The Amino-Terminal Portion of the Rieske Iron-Sulfur Protein Contributes to the Ubihydroquinone Oxidation Site Catalysis of the *Rhodobacter capsulatus* bc_1 Complex†

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ABSTRACT: The Rieske iron-sulfur (Fe-S) protein subunit of bc_1 complexes contains in its carboxyl-terminal part two highly conserved hexapeptide motifs (box I and box II) that include the four amino acid ligands of its [2Fe-2S] cluster. In the preceding paper [Liebl, U., Sled, V., Brasseur, G., Ohnishi, T., & Daldal, F. (1997**)** *Biochemistry 36,* 11675−11684], the effects of mutations at two of the nonliganding residues [threonine (T) 134 and leucine (L) 136 in the *Rhodobacter capsulatus* Rieske Fe-S protein] of box I have been described. In this work, interactions between the occupants of the Q_o site of the bc_1 complex (UQ/UQH_2 and the inhibitors stigmatellin and myxothiazol) and the [2Fe-2S] cluster of the Rieske Fe-S protein were probed by isolating photosynthesis-proficient (Ps^+) revertants of the Ps^- mutants L136R, -H, -D and -G. These revertants contained either a single substitution at the original position 136 or an additional mutation located in the amino-terminal part of the Fe-S protein at either position 44 or 46. The same-site revertants L136A and -Y grew well under photosynthetic conditions and contained highly active bc_1 complexes but exhibited modified EPR spectra both in the presence and in the absence of stigmatellin. Unexpectedly, they were highly resistant to stigmatellin (Sti^R) and hypersensitive to myxothiazol (Myx^{HS}) *in vivo*, demonstrating for the first time that mutations located in the Fe-S subunit confer resistance to stigmatellin. The [2Fe-2S] cluster of the same-site revertants responded weakly to the Q_{pool} redox state and had redox midpoint potential (E_{m7}) values (around 265 mV) lower than those of their wild type counterpart (about 310 mV). On the other hand, the second-site revertants L136H/V44L, L136G/V44F, and L136G/A46T, -V, or -P supported photosynthetic growth poorly, were Sti^R and Myx^{HS}, and contained barely active bc_1 complexes. Like the same-site revertants, they exhibited modified EPR spectra both in the presence and in the absence of stigmatellin and had perturbed Q_o site occupancy. In addition, they contained substoichiometric amounts of the Fe-S protein with respect to the other subunits of the bc_1 complex. The E_{m7} values of the [2Fe-2S] cluster of these double mutants were lower (around 245 mV) than that of the wild type strain but appreciably higher than those of their Ps^- parents (about 200 mV for L136G). In order to define the molecular nature of the suppression mediated by the second-site mutations, the single mutants V44L and -F and A46T and -V were constructed in the absence of the original mutations at position 136. These mutants behaved like a wild type strain with respect to their Ps^+ growth ability, inhibitor sensitivity, EPR spectra of their [2Fe-2S] cluster, and response to stigmatellin or to the Q_{pool} redox state. But surprisingly, the E_{m7} values of their [2Fe-2S] cluster were much higher (about 385 mV) than that of a wild type strain. These findings demonstrated for the first time that the amino-terminal part of the Rieske Fe-S protein encompassing residues 44 and 46 is important not only for the structure and function of the Q_o site of the bc_1 complex but also for the properties of its [2Fe-2S] cluster.

