at very low levels) among the box I mutants, but it was found in at least one substitution at each of the positions altered in the box II mutants (Figure 3C and data not shown). All strains containing the mutated Rieske gene on a plasmid overexpressed the cytochrome *b* and cytochrome c_1 subunits of the bc_1 complex even though they lacked stoichiometric amounts of the Rieske subunit (Figure 3A, for examples, H156LEU, -PHE, and -PRO). The properties of the Rieske-less mutants containing bc_1 subcomplexes and the biochemical properties of the remaining subunits in chromatophores are reported in the following paper (Davidson et al., 1992).

EPR spectroscopy was used to determine if any of the strains C138SER, C153SER, C155ASP, -SER, or -GLY, and H156LEU, -PHE, or -THR (containing low levels of the Rieske apoprotein) also contained a [2Fe-2S] cluster. Chromatophores were reduced with ascorbate and PMS, and the EPR spectra were recorded at a temperature of 15 K at 2-mW microwave power (Figure 4 shows representative spectra). The g_y (1.89) signal is readily observed in ascorbate-reduced samples of MT0-404, but among the mutant strains, it is seen only in C155SER, and at a much lower amount. The small size of the g_y signal observed in this mutant is in agreement with the small amount of Rieske apoprotein detected by immunoblotting (Figure 3C). A sample of MT0-404 membranes diluted to 5% was used to determine the limit of our ability to detect the g_y signal, since the immunoblotting data indicated that the Rieske protein was likely to be present at very low levels (Figure 3B,C). The g_y signal of the 5% control was easily seen under the conditions used (15 K, 2 mW), and it increased with microwave power up to 20 mW although power saturation was evident after 3 mW (data not shown). Since it was possible to maximize the g_y signal detection in the mutant strains by using high microwave power, we feel confident that 1% of the overproduced level of a wild-type Rieske signal could be detected by this means. It should be noted that the shape of the g_y signal in C155SER is affected by another signal, $g = 1.94$, which is present in all Rieske mutants shown in Figure 4. This signal is not related to the Rieske cluster since it is present in the bc_1^- strain MT-RBC1 and is not seen in the diluted wild-type overproducer MT0-404 where its amount is decreased by the $^1/_{20}$th dilution. It possibly reflects the presence of a small amount of the S1 cluster of succinate dehydrogenase ($g_y = 1.94$; Ohnishi, 1987).

Properties of C155SER Substitution. In C155SER, several characteristic features of the Rieske EPR spectrum are altered (Figure 5A). The g_y feature detected in ascorbate-reduced membranes (i.e., Q pool oxidized) has shifted slightly to $g =$ 1.90 from the $g = 1.89$ value of the wild-type strain, but, more strikingly, its g_x band is much shallower than the wild type,

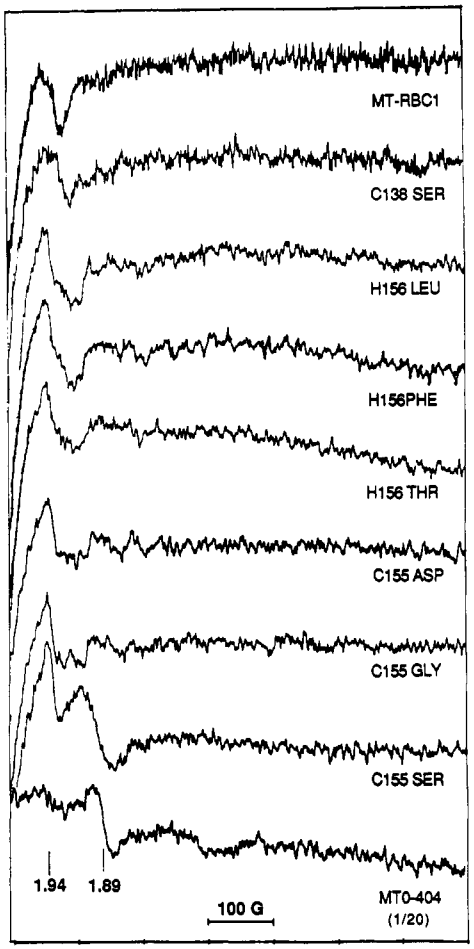

FIGURE 4: EPR spectra of site-directed Rieske mutants of *R. capsulatus*. Samples were reduced with ascorbate + PMS; each spectrum is an average of four scans. EPR conditions: T = 15 K; microwave power = 2 mW.

