Conserved Nonliganding Residues of the *Rhodobacter capsulatus* Rieske Iron-Sulfur Protein of the bc_1 Complex Are Essential for Protein Structure, Properties of the [2Fe-2S] Cluster, and Communication with the Quinone Pool†

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ABSTRACT: The iron-sulfur (Fe-S) protein subunit of the bc_1 complex, known as the Rieske protein, contains a high-potential [2Fe-2S] cluster ligated by two nitrogen and two sulfur atoms to its apoprotein. Earlier work indicated that in *Rhodobacter capsulatus* these atoms are provided by two cysteine (C133 and C153) and two histidine (H135 and H156) residues, located at the carboxyl-terminal end of the protein [Davidson, E., Ohnishi, T., Atta-Asafo-Adjei, E., & Daldal, F. (1992) *Biochemistry 31*, 3342−3351]. These ligands are part of the conserved sequences C_{133}THLGC_{138} (box I) and C_{153}PCHGS_{158} (box II) and affect the properties of the Fe-S protein and its [2Fe-2S] cluster. In this work, the role of amino acid side chains at positions 134 and 136, adjacent to the cluster ligands in box I, was probed by using site-directed mutagenesis and biophysical analyses. These positions were substituted with R, D, H, and G to probe the effect of charged, polar, large, and small amino acid side chains on the properties of the [2Fe-2S] cluster. Of the mutants obtained T134R, -H, and -G were photosynthetically competent (Ps^+) but contained Fe-S proteins with redox midpoint potentials (E_{m7}) 50−100 mV lower than that of a wild type strain. In contrast, T134D was Ps^- and contained no detectable [2Fe-2S] cluster, although it reverted frequently to Ps^+ by substitution of D with N. On the other hand, all L136 mutants were Ps^-, the EPR characteristics of their [2Fe-2S] cluster were perturbed, and they were unable to sense the Q_{pool} redox state or to bind stigmatellin properly. The overall data indicated that replacement of the amino acid side chain at position 134 of the Fe-S protein affects mainly the E_{m7} and oxygen sensitivity of the [2Fe-2S] cluster without abolishing its function, while substitutions at position 136 perturb drastically its ability to monitor the Q_{pool} redox state and its interaction with the Q_o site inhibitor stigmatellin. These two distinct phenotypes of box I T134 and L136 mutants are discussed with regard to the recently published three-dimensional structure of the water soluble part of the bovine heart mitochondrial Rieske Fe-S protein.

Ubiquinone:cytochrome (cyt)[1] *c* oxidoreductases (bc_1 complexes) are membrane-spanning energy-transducing enzymes that catalyze the two-electron oxidation of ubihydroquinone (UQH_2) and the one-electron reduction of cyt *c*. They are key components of respiratory electron transport chains in mitochondria and aerobic prokaryotes, and their functional equivalents, the cyt b_6f complexes, play similar central roles during photosynthetic energy transduction in chloroplasts [reviewed in Prince (1990), Knaff (1993), Trumpower and Gennis, (1994), Cramer et al. (1994), and Gray and Daldal (1995)]. In facultative phototrophic bacteria, the bc_1 complex is involved in both light-driven electron transfer and dark respiration but is essential only for photosynthetic (Ps) growth (Daldal et al., 1987; Gennis et al., 1993).

The subunit composition of bc_1 complexes varies with the organisms, and its simplest version is encountered in Gram-negative bacteria such as the *Rhodobacter* species (Gabellini & Sebald, 1986; Daldal et al., 1987; Davidson & Daldal, 1987a,b). It contains a cyt *b* subunit with two noncovalently bound protohemes (Meinhardt & Crofts, 1983; Robertson & Dutton, 1988; Robertson et al., 1993), a cyt c_1 with a covalently bound *c* type heme, an iron-sulfur (Fe-S) protein with a [2Fe-2S] cluster (commonly called the Rieske cluster; Rieske et al., 1964), and two UQ and UQH_2 processing domains (Robertson & Dutton, 1988; Salerno et al., 1990;

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[1] Abbreviations: bp, base pair; cyt, cytochrome; bc_1 complex, ubihydroquinone:cyt *c* oxidoreductase; cyt b_H, high-potential cyt *b* of the bc_1 complex; cyt b_L, low-potential cyt *b* of the bc_1 complex; DAB (diaminobenzidine), 3,3′,4,4′-tetraaminobiphenyl; DBH_2, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohydroquinone; EDTA, ethylenediaminetetraacetic acid; E_h, ambient redox potential; E_{m7}, equilibrium redox midpoint potential at pH 7.0; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; ESEEM, electron spin echo envelope modulation; Fe-S, iron-sulfur protein; MK, menaquinone; MKH_2, menahydroquinone; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Myx, myxothiazol; Sti, stigmatellin; PES, *N*-ethyldibenzopyrazine ethosulfate; PMS, *N*-methyldibenzopyrazine methosulfate; PMSF, phenylmethanesulfonyl fluoride; Ps, photosynthetic growth; Q_i, ubiquinone reduction site; Q_o, ubihydroquinone oxidation site; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tween 20, polyoxyethylenesorbitan monolaurate; UQ, ubiquinone; Q_{pool}, ubiquinone pool; UQH_2, ubihydroquinone; wt, wild type.

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Meinhardt & Ohnishi, 1991). In the bc_1 complex, UQH_2 oxidation is initiated by ferricyt c_2 and mediated by cyt c_1 and the [2Fe-2S] cluster, driving transmembrane electrical charge separation through two *b* hemes of cyt *b* according to the Q cycle mechanism (Mitchell, 1975).

The Fe-S protein subunit of the bc_1 complex is thought to be anchored to the membrane by a hydrophobic amino-terminal helix (Harnisch et al., 1986; Van Doren et al., 1993a; Wu & Niederman, 1995). Its carboxyl-terminal domain faces the periplasm and bears a [2Fe-2S] cluster (Li et al., 1981; Gonzalez-Halphen et al., 1988; Cocco et al., 1991), which is close to the polar head groups of the phospholipid bilayer (Ohnishi et al., 1989). Biophysical analyses have predicted a coordination of the [2Fe-2S] cluster by two nitrogen and two sulfur ligands (Gurbiel et al., 1991; Britt et al., 1991; Shergill & Cammack, 1994). The C-terminal region of the Fe-S protein contains two highly conserved hexapeptides, box I and box II, which in *Rhodobacter capsulatus* comprise the amino acid residues C_{133}THLGC$_{138}$ and C_{153}PCHGS$_{158}$, respectively (Figure 1). Site-directed mutagenesis of the H135, H156, C133, and C153 residues (*R. capsulatus* numbering) yielded nonfunctional bc_1 complexes with undetectable Fe-S apoprotein and no [2Fe-2S] cluster and suggested them as ligands to the [2Fe-2S] cluster (Davidson et al., 1992a; Van Doren et al., 1993a). In contrast, mutagenesis of the remaining cysteine (C138 and C155) residues of these boxes yielded mutants which contained very small amounts of the Rieske Fe-S protein with the perturbed [2Fe-2S] cluster (Davidson et al., 1992a; Ohnishi et al., 1994). These findings, in combination with the lack of sequence conservation in the Rieske-type [2Fe-2S] cluster containing bacterial dioxygenases (Mason & Cammack, 1992), have excluded them from being cluster ligands (Davidson et al., 1992a) (Figure 1). Considering that their substitution also greatly decreased the Fe-S protein, Davidson et al. (1992a) suggested that these residues form a disulfide bridge essential for the structure of this subunit, and for its assembly into the bc_1 complex.