Ubihydroquinone:cytochrome (cyt)[1] *c* oxidoreductases (or the bc_1 complexes) are constituents of electron transfer chains of bacteria, mitochondria, and chloroplasts (where it is known as the b_6f complex). In facultative photosynthetic bacteria like *Rhodobacter capsulatus*, the bc_1 complex is involved in both photosynthetic (Ps) and respiratory (Res) growth. It

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[1] Abbreviations: bp, base pair; cyt, cytochrome; bc_1 complex, ubihydroquinone: cyt *c* oxidoreductase; UQH_2, ubihydroquinone; UQ, ubiquinone; Q_{pool}, ubiquinone pool; Q_o, ubihydroquinone oxidation site; Q_i, ubiquinone reduction site; EPR, electron paramagnetic resonance; cyt b_H, high-potential cyt *b*; cyt b_L, low-potential cyt *b*; E_h, ambient redox potential; E_{m7}, equilibrium redox midpoint potential at pH 7.0; R, resistant; HS, hypersensitive; Ps^+, photosynthesis-proficient; Ps^-, photosynthesis-incompetent; [2Fe-2S], iron-sulfur cluster of the Rieske iron-sulfur (Fe-S) protein; DAB (diaminobenzidine), 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene, DAD); PMS, *N*-methyldibenzopyrazine methosulfate; PES, *N*-ethyldibenzopyrazine ethosulfate; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; Myx, myxothiazol; Sti, stigmatellin; Ant, antimycin A; DBH_2, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzoquinone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SHE, standard hydrogen electrode, WT, wild type parental strain.

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catalyzes the oxidation of ubihydroquinone (UQH_2) and reduction of cyt *c* and contributes to the establishment of a proton gradient, which is subsequently used for ATP production, active transport and taxis [for reviews, see Cramer and Knaff (1990), Prince (1990), Gennis et al. (1993), Cramer et al. (1994), Trumpower and Gennis (1994), and Gray and Daldal (1995)].

Bacterial bc_1 complexes are formed by a minimum of three proteins that contain four redox-active prosthetic groups. These are the cyt *b* polypeptide with two *b* hemes [designated b_L and b_H according to their relatively low and high redox midpoint potentials (E_{m7}) of approximately −90 and 50 mV, respectively, in *R. capsulatus* (Meinhardt & Crofts, 1983; Robertson et al., 1993)], the cyt c_1 with a covalently bound *c* type monoheme, and the iron-sulfur (Fe-S) protein with a [2Fe-2S] cluster, commonly referred to as the Rieske Fe-S protein. The structural genes for these subunits, *fbcFBC* (*petABC*), are available from various species [for a recent review, see Gray and Daldal (1995)]. These subunits form two distinct UQH_2/UQ binding sites, Q_o (or Q_p) where UQH_2 is oxidized and Q_i (or Q_n) where UQ is reduced. The Q_o site is localized on the positive side of the cytoplasmic membrane and is formed by both the cyt *b* and the Rieske Fe-S protein, while the Q_i site is on the negative side of the membrane and is mainly formed by the cyt *b* subunit [for a review, see Brandt and Trumpower (1994)]. A mechanism for coupling electron transfer to proton translocation catalyzed by the bc_1 complex was first described by the Q cycle scheme of Mitchell (1975). Recently, more detailed mechanistic models have been proposed for Q_o site catalysis (Crofts et al., 1983; Ding et al., 1992, 1995a; Brandt, 1996).

The Fe-S protein subunit was discovered by Rieske et al. (1964) and, later on, shown to be part of the bc_1 complex (Trumpower & Edwards, 1979). Its properties have been described in detail in the preceding paper (Liebl et al., 1997). Briefly, the Fe-S protein is thought to be attached to the membrane by an amino-terminal anchor and contains a [2Fe-2S] binuclear metal center ligated to its carboxyl-terminal part by two cysteine and two histidine residues (C133 and C153 and H135 and H156, respectively, in *R. capsulatus*). They are part of the two conserved hexapeptide sequences, box I and box II (C_{133}THLGC_{138} and C_{153}PCHGS_{158} in *R. capsulatus*; see Figure 1). The Rieske Fe-S protein has a unique electron paramagnetic resonance (EPR) spectrum with specific *g* values ($g_x = 1.8$, $g_y = 1.9$, and $g_z = 2.03$) and a high redox midpoint potential (E_{m7}) for its [2Fe-2S] cluster (between 150 and 300 mV in various species). Its g_x signal is of particular interest since its shape and *g* value are sensitive to the redox state of the Q_{pool} (Matsuura et al., 1983; Ding et al., 1992) and to the Q_o site inhibitors stigmatellin and myxothiazol (von Jagow & Ohnishi, 1985; Ding et al., 1992). These features have been used extensively to monitor UQ/UQH_2 and inhibitor binding to the Q_o site and to assess the effects of various mutations on Q_o site catalysis (Robertson et al., 1990; Ding et al., 1992, 1995a,b; Saribas et al., 1995).