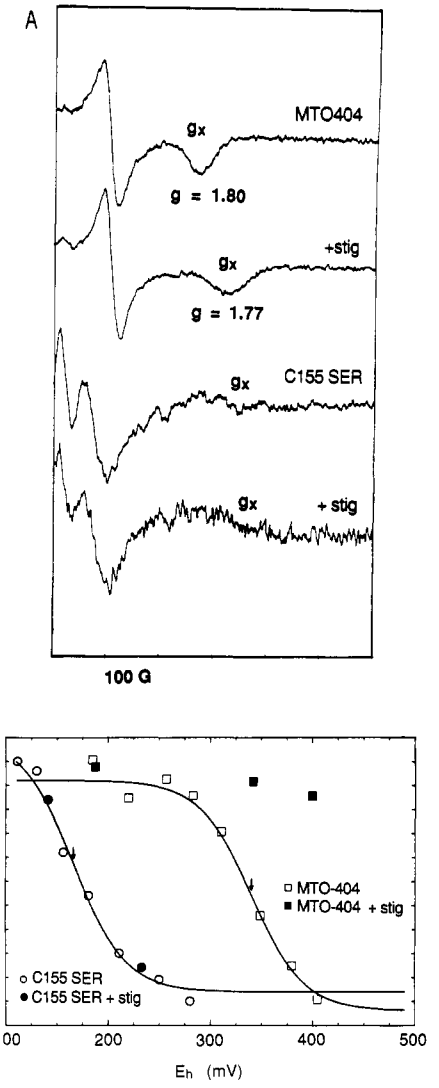

FIGURE 5: (A) EPR spectra of ascorbate-reduced chromatophores from wild-type (MT0-404) and C155SER in the presence ("+stig") or absence of stigmatellin (10 μM). EPR conditions: T = 12 K; power = 5 mW. (B) Redox titration of the g_y signal of wild-type (MT0-404) and C155SER chromatophores. EPR conditions: T = 12 K; power = 5 mW. Dyes were as described under Materials and Methods; the data points are fit to a Nernst curve, n = 1.

and, although difficult to measure with precision, it has a value of around g = 1.77, compared to the g = 1.80 value of the wild-type of Rieske protein. These features are similar to the position and shape of the wild-type g_x band when the Q pool is reduced, suggesting that sensing of the Q-pool redox state is impaired in this mutant. The redox titration of the g_y signal of C155SER shows that the redox midpoint potential of this Rieske protein was lowered by about 180 mV relative to wild type, to approximately 160 mV (Figure 5B).

The Q_o-site inhibitor stigmatellin has distinct effects on the Rieske EPR spectrum (von Jagow & Ohnishi, 1985). In the presence of this inhibitor, the g_x band of the wild-type overproducer MT0-404 is broadened and reduced in g value, from 1.80 to 1.77 (Figure 5A), and the redox midpoint of the cluster is shifted to values above 400 mV (Figure 5B) as has been previously reported (von Jagow & Ohnishi, 1985). Stigmatellin does not appear to alter the EPR spectrum (Figure 5A) and clearly does not shift the redox midpoint potential of the C155SER mutant Rieske protein (Figure 5B), and in this respect, this mutation can be said to cause resistance to stigmatellin. The C155SER mutation therefore affects the stability of the bc_1 complex Rieske subunit, the biophysical characteristics of its [2Fe-2S] cluster, and the integrity of the Q_o site.