The E_{m7} values of the Rieske [2Fe-2S] clusters are high [i.e., about 310 mV in *R. capsulatus*, and 100−165 mV in menahydroquinone (MKH_2)-oxidizing bc_1 complexes (Liebl et al., 1992)]. While the "Rieske-type" [2Fe-2S] clusters of bacterial dioxygenases (Mason & Cammack, 1992) show EPR features virtually identical to those of the [2Fe-2S] clusters of the bc_1 complexes, their E_{m7} values range from −155 to 0 mV (Geary et al., 1984; Rosche et al., 1995). Since their sequence comparison suggests a coordination similar to that of the Fe-S proteins of bc_1 complexes, the differences in the E_{m7} of the [2Fe-2S] clusters among these various Fe-S proteins cannot be attributed solely to different cluster coordinations (sulfur and nitrogen coordination in both the Rieske and Rieske-type proteins versus a sulfur only coordination in ferredoxins). Additional features provided by the protein environment, such as electrostatic effects of the surrounding amino acid residues (Gunner & Honig, 1991) or their involvement in a hydrogen bond network around the redox center, have been suggested as the basis for these E_{m7} differences (Backes et al., 1991; Davidson et al., 1992a; Riedel et al., 1995).

Recently, the structure of a water soluble fragment of the Fe-S protein from the bovine heart mitochondrial bc_1 complex containing an intact [2Fe-2S] cluster has been determined by X-ray diffraction analysis at a resolution of

FIGURE 1: Sequence alignment of the [2Fe-2S] cluster binding regions of various Rieske Fe-S proteins in bc_1 complexes and of Rieske-type Fe-S proteins of bacterial dioxygenases. Conserved residues are indicated in bold and labeled box I and box II. The *R. capsulatus* box I and II residues are shown underneath the alignment; those providing the [2Fe-2S] cluster ligands are circled, and residues T134 and L136 and their corresponding substitutions are indicated. The numbering corresponds to the *R. capsulatus* Fe-S protein, and UQ, MK, and PQ indicate ubi-, mena- and plastoquinone, respectively. The Fe-S proteins are from *R. capsulatus* (R.c.) (Davidson & Daldal, 1987a), *R. sphaeroides* (R.s.) (Yun et al., 1990), *Bradyrhizobium japonicum* (B.j.) (Thöny-Meyer et al., 1989), *Paracoccus denitrificans* (P.d.) (Kurowski & Ludwig, 1987), *Chlorobium limicola* (C.l.) (Schütz et al., 1994) *Bacillus subtilis* (B.s.) (Yu et al., 1995), *Bacillus stearothermophilus* (B.st.) (Sone, 1996), bovine (bo.) (Schägger et al., 1987), *S. cerevisiae* (S.c.) (Beckman et al., 1987), spinach (sp.) (Steppuhn et al., 1987), benzene 1,2-dioxygenase from *Pseudomonas putida* (ben.) (Kurkela et al., 1988), and 2-halobenzoate 1,2-dioxygenase from *Pseudomonas cepacia* 2CBS (halo.) (Haak et al., 1995).

1.5 Å (Iwata et al., 1996; Link & Iwata, 1996). This structure confirmed the findings previously inferred from mutagenesis data with regard to the nature of [2Fe-2S] cluster ligands and demonstrated the presence of the proposed disulfide bridge (Davidson et al., 1992a; Iwata et al., 1996). A wealth of detailed information about the spatial conformation of the conserved residues in the box I and box II wrapping around the [2Fe-2S] cluster is now available. Yet, little is known about their influence on the properties of the [2Fe-2S] cluster and their interactions with UQ/UQH_2 at the Q_o site. In earlier studies several respiratory-deficient mutants, such as G133D, P136L, and P149L in the *Saccharomyces cerevisiae* Fe-S protein, have been shown to exhibit diminished electron transfer activities, decreased E_{m7} values, and partial losses of the [2Fe-2S] cluster and the apoprotein (Gatti et al., 1989; Beckman et al., 1989). These findings suggested that positions 133, 136, and 149 (corresponding to 137, 140, and 154 in *R. capsulatus*) might be important for the conformation of the peptide loop around the [2Fe-2S] cluster and for its stability. Of these mutations, the G133D (133 in *S. cerevisiae* and 137 in *R. capsulatus*) substitution has also been obtained in the *Rhodobacter sphaeroides* Fe-S protein

and yielded a poorly functional bc_1 complex with a decreased Q_o site turnover rate (Van Doren et al., 1993a). Recently, additional temperature sensitive respiration-deficient mutants of the *S. cerevisiae* Fe-S protein (G127S, T130A, V135A, P136S, and G152D and -S, corresponding to positions 131, 134, 139, 140, and 157, respectively, in *R. capsulatus*) have been described (Graham et al., 1993), but not yet studied [note that the position numbers in Gatti et al., (1989) refer to the mature Fe-S protein while those in Beckman et al. (1989) and Graham et al. (1993) refer to the premature form of the same protein].

In the present work, the conserved amino acid residues T134 and L136, located immediately adjacent to the cluster ligands C133 and H135, were studied in order to define their influence on the properties of the [2Fe-2S] cluster and on the Q_o site. These positions were substituted with R, D, H, and G using site-directed mutagenesis, and the properties of the mutants obtained were examined in detail. The overall data indicate that mutations at position 134 of the Fe-S protein affect predominantly the E_{m7} value and oxygen sensitivity of the [2Fe-2S] cluster, while those at position 136, in addition, impair UQH_2 oxidation at the Q_o site of the bc_1 complex.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Growth of *Escherichia coli* strains in LB medium, supplemented with appropriate antibiotics, has been described (Daldal et al., 1989). Wild type and mutant *R. capsulatus* strains were grown in enriched MPYE, minimal Medium A (Sistrom, 1960), or "mixed medium" (50% MPYE plus 50% Medium A) either chemoheterotrophically (Res) at 35 °C in the dark at 150 rpm on a rotary shaker (New Brunswick) or photoheterotrophically (Ps) under anaerobic conditions at 35 °C in continuous light. The *R. capsulatus* strain pMTS1/MT-RBC1 has a chromosomal deletion−insertion of the *fbc* operon replaced by a spectinomycin resistance (Spe^R) cassette (Atta-Asafo-Adjei & Daldal, 1991) complemented *in trans* with the plasmid pMTS1 containing a kanamycin resistance (Kan^R) gene and a wild type copy of the *fbc* operon. It overproduces the bc_1 complex by about 5−8-fold under respiratory and about 4-fold under phototrophic growth conditions.