Previously, mutagenesis of the Rieske Fe-S protein has been undertaken using both yeast (Beckman et al., 1987; Gatti et al., 1989; Graham et al., 1993) and bacteria (Davidson et al., 1992; Van Doren et al., 1993), in particular to identify the cysteine and histidine residues involved in the ligation of its [2Fe-2S] cluster. Two histidine residues have previously been shown to ligate one of the iron atoms of the cluster (Gurbiel et al., 1991) at the Nδ positions of the imidazole rings (Gurbiel et al., 1996). The three-dimensional structure of the soluble portion of the bovine Rieske Fe-S protein (Iwata et al., 1996) confirmed that Cys133, Cys153, His135, and His156 (*R. capsulatus* numbering) are the ligands of the [2Fe-2S] cluster and that a disulfide bridge is formed between the Cys138 and Cys158 residues (*R. capsulatus* numbering), as proposed earlier by Davidson et al. (1992). It also revealed that the [2Fe-2S] cluster is located at one end of the Fe-S polypeptide and that its histidine ligands are solvent-exposed. On the basis of these observations, it was proposed that the Nϵ positions of the imidazole rings may undergo redox-dependent protonation and deprotonation at the Q_o site (Link et al., 1996a). The structure of the Rieske Fe-S protein also revealed that the nonliganding residue Leu136 of box I sticks out of this protein. On the other hand, Liebl et al. (1995, 1997) have found out that the L136R, -H, -D, and -G mutations yielded assembled but non functional bc_1 complexes, which contained modified [2Fe-2S] clusters. More recently, L136 was also proposed to be a structural determinant for binding of stigmatellin to the Q_o site of the bc_1 complex (Link & Iwata, 1996).

In the present work, Ps^+ revertants of the Ps^- L136 mutants were isolated to better define the role and properties of the Rieske Fe-S protein in Q_o site catalysis. The analysis of these mutants indicated that the same-site revertants L136A and -Y conferred Sti^R and Myx^{HS} while the non-functional L136G and -H substitutions are suppressed by second-site mutations located at position 44 or 46 in the amino-terminal part of the Rieske Fe-S protein. In addition, these mutations modulated the E_{m7} of the [2Fe-2S] cluster between 240 and 380 mV without causing any drastic loss of the bc_1 complex activity. The overall findings demonstrated for the first time that position 136 of the Rieske Fe-S protein can confer resistance to stigmatellin and that the amino-terminal portion of this subunit is critical for the structure and function of the Q_o site of the bc_1 complex.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *Escherichia coli* strains were grown in LB medium, supplemented with appropriate antibiotics as described previously (Gray et al., 1992). Wild type and mutant *R. capsulatus* strains were grown in MPYE, Medium A (Sistrom, 1960), or "mixed medium" (1 volume of Medium A plus 1 volume of MPYE) either chemoheterotrophically (Res) or photoheterotrophically (Ps), and growth rates were determined as reported earlier (Daldal et al., 1989). The *R. capsulatus* strain pMTS1/MT-RBC1 is kanamycin resistant (Kan^R) and overproduces the wild type bc_1 complex by about 5−8-fold, as described previously (Gray et al., 1992).