Comparison of Amino Acid Sequences of Rieske and Dioxygenase Iron–Sulfur Proteins: A Similar [2Fe-2S] Binding Motif? The dioxygenases typically contain three subunits [see Geary et al. (1984)], featuring an NADH binding flavosubunit, a "ferredoxin-type" cluster-containing subunit, and another subunit containing a mononuclear Fe center as well as a "Rieske-type" cluster with biophysical characteristics similar to those of the Rieske proteins (Geary et al., 1984). The amino acid sequences of the naphthalene (Kurkela et al., 1988), benzene (Irie et al., 1987), and toluene (Zylstra & Gibson, 1989) dioxygenases of *Pseudomonas putida* show strong similarities to the box I and box II regions of the Rieske proteins in that there are two conserved stretches, each featuring one cysteine and one histidine (Figure 6). When these regions of the dioxygenase sequences are aligned with that of the *R. capsulatus* Rieske protein by matching Cys153 and His156 of box II, then Cys133 and His135 of box I coincide with a Cys and His in the dioxygenase sequence, with the spacing between these boxes also being conserved. This alignment reveals that, while Cys138 and Cys155 are not conserved in dioxygenases, additional homologies outside of boxes I and II are also present. These include Gly137, Gly157, Gly164, and Pro170 (in *R. capsulatus* numbering) which are absolutely conserved among all Rieske proteins (Figure 1). Remarkably, Cys133, His135, Cys153, His156, Gly157, Gly164, and Pro170 are also conserved when the Rieske protein is compared to both "Rieske-type" and "ferredoxin-type" [2Fe-2S] subunits of

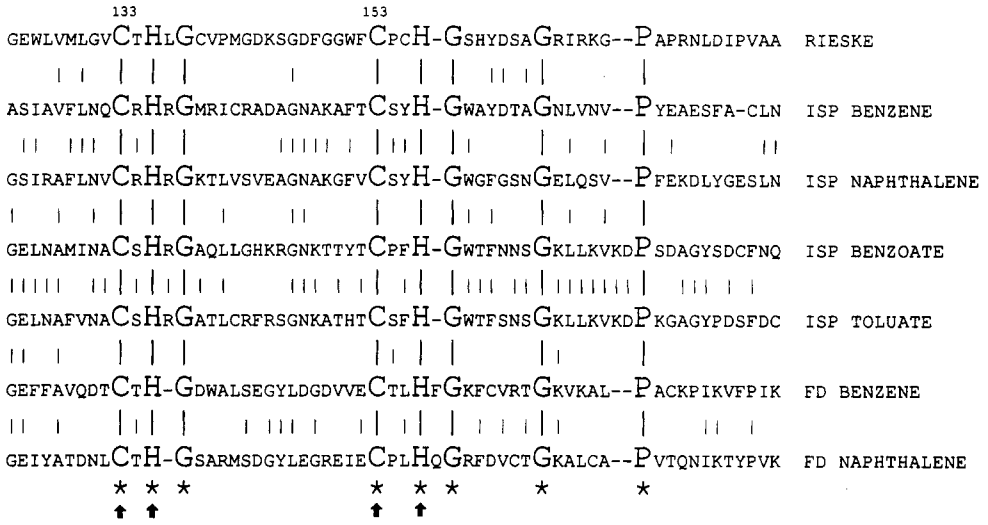

FIGURE 6: Alignment of the *R. capsulatus* putative [2Fe-2S] Rieske cluster binding region with similar proposed binding regions from the "Rieske-type" FeS proteins of benzene (Kurkela et al., 1988), naphthalene (Irie et al., 1987), and toluate dioxygenases from *Pseudomonas putida*, of benzoate dioxygenase from *Acinetobacter calcoaceticus* (Neidle et al., 1991), and of the dioxygenase ferredoxins from *P. putida*. Completely conserved residues are printed in the larger font and indicated by asterisks. Proposed ligands to the [2Fe-2S] clusters are indicated by arrows.

benzene and naphthalene dioxygenases (Figure 6).