Genetic Methods. Site-directed mutagenesis was performed as described (Atta-Asafo-Adjei & Daldal, 1991), using the uracilated phage M13-73R2BC1, which contains the appropriate fragment of the *fbc* operon as a template and mutagenic oligonucleotides *fbc*F-T134 (5′-GCCGAGGTGG-(C/T)(G/C)GCACACGC-3′) and *fbc*F-L136 (5′-CACG-CAGCCG(C/T)(C/G)GTGGGTGC-3′) as primers. After mutagenesis and sequencing of the single-stranded phage DNA, the approximately 530 bp *Bst*XI-*Eco*RI fragment carrying the desired mutation in *fbcF* (*petA*) encoding the Fe-S protein was cloned from the replicative form of phage M13-73R2BC1 into the conjugation-proficient plasmid pMTS1, yielding pF:T134R, D-, -H, and -G and pF:L136R, -D, -H, and -G. These plasmids were introduced into *R. capsulatus* strain MT-RBC1 by selecting for Kan^R transconjugants, and the mutations were reconfirmed by sequencing of the DNA isolated from them. Spontaneous Ps^+ revertants of pF:T134D/MT-RBC1 were isolated on MPYE plates containing kanamycin. They were purified three times under Ps growth conditions, and their plasmid content was isolated and the DNA region between nucleotides 1040 and 1160 covering most of *fbcF* (Davidson & Daldal, 1987a) sequenced to determine the location and molecular nature of the reversion mutation.

Biochemical Techniques. Cells were suspended in 50 mM MOPS (pH 7.0) with 100 mM KCl and 1 mM EDTA and ruptured via two passages in a French pressure cell at 18 000 psi in the presence of 1 mM PMSF. For mutants with an unstable [2Fe-2S] cluster, only one passage was performed in the presence of 20 mM sodium ascorbate (pH 7.0) and 20% glycerol. Intracytoplasmic (chromatophore) membranes were isolated by differential centrifugation and washed twice as described previously (Atta-Asafo-Adjei & Daldal, 1991). Protein concentrations were determined by the method of Lowry et al. (1951); SDS−PAGE gels and samples for protein gels were prepared as described in Davidson et al. (1992a). Western blotting and immunodetection were performed using rabbit polyclonal antibodies raised against the *R. capsulatus* Fe-S protein in the presence of Tween 20 (Sigma, St. Louis, MO) as a blocking agent (Batteiger et al., 1982). Horseradish peroxidase-conjugated anti-rabbit secondary antibodies from BioRad (Richmond, CA) and metal ion-enhanced diaminobenzidine (DAB) staining was used to detect the immune complexes (Hsu & Sobane, 1982). The enzymatic activity of the bc_1 complex was assayed by measuring DBH_2-dependent reduction of horse heart cyt *c* as described in Atta-Asafo-Adjei and Daldal (1991).

EPR Spectra and Redox Titrations. EPR spectra were recorded using a Bruker ESP-300E spectrometer equipped with an Oxford Instruments ESR-9 helium cryostat. Redox titrations of the [2Fe-2S] cluster were performed anaerobically in near darkness as described by Dutton (1978). Chromatophore membranes were resuspended in MOPS/KCl buffer in the presence of the following redox mediators: indigo tetrasulfonate, duroquinone, *N*-methyldibenzopyrazine methosulfate (PMS), 1,2-naphthoquinone 4-sulfonate, tetrachlorohydroquinone, and *N*, *N*, *N*′, *N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (each at 50 μM), 40 μM 1,2-naphthoquinone, and 10 μM pyocyanine. Aliquots were drawn at appropriate redox potentials between +450 and −40 mV, frozen immediately, and stored in liquid nitrogen until the EPR spectra were recorded. Stigmatellin and myxothiazol were added from DMSO stocks to final concentrations of 20 and 50 μM, respectively. EPR conditions were as follows: temperature, 20 K; microwave power, 2 mW; modulation amplitude, 12.5 G; modulation frequency, 100 kHz; and microwave frequency, 9.45 GHz.

Molecular Modeling was carried out at the Molecular Modeling Facilities of the Cancer Center of the University of Pennsylvania. The coordinates for the bovine Rieske protein (PDB accession number 1RIE) were kindly provided by T. Link (University of Frankfurt, Germany) prior to publication. Molecular modeling and minimizations were performed using Indy and Power Challenge Silicon Graphics stations, respectively. Insight II with a Biopolymer module was used for the addition of the [2Fe-2S] cluster and amino acid replacements; Discover was used for structure minimization with 500 steepest descent iterations and between 1000 and 7000 iterations of the conjugate descent algorithm, until convergence (derivative of <0.001) was reached. The structure of the Fe-S protein without the water molecules was first minimized after the addition of the [2Fe-2S] cluster

Table 1: Characteristics of *R. capsulatus* Rieske Fe-S Protein Mutants

strain	mutation	pheno-type[a]	doubling time (min)[a]	bc_1 complex activity[b] (%)	reversion frequency[c]
pMTS1	ACC (wt)	Ps^+	170	100	na[d]
pF:T134R	ACC → CGC	Ps^+	170	24	na
pF:T134H	ACC → CAC	Ps^+	240	10	na
pF:T134G	ACC → GGC	Ps^+	320	10	na
pF:T134D	ACC → GAC	Ps^-	na	2	1×10^{-7}
pF:T134N	GAC → AAC	Ps^+	185	16	na
pF:L136R	CTC → CGC	Ps^-	na	5	1×10^{-7}
pF:L136H	CTC → CAC	Ps^-	na	4	7×10^{-6}
pF:L136G	CTC → GGC	Ps^-	na	4	1×10^{-5}
pF:L136D	CTC → GAC	Ps^-	na	2	6×10^{-8}
MT-RBC1	Δ(*fbcFBC*)	Ps^-	na	<1	na

[a] Ps^+ and Ps^- indicate photosynthetic competence or incompetence, respectively. Doubling times are for photosynthetic growth in MPYE medium. [b] bc_1 complex activity was determined by measuring the DBH_2-dependent cyt *c* reductase activity (100% corresponds to approximately 5100 nmol of horse heart cyt *c* reduced per minute per milligram of chromatophore membranes, using an ϵ_{550} of 20 mM^{-1} cm^{-1} for cyt *c*). It is expressed as a percentage of the activity found in pMTS1/MT-RBC1 overproducing the wild type bc_1 complex. [c] Reversion frequency as measured by the ratio of Ps^+ to Ps^- colonies on MPYE plates. [d] na, not applicable.

and then subjected to amino acid replacement at position 134 and reminimized. The structures thus generated were compared to each other by superimposition of their backbones.

Chemicals. All chemicals were obtained as described previously (Gray et al., 1994).