Genetic Methods. Spontaneous Ps^+ revertants of the L136R, -H, -D, and -G mutants were obtained on MPYE plus kanamycin plates over several days of incubation under selective conditions. Revertants forming both large and small colonies were retained to increase the probability of obtaining different classes of revertants. All Ps^+ colonies thus obtained were able to form visible colonies after 2−3 days of Ps growth and were purified extensively under the selective growth conditions. Their plasmid DNA was extracted and sequenced to determine the location and nature of the

mutation(s) either manually using [α-^{35}S]ATP and "Sequanase" (U.S. Biochemicals, Inc.) or with an automated DNA sequencer using a dye terminator cycle sequencing kit (Amplitaq-FS from Applied Biosystems Inc.) according to the specifications of the suppliers. The DNA sequence of *fbc*F/*pet*A was determined on both strands between nucleotide positions 700 and 1350 (Davidson & Daldal, 1987). In each case, the completely sequenced *Bst*XI-*Apa*LI or *Bst*XI-*Eco*RI DNA fragment containing the mutation thus defined was exchanged with its counterpart either on pMTS1 carrying the wild type or on pF:L136H or -G carrying the original Ps$^-$ allele of *fbcF* (Figure 1), allowing segregation of the second-site mutations away from the initial mutations. The plasmids thus obtained were conjugated into the *R. capsulatus* strain MT-RBC1, and the mutations being studied were reconfirmed by DNA sequencing.

Biochemical and Biophysical Techniques. Chromatophore membranes were prepared as described earlier (Atta-Asafo-Adjei & Daldal, 1991) in 50 mM MOPS (pH 7.0), 100 mM KCl, 1 mM EDTA, and 1 mM PMSF using a French Pressure cell, except that they were washed three times before use for kinetic spectroscopy. The bacteriochlorophyll concentration was determined spectrophotometrically using an extinction coefficient ϵ_{775nm} of 75 mM^{-1} cm^{-1}, and protein concentrations were measured according to Lowry et al. (1951). SDS−PAGE and Western blot analysis using monoclonal or polyclonal antibodies raised against the *R. capsulatus* Rieske Fe-S protein and determination of the steady state activity of the bc_1 complex were performed as described in the preceding paper (Liebl et al., 1997).

Flash-activated time-resolved kinetics to monitor cyt *b* reduction and cyt *c* re-reduction were performed as described in Ding et al. (1995a), except that a single-beam spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania) was used. Chromatophore samples were calibrated using the reaction center concentration determined by measuring the optical absorption difference between 605 and 540 nm at an E_h of 370 mV and an extinction coefficient of 29 mM^{-1} cm^{-1}. Cyt *b* and *c* kinetics were measured at an E_h of 100 mV, in the presence of 1.5 μM *N*-methyldibenzopyrazine methosulfate (PMS), 1.5 μM *N*-ethyldibenzopyrazine ethosulfate (PES), 1.5 μM 2-hydroxy-1,4-naphthoquinone (OHQN), 7 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene, DAD), 10 μM $FeCl_3$/EDTA, 1.5 μM pyocyanine, and 3 μM valinomycin. The bc_1 complex inhibitors antimycin (10 μM), myxothiazol (5 μM), and stigmatellin (10 μM) were used as needed. Traces were recorded at 550 and 560 nm for cyt *c* and cyt *b* kinetics, respectively, from which those obtained at appropriate isosbestic wavelengths (540 and 570 nm for cyt *c* and cyt *b* kinetics, respectively) were subtracted. The electron transfer rates were calculated as in Gray et al. (1994) by fitting the data to a single-exponential curve.

EPR spectroscopy was performed as described in the preceding paper (Liebl et al., 1997) using a Bruker ESP-300E instrument equipped with an Oxford Instruments ESR-9 helium cryostat. Redox titrations of the Rieske [2Fe-2S] cluster in chromatophore membranes were conducted potentiometrically according to Dutton (1978), in the presence of 40 μM tetrachlorohydroquinone, 20 μM *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), 40 μM 1,2-naphthoquinone-4-sulfonate, 40 μM 1,4-naphthoquinone-2-sulfonate, 40 μM *N*-methyldibenzopyrazine methosulfate (PMS), 40 μM duroquinone, and 10 μM pyocyanine. Reductive and oxidative titrations were conducted with dithionite and ferricyanide, respectively, as described in the preceding paper (Liebl et al., 1997). Stigmatellin (40 μM) and myxothiazol (60 μM) were added from stock solutions prepared in DMSO. EPR recording conditions were as follows: sample temperature, 20 K; microwave power, 5 mW; modulation amplitude, 12.5 G; modulation frequency, 100 kHz; microwave frequency, 9.45 GHz.