DISCUSSION

In this work, site-directed mutagenesis was used in combination with biochemical analyses of the mutants obtained to define the ligands of the [2Fe-2S] cluster of the Rieske protein of the bc_1 complex. The overall data yielded important information with respect to the specific interactions of the [2Fe-2S] cluster with its apoprotein. First, it was found that the H159ALA or -SER substitutions do not greatly affect the cell's ability to make the Rieske protein or the biophysical properties of the resulting [2Fe-2S] cluster, namely, the redox midpoint (Figure 2B), the effect of the redox state of the membrane Q_{pool} on the g_x feature of the EPR signal (Figure 2A), and the pK on the oxidized cluster observed at higher pH. Thus, this residue located immediately downstream from box II (position 159 in *R. capsulatus*) does not provide a nitrogenous ligand to the Rieske [2Fe-2S] cluster; this is consistent with its lack of absolute conservation (Figure 1). If the two nitrogen ligands of the [2Fe-2S] cluster are provided by the His residues located at the carboxyl-terminal end of the Rieske apoprotein (Britt et al., 1991; Gurbiel et al., 1991), these should be the universally conserved His135 and His156 of boxes I and II, respectively. This conclusion is consistent with the results of the mutagenesis of these residues which indicated that their substitutions lead to the loss of the cluster and degradation of the mutant subunits. Several substitutions at position H156 yielded a small but detectable amount of Rieske apoprotein devoid of any [2Fe-2S] cluster (Figures 3 and 4). The immunoblotting data suggest that any cluster present in the membranes of these mutants would have been at an amount comparable to that seen with C155SER (see below), and thus within the limits of our detection capabilities.

Of the four cysteines (C133, -138, -153, and -155) mutagenized, only the C138SER, C153SER, and C155SER mutations yielded small amounts of Rieske apoproteins (Figure 3). Among them, only C155SER contained a [2Fe-2S] cluster (as detected by EPR spectroscopy, Figure 4) but at less than 5% of the amount present in a wild-type overproducer strain. The ability of the plasmid-containing strains to overproduce the bc_1 complex was therefore crucial to detection of this C155SER signal; had the mutation been chromosomal, the cluster would never have been detected. The presence of a [2Fe-2S] cluster in this mutant suggests that C155 is not a ligand to the cluster. This is supported by C155 being located next to a likely nitrogen ligand, H156. Recent mutagenesis studies (cited below) indicate that alternative possibilities cannot be rigorously eliminated. First, C155 may be a ligand in the wild-type protein, but one of the remaining Cys or His residues of box II may substitute poorly for it in C155SER. Such behavior has been observed with the [4Fe-4S] ferredoxin of *Azotobacter vinelandii*, where mutagenesis of a cysteine ligand to alanine did not eliminate the cluster (Martin et al., 1990). X-ray crystallographic analysis of the mutant protein revealed that an adjacent cysteine residue has been promoted to a ligand of the cluster, by twisting the peptide backbone conformation (Martin et al., 1990). Second, the serine residue replacing Cys155 may also be able to function as a ligand (although much less efficiently). In the *E. coli* fumarate reductase, site-directed mutagenesis of three conserved cysteine residues (considered to be ligands to a [2Fe-2S] cluster) to serines still produced clusters with altered EPR spectra and redox midpoint potentials (Werth et al., 1990).