RESULTS

Rationale and Choice of Substitutions. Broad variation of the E_{m7} values is known among the [2Fe-2S] clusters of Rieske proteins (100−300 mV in bc_1 complexes) and of Rieske-type proteins (as low as −150 mV in bacterial dioxygenases). However, since their clusters have identical ligands, namely two cysteines and two histidines, additional features are suggested to modulate their physicochemical properties. This notion led us to examine the role of the polar T134 and hydrophobic L136 amino acid residues located adjacent to the cluster ligands C133 and H135 (Figure 1). Using site-directed mutagenesis, various substitutions were constructed to analyze the effect of charge [positive (R) and negative (D and H)] and size (G) of the amino acid side chain on the properties of the Rieske Fe-S protein and its [2Fe-2S] cluster.

Phenotypic Characterization of the T134 and L136 Substitutions. Considering the fact that the Ps growth of *R. capsulatus* requires an active bc_1 complex (Daldal et al., 1987), the mutants obtained by substitution of positions 134 and 136 were first tested for their ability to support Ps growth (Table 1). The T134R, -H, and -G substitutions were Ps^+ and sensitive to the Q_o site inhibitors myxothiazol and stigmatellin (cell growth was completely inhibited on MPYE plates containing these inhibitors at 5×10^{-6} M), as it had been observed for the wild type strain pMTS1/MT-RBC1 overproducing the bc_1 complex. The doubling time of T134R was similar to that of a wild type strain, while those of T134H and T134G were 1.5 and 2 times shorter, respectively (Table 1). On the other hand, T134D was Ps^-, although it reverted to a Ps^+ phenotype readily (see below). The L136R, -H, -G, and -D substitutions were Ps^- and exhibited variable reversion frequencies [Table 1 and see also the following paper by Brasseur et al. (1997)].

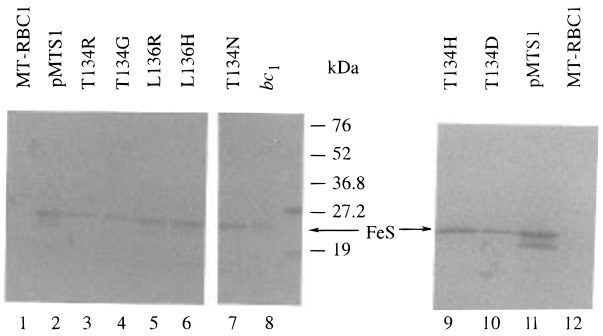

FIGURE 2: SDS−PAGE/immunoblot analyses of various *R. capsulatus* Fe-S protein mutants. Approximately 70 μg of chromatophore membrane proteins was loaded in each lane, and the Fe-S protein subunit of the bc_1 complex was detected (Materials and Methods) using subunit specific polyclonal antibodies raised against the purified *R. capsulatus* protein. Lane 8 contains the purified bc_1 complex as a control, and the arrow indicates the Rieske Fe-S protein. MT-RBC1 (lanes 1 and 12) carries a deletion covering the *fbcFB*C operon and hence lacks the bc_1 complex, while pMTS1 (lanes 2 and 11) overproduces the wild type bc_1 complex and is the parent of the mutants shown in the other lanes. Molecular mass markers are also indicated.

Biochemical Characterization of Mutant bc_1 Complexes. Optical absorption difference spectra (dithionite- or ascorbate-reduced *minus* ferricyanide-oxidized) indicated that all substitutions at positions 134 and 136 of the Fe-S protein overproduced the cyt *b* and c_1 subunits like their parent pMTS1/MT-RBC1 (data not shown). In addition, SDS−PAGE analysis of their chromatophore membranes revealed that they contained all three subunits of the bc_1 complex (data not shown). Thus, unlike the mutations that affect the liganding residues of the Fe-S cluster studied previously (Davidson et al., 1992a,b), they did not abolish the assembly of the Fe-S protein into the bc_1 complex. Nonetheless, immunoblot analyses using polyclonal antibodies specific for the Fe-S protein of *R. capsulatus* indicated that some of the mutants contained a substoichiometric amount of the Fe-S apoprotein in comparison with the other subunits of the bc_1 complex (Figure 2). In addition, of the two differently migrating wild type Fe-S protein conformers (24 and 22 kDa bands on SDS−PAGE), only the upper one was readily detectable in several of the T134 substitutions (Figure 2, e.g., lane 3). The significance of this observation, if any, remains unknown at the present time.

As expected, the DBH_2:cyt *c* reductase activities detected in chromatophore membranes of the Ps^- mutants T134D and L136R, -D, -H, and -G were very low (between 2 and 5% of that of a wild type strain) (Table 1), confirming that the corresponding bc_1 complexes were functionally defective. Unexpectedly, this activity was also very low in the Ps^+ mutants T134R, -H, and -G, when membranes were prepared using cells grown semiaerobically, suggesting that the corresponding mutant bc_1 complexes may be labile. Indeed, when the T134R, -H, and -G mutants were grown under Ps growth conditions, and their chromatophore membranes were prepared in the presence of 20 mM sodium ascorbate at pH 7.0, their DBH_2:cyt *c* reductase activities increased to about 24, 10, and 10% of that of a wild type strain, respectively. The drastic loss of this enzymatic activity in the mutant chromatophores prepared in the absence of a reducing agent

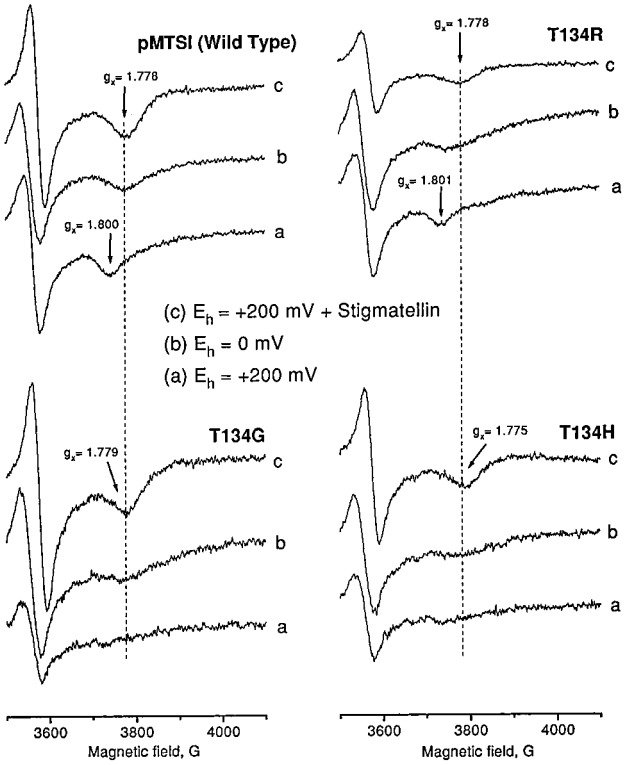

FIGURE 3: EPR spectra of the [2Fe-2S] cluster of the Rieske Fe-S protein from the strain pMTS1/MT-RBC1 overproducing the wild type bc_1 complex and its mutant derivatives T134R, -G, and -H. The Q_o site occupancy in these strains was monitored by using chromatophore membranes poised at redox potentials (E_h) of 200 and 0 mV (traces a and b, respectively) at pH 7.0 (Materials and Methods). The effect of stigmatellin on the EPR line shape is shown for each mutant (traces c). Due to the variable degree of autoxidation of mutant samples, the EPR signals are only shown to illustrate the differences between the mutants and are not to be compared quantitatively. The g_x signals corresponding to the oxidized and reduced Q_{pool} states are best seen in T134R, while that induced by stigmatellin is observed in all cases.