Molecular modeling was carried out in the Molecular Modeling Facilities of the Cancer Center of the University of Pennsylvania as described in the preceding paper (Liebl et al., 1997). The coordinates for the bovine Fe-S protein (current PDB accession number of 1RIE) were kindly provided by T. Link (University of Frankfurt, Germany) prior to publication.

Chemicals. All chemicals were as described in Gray et al. (1994).

RESULTS

Isolation of Ps$^+$ Revertants from the L136R, -H, -D, and -G Mutants of the Fe-S Protein Subunit of the bc_1 Complex. The work described in the preceding paper (Liebl et al., 1997) established that the conserved nonliganding residue L136 of the Rieske Fe-S protein is critical for the function of the Q_o site of the bc_1 complex, hence the Ps growth of *R. capsulatus*. Here, Ps$^+$ revertants were selected from the Ps$^-$ mutants L136R, -H, -D, and -G of the Fe-S protein to further probe the nature of possible interactions between various amino acid residues contributing to the structure and function of the Q_o site. A total of 42 independent Ps$^+$ revertants (seven, six, seven, and 22 from L136R, -H, -D, and -G, respectively) were retained, and 23 of them (five, six, five, and seven from L136R, -H, -D, and -G, respectively) were analyzed (Figure 1 and Table 1). Plasmid DNA was isolated from these revertants, and in most cases, the entire *fbcF* gene was sequenced to define the nature and location of the reversion mutations. Of the L136H derivatives, three were true revertants (His to Leu) and the remaining revertants kept the original mutation at position 136. DNA sequence analysis revealed that one of them contained a Val to Leu substitution at position 44 of the Fe-S protein (Figure 1 and Table 1). All seven L136G revertants retained the original mutation at position 136, and their analysis uncovered one substitution (Val to Phe) at position 44 and six [two (Ala to Val), three (Ala to Thr), and one (Ala to Pro)] at position 46. In the case of L136D, all five revertants were located at the original position 136, and four of them substituted Asp with Ala and one with Tyr. Of the five L136R derivatives, two were true revertants (Arg to Leu) while two substituted Arg with Ala and one with Tyr (Figure 1). In all cases, the reversion mutations were confined to the Rieske Fe-S protein, and none was found outside of *fbc*F.

In summary, genetic analysis of the Ps$^+$ revertants of L136R, -H, -D, and -G substitutions yielded two groups of revertants. They were the same-site revertants, like L136A and -Y, which contained a single mutation at the original position 136 in the carboxyl-terminal part of the Fe-S protein, and the second-site suppressor mutants, like L136H/V44L, L136G/V44F, and L136G/A46T, -V, or -P, which contained, in addition to the original mutation at position 136, a second compensatory mutation at either position 44 or 46 in the

Table 1: Characteristics of L136 Revertants of *R. capsulatus* Rieske Fe-S Protein Mutants