The mutagenesis experiments have unequivocally eliminated H159 from consideration as a ligand, and suggested that C155 is unlikely to be involved. If boxes I and II were to provide one cysteine and one histidine each as ligands to the [2Fe-2S] cluster, then C153 is the most likely candidate from box II. However, these experiments could not define which of the two cysteines of box I is the ligand since C133 mutants had no apoprotein and C138 mutations did not allow reliable detection of a Rieske-related EPR signal. To identify this remaining ligand, the amino acid sequence of *R. capsulatus* Rieske protein was compared to those of the bacterial dioxygenase Fe-S proteins, frequently referred to as "Rieske-type" proteins. The EPR spectra of the Rieske and "Rieske-type" clusters are similar, with an average g value of 1.91, compared to 1.96 for typical ferredoxin-type clusters, featuring a more anisotropic EPR spectrum ($g_{x,y,z}$ = 1.80, 1.90, and 2.02, respectively). If the [2Fe-2S] clusters of the Rieske proteins and the "Rieske-like" iron–sulfur proteins of dioxygenases use a similar (two Cys/two His) liganding pattern, then the nonconserved Cys138 and Cys155 (in *R. capsulatus* protein, Figure 6) are not involved in binding the cluster. Additional "Rieske-type"

sequences (for benzoate and toluate dioxygenase iron–sulfur proteins from *Acinetobacter calcoaceticus* and *Pseudomonas putida*, respectively) have been reported very recently (Neidle et al., 1991). These enzymes are related to the aromatic ring-hydroxylating dioxygenases although they differ in some ways; for example, their ferredoxin proteins are of the "classical" [2Fe-2S] type, with four Cys ligands. The deduced amino acid sequences also show the presence of a subunit containing the conserved pattern of His and Cys residues found in the Rieske-type iron–sulfur proteins of the aromatic dioxygenases and Rieske proteins (Neidle et al., 1991). The above data favor the C133, H135, C153, and H156 residues as the four ligands of the [2Fe-2S] cluster.

A similar site-directed mutagenesis study of the equivalent residues in the yeast Rieske protein showed that the [2Fe-2S] cluster was lost after changing the totally conserved Cys and His residues to Ser and Arg, respectively (Graham & Trumpower, 1991) but this study did not provide any information on the identity of the ligands. This group, on the basis of their earlier analysis of temperature-sensitive mutations of the Rieske protein, has proposed a liganding pattern similar to that described here (Beckman et al., 1987) with the exception that, due to different effects of two mutations proximal to both box I and box II, they suggest C138 (*R. capsulatus* numbering) as the box I Cys ligand; this is in contrast to C133 proposed here on the basis of homology with the dioxygenases.

Both the Rieske and dioxygenase "Rieske-type" clusters have relatively high E_m's in comparison to the well-studied [2Fe-2S] ferredoxins (Fee et al., 1986). Rieske E_m's are typically from +150 to +300 mV, dioxygenase "Rieske-type" cluster E_m's range between −155 and 0 mV, and those of ferredoxins typically range from −300 to −460 mV. This points out that factors other than the nature of the ligands [see Kassner and Yang (1977)] need to be invoked to account for the differences in the redox midpoint potentials of the dioxygenase "ferredoxin-like" cluster (E_m = −150 mV; Geary et al., 1984), the dioxygenase "Rieske-like" cluster (E_m = −150 to 0 mV; Geary et al., 1984), and the bc_1 complex Rieske cluster (E_m = +300 mV), all of which are [2Fe-2S] clusters.

The reversion frequencies of some of the mutants obtained in this work (Table I) have interesting implications for alternate liganding of the [2Fe-2S] cluster of the Rieske protein. Both the C155ASP and H156LEU mutations involved two base-pair changes from the corresponding wild-type codons, and do not yield spontaneous Ps^+ revertants even though the C155ASP mutation could theoretically revert to His (GAC to CAC) and H156PHE to Cys (TTC to TGC) by single base-pair changes. This implies, but does not demonstrate, that the exchange of a cysteine ligand by an histidine at these positions would not result in a functional Rieske protein.