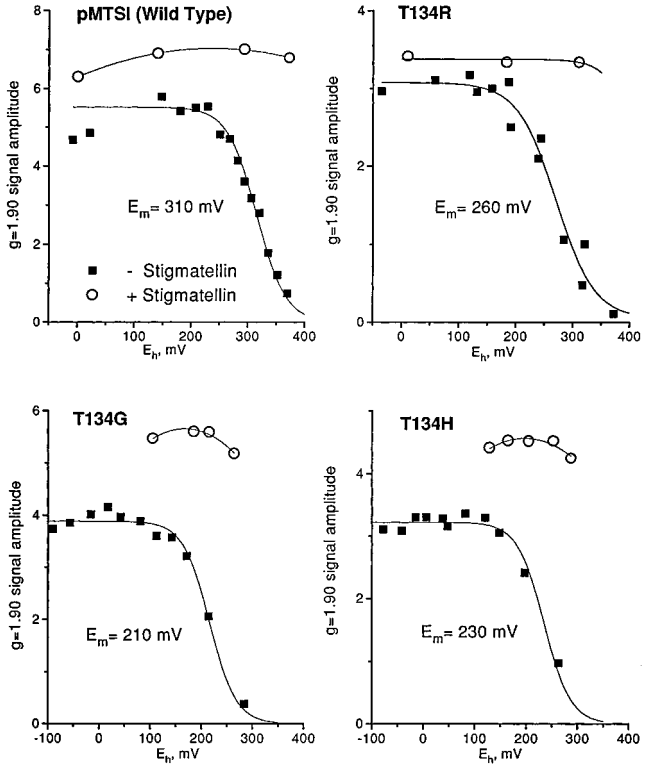

FIGURE 4: Potentiometric titrations of the [2Fe-2S] cluster of the Rieske Fe-S protein subunit of the bc_1 complex in various T134 substitutions. In all cases, the amplitude of the g_y = 1.90 signal was determined at various E_h values in the presence or absence of stigmatellin. The data points were collected between −40 and 400 mV and fit to an n = 1 Nernst equation, and the E_{m7} values determined are indicated.

like ascorbate indicated that their bc_1 complexes are oxidatively damaged readily upon exposure to air.

Biophysical Characterizations of Mutant bc_1 Complexes. The EPR spectrum of the [2Fe-2S] cluster of the Rieske Fe-S protein is sensitive to the redox state of the Q_{pool} and to the UQ/UQH_2 occupancy of the Q_o site (Matsuura et al., 1983; Robertson et al., 1993; Ding et al., 1992). Moreover, the inhibitors stigmatellin and myxothiazol are known to interact specifically with this site where they impede UQH_2 oxidation (Takamiya & Dutton, 1979; De Vries et al., 1983; Ohnishi et al., 1988). Thus, EPR spectroscopy was used to probe the effect of the various substitutions at positions 134 and 136 on the properties of the [2Fe-2S] cluster, the binding of the inhibitors, and the degree of occupancy of the Q_o site. In chromatophore membranes of T134D, no EPR signal characteristic of the Rieske [2Fe-2S] cluster was observed after ascorbate or dithionite reduction, explaining its Ps^- phenotype, although some Fe-S apoprotein could be detected by specific antibodies (Figure 2, lane 10). Small and variable amounts of the [2Fe-2S] cluster signal were detected in the chromatophore membranes of the Ps^+ mutants T134R, -H, and -G when chromatophore membranes were prepared in the absence of sodium ascorbate from cells grown by respiration (data not shown). However, when Ps-grown cells were used, and the chromatophore membranes were prepared in the presence of sodium ascorbate, appreciable (20−60% of the wild type) amounts of EPR detectable [2Fe-2S] cluster were found in all cases (Figure 3, traces a). The need for a reducing agent, which is not required to protect the wild type [2Fe-2S] cluster under standard assay conditions, further confirmed that the [2Fe-2S] cluster in the mutant Fe-S proteins was more prone to rapid oxidative damage upon exposure to air.

The T134R, -H, and -G mutants bound stigmatellin, as indicated by a characteristic alteration of their EPR spectra (Figure 3, traces c) (von Jagow & Ohnishi, 1985). Moreover, expected spectral changes (readily observed as a shift of the g_x trough toward higher magnetic field values) were observed upon lowering the E_{h7} from 200 to 0 mV (Figure 3, traces a and b), reflecting the ability of the [2Fe-2S] cluster to monitor the redox state of the Q_{pool}. Finally, potentiometric titrations of the EPR g_y signal of the [2Fe-2S] cluster of these mutants were performed in the E_h range of −40 to 400 mV, in the presence and absence of stigmatellin (Figure 4). These measurements indicated that the E_{m7} values of the [2Fe-2S] cluster in the mutants were significantly lower (260, 230, and 210 mV for T134R, -H, and -G substitutions, respectively) than the redox midpoint potential of a wild type strain (310 mV), which is in agreement with their increased oxidative damage. Furthermore, stigmatellin-induced shifts of the redox midpoint potential (E_{m7}) of the [2Fe-2S] cluster in these mutants were similar to those seen in a wild type strain (Figure 4). Therefore, even though the T134R, -H, and -G substitutions lowered the E_{m7} value of the [2Fe-2S] cluster by as much as 100 mV, they affected neither the communication of the [2Fe-2S] cluster with the Q_{pool} nor its response to stigmatellin.