strain	mutation	Ps phenotype[a] (doubling time), inhibitor, phenotype[b]	cytochrome content[c] [cyt *b*] (%)	[cyt *c*] (%)	FeS g_y amplitude[d] (%)	DBH_2:cyt *c* reductase activity (TN)[e] (%)
WT	136:CTC	Ps^+ (167 min), Sti^S, myx^S	100	100	100	100
L136A	136:GCC	Ps^+ (205 min), Sti^R, Myx^{HS}	94	94	80	26
L136Y	136:TAC	Ps^+ (200 min), Sti^R, Myx^{HS}	93	93	93	20
L136H/V44L	136:CAC/44:GTC → CTC	Ps^+ (190 min), Sti^R, Myx^{HS}	100	100	53	15[f]
L136G/V44F	136:GGC/44:GTC → TTC	Ps^+ (240 min), Sti^R, Myx^{HS}	85	81	24	0.4
L135G/A46T	136:GGC/46:GCG → ACG	Ps^+ (213 min), Sti^R, Myx^{HS}	97	90	39	3.6
L136G/A46V	136:GGC/46:GCG → GTG	Ps^+ (215 min), Sti^R, Myx^{HS}	92	91	52	6.5
L136G/A46P	136:GGC/46:GCG → CCG	Ps^+ (204 min), Sti^R, Myx^{HS}	nd[g]	nd	nd	nd
V44L	44:GTC → CTC	Ps^+ (nd), Sti^S, Myx^S	nd	nd	105	nd
V44F	44:GTC → TTC	Ps^+ (195 min), Sti^S, Myx^S	112	106	68	4
A46T	46:GCG → ACG	Ps^+ (190 min), Sti^S, Myx^S	85	83	122	70
A46V	46:GCG → GTG	Ps^+ (181 min), Sti^S, Myx^S	95	87	117	103

[a] Ps^+ indicates photosynthetic competence. [b] Sti^S and Myx^S refer to stigmatellin and myxothiazol sensitivity *in vivo*, respectively. Sti^R and Myx^{HS} indicate resistance and hypersensitivity to stigmatellin and myxothiazol *in vivo*, respectively. [c] One hundred percent corresponds to the cyt *b* and cyt *c* concentrations of 3.43 and 2.78 nmol/(mg of protein), respectively, in the wild type strain, as determined by dithionite-reduced *minus* ferricyanide-oxidized spectra. [d] Quantification of the Rieske Fe-S protein was conducted by monitoring the peak to peak amplitude of the EPR $g_y = 1.90$ signal which was normalized against the total cyt *b* content. [e] One hundred percent turnover number (TN) is 32 s^{-1} (moles of cyt *c* reduced per mole of cyt *b* per second) for the wild DBH_2−cyt *c* reductase activity measured at 25 °C and 550 nm, using the horse heart cytochrome *c* as the electron acceptor. [f] In this case, the chromatophores were obtained from photosynthetically grown cells and prepared in the presence of ascorbate to decrease autoxidation. [g] nd, not determined.

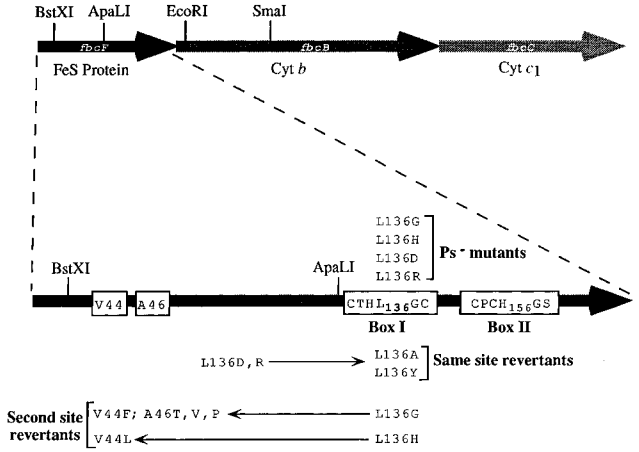

FIGURE 1: Revertants of L136R, -D, -H, and -G mutants of the *R. capsulatus* Rieske Fe-S protein subunit of the bc_1 complex. The *fbcFBC* operon, encoding the Rieske Fe-S protein and the cyt *b* and cyt c_1 subunits of the bc_1 complex in *R. capsulatus*, is depicted with the restriction sites used to construct various mutants. The conserved box I and box II sequences containing the four ligands of the [2Fe-2S] cluster (C133, H135, C153, and H156) as well as the two cysteine residues (C138 and C155) forming a disulfide bridge are shown. The same-site revertants at position 136 in the carboxyl-terminal portion and the second-site suppressor mutations at positions 44 and 46 in the amino-terminal portion of the Rieske Fe-S protein are indicated.

amino-terminal region of the Rieske Fe-S protein (Figure 1 and Table 1).