The Rieske Cluster of C155SER. The biophysical characteristics of the Rieske cluster in a protein with a C155SER mutation are different from the normal Rieske protein. The g_x feature, in particular, is at a lower g value (approximately 1.77 vs 1.80 at +150 mV) and is much shallower (Figure 5). This type of shallow, and low g value, g_x feature has previously been observed with some cytochrome b mutations [e.g., F144L and G158D (Daldal et al., 1989)] which resulted in low rates of QH_2 oxidation at the Q_o site (Robertson et al., 1990). The Q_o site in these mutants is thought to have a lower affinity for quinones due to the effects of the mutations on cytochrome b. This type of g_x signal is also seen in wild-type membranes from which most of the quinones had been extracted (Ding et al., 1992) and is thought to be indicative of an unoccupied, or "empty", Q_o site. The yeast mutation G133ASP (137 in *R. capsulatus*) in box I also resulted in a lowered midpoint potential and a flat, "empty", g_x signal (Gatti et al., 1989). These observations suggest that boxes I and II of the Rieske protein are involved in sensing the redox state of the Q pool and thus may be part of the Q_o site. A possible interpretation of the Rieske C155SER mutation is that it "opens up" the Rieske protein, making it less stable and rendering the cluster more accessible to solvent, thus reducing the redox midpoint potential and modifying the Q_o site.

Is There a Disulfide Bond in the Rieske Protein? A striking aspect of our results is that any mutation affecting the universally conserved potential ligands at positions C133, -138, -153, and -155 and H135 and -156 (Figure 1) of the Rieske protein of *R. capsulatus* leads to a drastic decrease of the in vivo steady-state levels of the Rieske subunit in chromatophore membranes. Site-directed mutagenesis has recently been used to alter putative ligands of the iron–sulfur clusters in several other enzymes: the *E. coli* fumarate reductase (Werth et al., 1990) and dimethyl sulfoxide (DMSO) reductase (Rothery & Weiner, 1991); the *Bacillus subtilis* glutamine-phosphoribosylpyrophosphate amidotransferase (Makaroff et al., 1986); and the *Azotobacter* [4Fe-4S] ferredoxin (Martin et al., 1990). These proteins have generally been more resilient to mutations than the Rieske protein. The contrasting situation observed here with the Rieske protein clearly shows that while some of these six universally conserved residues are involved in liganding the [2Fe-2S], they are all also extremely important for maintaining the structure of the Rieske protein. Our predicted nonligands, C138 and C155, which are not conserved in the dioxygenases, are clearly important in this respect. One possible reason for the universal conservation of these residues in Rieske proteins is that they participate in the formation of a disulfide linkage. The possibility of a disulfide bond involving the Rieske protein has previously been suggested for the spinach b_6f complex (El-Demerdash et al., 1988). If there is a disulfide linkage between C138 and C155, the phenotype of the C155SER mutation (which would eliminate such a disulfide bond) would illustrate that the bond is necessary for maintenance of the Q_o site. A disulfide bond is unusual in proteins containing Fe-S clusters. Only ferredoxin II (a single-cluster ferredoxin) from *Desulfovibrio gigas* is known to contain a disulfide bond (Kissinger et al., 1989). It occurs in the region of the protein where, in ferredoxins containing two clusters, the second Fe-S cluster was originally bound (Fukuyama et al., 1988). Since the single-cluster ferrodoxins are thought to have arisen by the loss of a cluster from the double-cluster-containing forms (Fukuyama et al., 1988; Beinert, 1990), it is possible that the disulfide bond found in *D. gigas* ferredoxin II is not required for the stability of this protein.

Model of Rieske Structure. The work of Gurbiel et al. (1989, 1991) and Britt et al. (1991) has indicated that the histidines are ligated to the same Fe atom of the cluster. It is also known that the Fe–Fe axis is in the plane of the membrane (Salerno et al., 1979; Prince, 1983). These data, together with the liganding roles attributed to the specific residues, indicate the box I and box II regions of the Rieske protein must be in the membrane plane and in a parallel orientation for the anticipated cysteines and histidines to ligate their respective Fe atoms in the cluster. Therefore, a simple loop between boxes I and II will not suffice, and the intervening region between the conserved boxes must twist back upon itself to bring them parallel to each other, as shown in Figure 7. Although this model is consistent with the available data, it should be emphasized that other possibilities cannot yet be