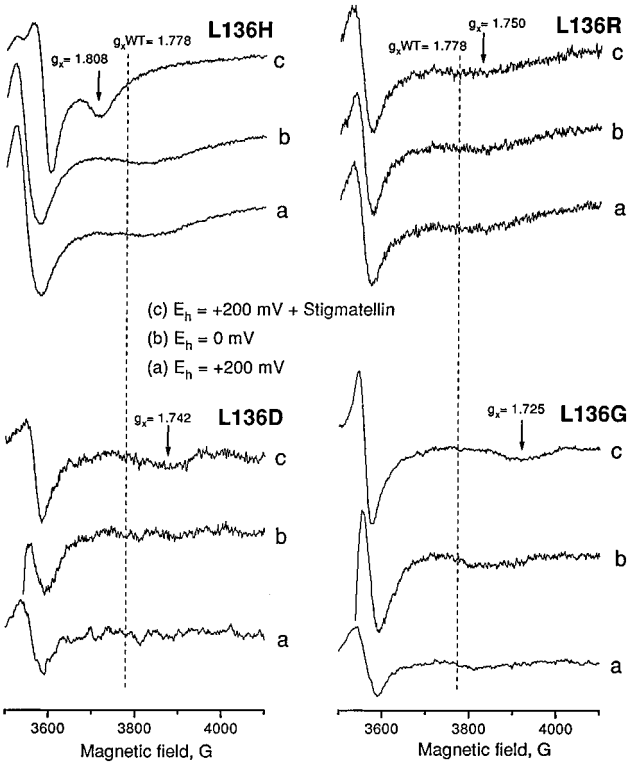

FIGURE 5: EPR spectra of the [2Fe-2S] cluster of the Rieske Fe-S protein from the L136H, -R, -D, and -G mutants. The experimental conditions used are identical to those used with T134 mutants shown in Figure 3. Note that none of these substitutions is able to monitor the Q_o site redox state, and while L136R does not respond to stigmatellin, L136H, -D, and -G respond to it, although differently than a wild type strain (Figure 3). The amplitudes of the EPR signals are not to be compared quantitatively.

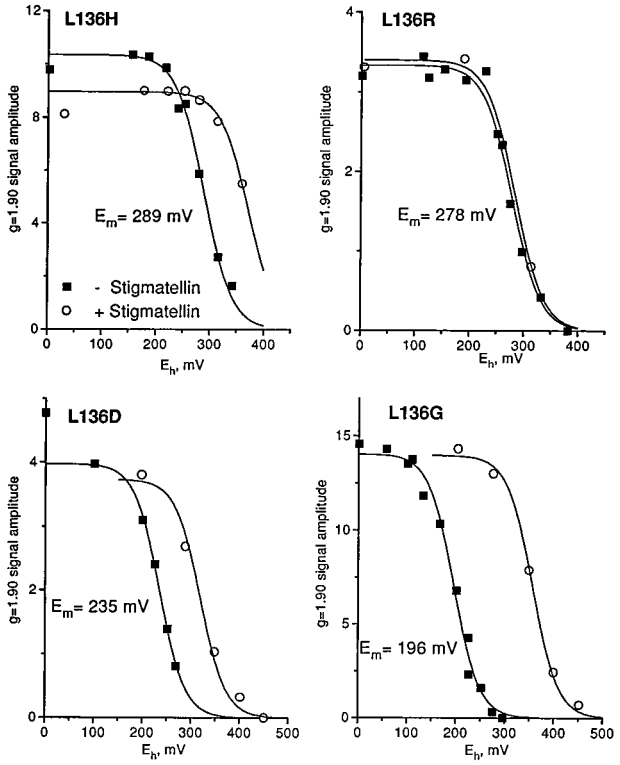

FIGURE 6: Potentiometric titrations of the [2Fe-2S] cluster of the Fe-S protein subunit of the bc_1 complex in various L136 substitutions. The data were obtained as described in Figure 4, and the E_{m7} values determined are indicated. Note that stigmatellin does not affect the redox midpoint potential of L136R and has less pronounced effects in other substitutions compared to a wild type bc_1 complex.

The EPR spectra of the [2Fe-2S] cluster of the L136H, -R, -D, and -G mutants were drastically altered (Figure 5). The g_x trough was flattened and shifted to a higher magnetic field, which resembled the changes observed when the Q_{pool} of the wild type chromatophore membranes was extracted with solvents (Ding et al., 1992). In these mutants, no readily discernible spectral differences could be detected upon lowering the E_{h7} from 200 to 0 mV, indicating that their [2Fe-2S] cluster could not monitor the Q_{pool} redox state (Figure 5, traces a and b). In addition, the interactions between stigmatellin and the Q_o site were modified to different degrees. While L136R did not respond at all to this inhibitor, L136G, L136D, and L136H exhibited weak but observable spectral changes (Figure 5, traces c). In particular, in L136H, the stigmatellin-induced narrowing of the g_x trough ($g = 1.808$) in L136H was quite pronounced and a shift toward a lower field compared to wild type ($g = 1.778$) was observed, while in L136G and -D mutants, the g_x trough shifted toward a higher magnetic field to $g = 1.725$ and 1.742, respectively (Figure 5, traces c). Potentiometric titrations of the EPR g_y signal indicated that the E_{m7} values of the [2Fe-2S] cluster in these mutants were also lower (289, 278, 235, and 196 mV in L136H, -R, -D, and -G, respectively) than the redox midpoint potential of a wild type strain (310 mV) (Figure 6), but could be raised to higher values upon the addition of stigmatellin in L136H, D and G, respectively. Interestingly, the E_{m7} value of L136R did not change in the presence of this inhibitor, indicating that among the side chains tested, R was the most deleterious for the recognition of stigmatellin by the Q_o site. Thus, the mutations at position 136, unlike those at 134, perturbed drastically UQH_2 oxidation and modified the binding of stigmatellin to the Q_o site of the bc_1 complex, abolishing the communication of the [2Fe-2S] cluster of the Fe-S protein with the Q_{pool}.

Ps^+ Revertants of the T134D Substitution. Ps^+ revertants from the Ps^- mutant T134D were sought to gain further insights into the role of the amino acid side chain at this position of the Fe-S protein. Nucleotide sequence determination of the Fe-S protein structural gene (*fbcF/petA*) in three such Ps^+ revertants revealed that they all substituted D (GAC) with N (AAC) (Table 1). Thus, elimination of a negatively charged side chain from position 134 appears to be a frequent reversion event. On the other hand, no second site suppressor located elsewhere in the bc_1 complex was readily encountered with T134D, unlike with the L136H and -G substitutions described in the following paper (Brasseur et al., 1997). Detailed analysis of the T134N revertant revealed that its Ps growth ability (doubling time of 185 min in MPYE medium) (Table 1) and inhibitor (stigmatellin and myxothiazol) sensitivity were similar to those of the wild type strain pMTS1/MT-RBC1. T134N also contained parental levels of the cyts *b* and c_1 and the Fe-S apoprotein (Figure 2) in chromatophore membranes. Its DBH_2:cyt *c* reductase activity was low (about 16% of that of a wild type strain) and only reliably detected when the chromatophore membranes were prepared from Ps-grown cells in the presence of 20 mM ascorbate. Its EPR and autoxidation characteristics were also similar to those observed with the other substitutions at this position described above (data not shown). Thus, a comparison of T134D (Ps^-) with T134N

(Ps^+) indicates that the presence of the negatively charged aspartic acid at this position is deleterious for the [2Fe-2S] cluster even under anaerobic conditions.