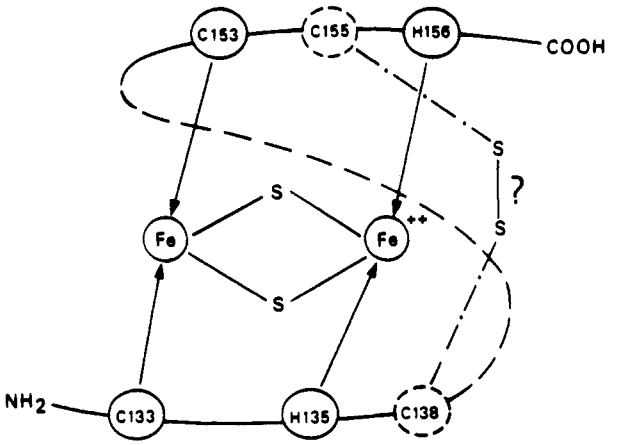

FIGURE 7: Model for the [2Fe-2S] cluster binding region of the *R. capsulatus* Rieske protein. The proposed ligands are circled, and S–S represents the putative disulfide bridge between the conserved, non-liganding residues. NH_2 and COOH represent the amino and carboxyl ends of the protein.

completely excluded, and therefore should not be considered definitive until the resolution of the structure of the Rieske protein.

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Rhodobacter capsulatus Mutants Lacking the Rieske FeS Protein Form a Stable Cytochrome bc_1 Subcomplex with an Intact Quinone Reduction Site†

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ABSTRACT: The ubiquinol–cytochrome *c* oxidoreductase (or bc_1 complex) of *Rhodobacter capsulatus* consists of three subunits: cytochrome *b*, cytochrome c_1, and the Rieske iron–sulfur protein, encoded by the *fbcF*, *fbcB*, and *fbcC* genes, respectively. In the preceding paper [Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E., & Daldal, F. (1992) *Biochemistry* (preceding paper in this issue)], we have observed that the apoproteins for cytochromes *b* and c_1 are fully present in the intracytoplasmic membrane of *R. capsulatus* mutants containing low amounts of, or no, Rieske apoprotein. Here we present evidence that the redox midpoint potentials of cytochromes *b* and c_1, as well as their ability to bind antimycin and stabilize a semiquinone at the Q_i site, are unaffected by the absence of the Rieske subunit. This is the first report describing a mutant containing a stable bc_1 subcomplex with an intact Q_i site in the chromatophore membranes, and provides further evidence that a functional quinone reduction site can be formed in the absence of a quinol oxidation (Q_o) site. Additional mutants carrying *fbc* deletions expressing the remaining subunits of the cytochrome bc_1 complex were constructed to investigate the relationship among these subunits for their stability in vivo. Western blot analysis of these mutants indicated that cytochromes *b* and c_1 protect each other against degradation, suggesting that they form a two-protein subcomplex in the absence of the Rieske protein subunit.

The ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex)[1] of the photosynthetic bacterium *Rhodobacter capsulatus* is an important electron-transfer component involved in both photosynthetic and respiratory growth (Dutton, 1986; Cramer & Knaff, 1990). It is located in the intracytoplasmic membrane and consists of three protein subunits: cytochrome *b*, cytochrome c_1, amd the Rieske iron–sulfur protein, containing characteristic redox-active prosthetic groups [e.g., see Trumpower (1990)]. The primary structure of these subunits has been established through the cloning and sequencing of the *fbc* (*pet*) operon which encodes all three proteins (Gabellini

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[1] Abbreviations: bp, base pair(s); bc_1 complex, ubiquinol–cytochrome *c* oxidoreductase; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; cyt, cytochrome; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; E_m, redox midpoint potential; EPR, electron paramagnetic resonance; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, phenazine methosulfate; Q_o, quinol oxidation site; Q_i, quinone reduction site; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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