DISCUSSION

In this work, we have studied the structural and functional roles of the conserved amino acid residues T134 and L136, which are adjacent to the box I cluster ligands of the Fe-S protein subunit of the bc_1 complex. Substitutions of these residues with selected amino acids yielded mutant bc_1 complexes that were assembled at least partly so that their properties could be analyzed in detail. Prior to this work, no specific information was available on the role of these residues, in particular with respect to the properties of the [2Fe-2S] cluster of the Rieske Fe-S protein or the Q_o site of the bc_1 complex, with the exception of temperature sensitive mutation T160A (corresponding to T134 in *R. capsulatus*) reported in *S. cerevisiae* (Graham et al., 1993), which has not been characterized.

The most striking finding with respect to position 134 is that, although many of the substitutions tested yielded mutant bc_1 complexes which were able to support photosynthetic growth (Table 1), they exhibited barely detectable amounts of [2Fe-2S] cluster and cyt *c* reductase activity under our standard assay conditions. However, when chromatophore membranes were prepared in the presence of a reducing agent such as sodium ascorbate from anaerobically (Ps) grown cells, greatly increased amounts of [2Fe-2S] cluster and a higher cyt *c* reductase activity were obtained. Potentiometric titration of the mutant complexes indicated that the E_{m7} values of their [2Fe-2S] cluster were about 50−100 mV lower than that of a wild type complex, which is in agreement with their increased oxidative damage upon exposure to air. Therefore, a major role of the amino acid side chain at position 134 seems to control and fine tune the E_{m7}, and thereby the oxygen sensitivity of the [2Fe-2S] cluster. Interestingly, these mutants revealed that an Fe-S protein with an E_{m7} value of around 210 mV was still able to support anoxygenic photosynthesis. However, whether a bc_1 complex with an oxygen labile Fe-S protein can support respiratory growth of *R. capsulatus* in the absence of the alternate oxidase pathway, or in a strictly respiratory organism such as yeast, remains to be seen. In this respect, note that the *R. sphaeroides* mutant G133D (position 137 in *R. capsulatus*) is Ps^+ albeit its partly inactive Q_o site (Van Doren et al., 1993b), while its yeast counterpart (G133D; Gatti et al., 1989) is partially respiratory-deficient. The [2Fe-2S] cluster of the latter mutant has an E_{m7} value about 100 mV lower than that of a wild type protein and is inactive, possibly due to increased oxidative damage on the mutant bc_1 complex.

The structure of the soluble portion of the bovine heart mitochondrial Rieske Fe-S protein indicated that the side chain of T134 is directed toward the inside of the protein (Figure 7) and is tightly packed against the so-called "Pro" loop formed of seven residues from position 174 to 180, and covering the [2Fe-2S] cluster from one side (Iwata et al., 1996). It has been postulated that this "proline crown" motif is important for the stability of the [2Fe-2S] cluster, since mutations in the fully conserved stretch $G_{174}PAP_{177}$ (bovine numbering, $G_{169}PAP_{172}$ in *R. capsulatus*) affected the cluster stability (Graham et al., 1993). In earlier studies, moderate changes in the E_{m7} values of the [2Fe-2S] cluster of the Rieske Fe-S protein similar to those observed in the T134 substitutions have been noticed with the G133D, P136L, and P149L (G137, P140, and P154 in *R. capsulatus*, respectively) mutations in yeast (Gatti et al., 1989). Considering the fact that the Fe-S proteins from these two species are exceedingly similar in their cluster-containing C-terminal portions, the molecular effect of these mutations could be to perturb the disulfide bridge or the hydrogen bonding network surrounding the [2Fe-2S] cluster (Iwata et al., 1996). Such a change would render the cluster more accessible to solvent, changing its E_{m7} value and increasing its oxidative damage, as observed here. Figure 7 represents a molecular depiction of the T134G, -H, and -N mutations using the three-dimensional structure of the bovine soluble Rieske Fe-S protein, and illustrates the close proximity of position 134 of box I to the [2Fe-2S] cluster and its ligands C133 and H135.

Among the five substitutions (R, H, D, N, and G) at position 134 of the Fe-S protein, only the negatively charged D residue completely abolished UQH_2 oxidation at the Q_o site of the bc_1 complex. Why the Ps^- mutant T134D lacks the [2Fe-2S] cluster of the Rieske protein even under anaerobiosis is unknown. However, the high frequency with which the aspartic acid residue reverts to an asparagine is consistent with the idea that a negatively charged side chain at this position affects drastically the stability of this redox center. In the available Rieske Fe-S protein structure, the Oγ atom of T140 (T134 in *R. capsulatus*) forms a hydrogen bond to the backbone nitrogen of L178 (bovine numbering) located in the Pro loop (Iwata et al., 1996), yielding a tight inward packing of this residue in close proximity to the positively charged side chains at positions R170, R172, and K173 (R165, R167, and K168 in *R. capsulatus*, respectively) (T. Link, personal communication). Thus, a possible ionic interaction between an aspartate at position 134 and a lysine at position 168 may explain why the negatively charged T134D substitution in the *R. capsulatus* Rieske Fe-S protein is deleterious to the [2Fe-2S] cluster and the Q_o site of the bc_1 complex.

Prior to this work, very little information was available on the role of the amino acid side chain of position L136 in Q_o site catalysis. Unlike the T134 substitutions, all four L136 mutations (R, H, D, and G) analyzed here yielded Ps^- mutants, contained substoichiometric amounts of the Rieske Fe-S protein, exhibited altered EPR spectra revealing major perturbations of the [2Fe-2S] cluster geometry, and had severely impaired Q_o sites [see also the following paper by Brasseur et al. (1997)]. Consequently, they were unable to sense the redox state of the Q_{pool} and had impaired interactions with both UQ/UQH_2 and stigmatellin. In particular, note that in the L136R mutation stigmatellin had no effect on the E_{m7} value of the [2Fe-2S] cluster, while it raised the redox midpoint potential only slightly in the remaining L136 substitutions. A similar lack of response to stigmatellin has been seen with the C138S mutation (E_{m7} of 160 mV) of the Rieske Fe-S protein during our earlier work on the determination of the ligands of the [2Fe-2S] cluster (Davidson et al., 1992a).

The bovine heart Rieske Fe-S protein structure indicates that L142 (corresponding to L136 in *R. capsulatus*) sticks out of the protein surface and is part of a hydrophobic domain at one end of the protein where the [2Fe-2S] cluster is located and that the backbone amide group of this leucine residue is

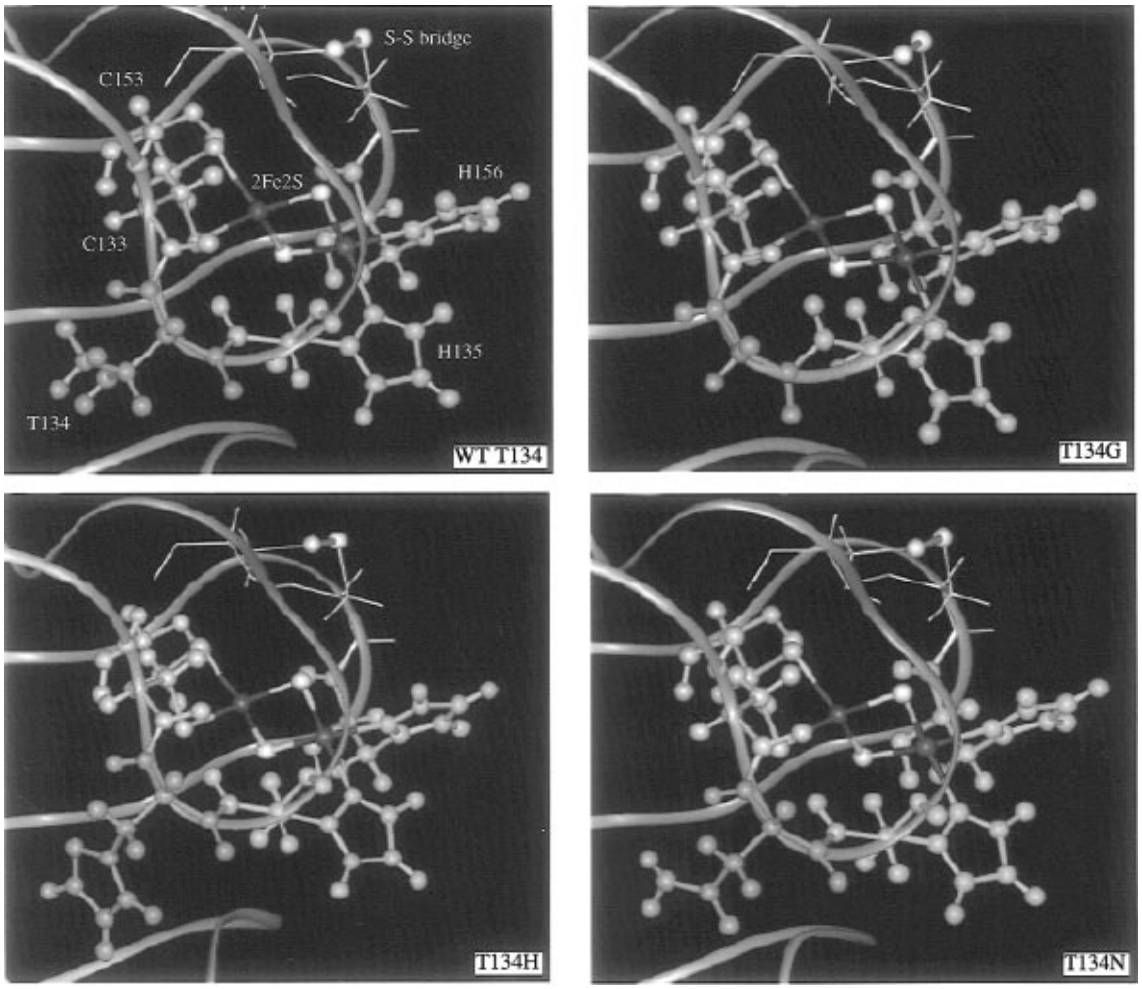

FIGURE 7: Model of the [2Fe-2S] cluster binding region of the *R. capsulatus* Fe-S protein in the wild type and various T134 substitutions. The figure is intended to depict the three-dimensional conformation of T134 using the atomic coordinates of the bovine Rieske Fe-S protein (Iwata et al., 1996). This position was substituted with glycine, histidine, and asparagine to depict various mutations. Iron and sulfur atoms of the [2Fe-2S] cluster are colored in red and yellow, respectively. The two histidine ligands corresponding to His135 and His156 in *R. capsulatus* are almost perpendicular to each other and are shown in blue. The Cys133 and Cys153 ligands are in pink, and Cys138 and Cys155 forming the disulfide bridge are yellow. The green residue highlights position 134 (140 in the bovine Fe-S protein) where the mutations are displayed.

linked to the [2Fe-2S] cluster via a hydrogen bond (Iwata et al., 1996) [for a molecular depiction of the L136 mutations, see the following paper by Brasseur et al. (1997)]. The hydrophobic surface surrounding the redox cluster has long been implicated in forming the Q_o site of the bc_1 complex through protein−protein interactions between the Fe-S protein and cyt *b* as well as in effective binding of UQ/UQH_2. Thus, decreased E_{m7} values, increased autoxidation, and substoichiometric association of the Fe-S protein subunit with the bc_1 complex could be expected from the substitution of position 136 with residues of less hydrophobicity than the wild type leucine. However, since a decrease of about 100 mV of the E_{m7} value of the [2Fe-2S] cluster is not prohibitive to Q_o site catalysis as seen with the T134 substitutions, the loss of enzymatic activity in the L136 substitutions cannot be explained by their lowered E_{m7} values. A possible distortion of the backbone amide group, affecting the H bonding and thus modifying the cluster redox properties and the binding of stigmatellin in these mutants, could account for the observed changes [Figure 7 and the following paper by Brasseur et al. (1997)]. In any event, the hydrophobic nature of the amino acid side chain at position 136 of the Fe-S protein is an essential requirement for the bc_1 complex to be able to communicate efficiently with the Q_{pool} to sense its redox state and to yield a functional Q_o site. This proposal is consistent with the finding that the L136A and -Y substitutions, which were obtained as Ps^+ revertants of the L136R and -D mutations in *R. capsulatus* (Brasseur et al., 1997), yield functional bc_1 complexes.

It has been suggested that the pH dependence of the E_{m7} value of the Fe-S protein arises from the protonation−deprotonation of the amino acid residues in contact with the metal cluster (Kuila & Fee, 1986; Liebl et al., 1992; Link et al., 1992; Link, 1994). The structure of the reduced form of the water soluble fragment of the bovine heart Rieske Fe-S protein revealed that the hydrogen atoms at Nϵ positions of the histidine ligands with respect to Fe(II) are fully solvent-exposed, are not part of the hydrogen bond network around the cluster, and could undergo redox-dependent protonation−deprotonation (Iwata et al., 1996). However, recent ESEEM and ENDOR data on the Rieske-type [2Fe-2S] clusters of bacterial dioxygenases (Riedel et al., 1995) indicated virtually identical N couplings between these proteins independently of whether they exhibit pH-dependent E_{m7} changes. Future studies of *R. capsulatus* T134H and L136H mutants containing additional histidine residues next to the [2Fe-2S] cluster may further address this open issue.

In summary, this work revealed that the properties of the [2Fe-2S] cluster changed drastically when the residues T134 and L136 of the *R. capsulatus* Rieske Fe-S protein were modified. While the replacement of T134 affected mainly the E_{m7} value of the [2Fe-2S] cluster and its oxygen sensitivity without abolishing the Q_o site function, the substitution of L136 perturbed drastically the ability of the Rieske Fe-S protein to sense the Q_{pool} redox state and to interact with stigmatellin. Clearly, the former position is important for minimizing the oxidative damage to the Fe-S protein, and the latter is essential for proper communication with the Q_{pool} and for an active Q_o site. We are hopeful that a greater understanding of the Q_o site function will be attained when the high-resolution structure of the bc_1 complex is completed.

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