Phenotypical and Biochemical Characterizations of L136A and -Y Revertants. The same-site revertants L136A and -Y were Ps^+, but their doubling times were slightly longer than that of the wild type strain pMTS1/MT-RBC1 (about 200 versus 170 min in MPYE medium, respectively). They were Sti^R and Myx^{HS} *in vivo* (over 50 times more resistant and between 10 and 100 times more sensitive than a wild type strain on MPYE medium, respectively) (Table 1). Moreover, the bc_1 complex activity found in the chromatophore membranes of these mutants was about twice as sensitive to myxothiazol as that of a wild type strain *in vitro* (as estimated by determining the concentration of inhibitor per mole of cyt *b* necessary to inhibit 50% of the DBH_2:cyt *c* reductase activity, data not shown). Why these mutants behave so differently with respect to Myx^{HS} under *in vivo* and *in vitro* assay conditions is unknown.

Dithionite-reduced *minus* ferricyanide-oxidized optical absorption difference spectra revealed that chromatophore membranes of L136A and -Y contained wild type amounts of *b* and *c* type cytochromes associated with the bc_1 complex (Table 1). SDS−PAGE and immunological analyses with subunit specific polyclonal antibodies also confirmed these findings (data not shown) and further indicated that these revertants contained near stoichiometric amounts of the Fe-S protein subunit (Figure 2, lanes 5 and 6; Table 1). However, the steady state bc_1 complex activities detected in L136A and -Y revertants were between 20 and 26% of that of a wild type strain as measured in turnover numbers (moles of cyt *c* reduced per mole of cyt *b*/per second), in agreement with their slightly slower growth rate (Table 1).

Biophysical Characterizations of L136A and -Y Revertants. Flash-induced single-turnover transient kinetics were examined to assess the effects of the L136A and -Y substitutions

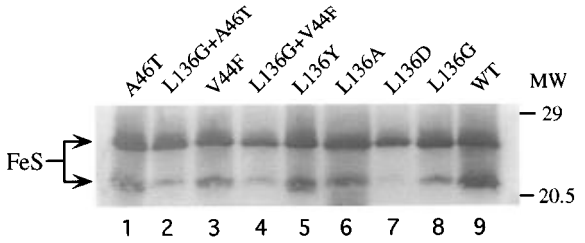

FIGURE 2: Western Blot analysis of the chromatophore membranes from various mutants of the Rieske Fe-S protein subunit of the bc_1 complex. Polyclonal antibodies raised against the purified Rieske Fe-S protein from *R. capsulatus* were used to detect the two conformers of the Fe-S protein (lower and upper bands indicated by arrows) (Robertson et al., 1993). Fifty micrograms of total chromatophore membrane proteins heated 10 min at 37 °C was loaded per lane on a 17% SDS−PA gel: lanes 1 and 3, single mutants A46T and V44F, respectively; lanes 2 and 4, second-site revertants L136G/A46T and L136G/V44F, respectively; lanes 5 and 6, same-site revertants L136Y and L136A, respectively; lanes 7 and 8, Ps$^-$ mutants L136D and L136G, respectively; and lane 9, pMTS1/MT-RBC1 used as a wild type (WT) control. No signal was detected in the bc_1 complex deletion strain [not shown, see also Liebl et al. (1997)], and the molecular mass markers (MW) of 29 and 20.5 kDa are indicated on the right.

Table 2: Flash-Induced Single-Turnover Kinetic Rates of Various L136 Revertants[a]

strain	cyt *c* re-reduction rate (s^{-1})[b]	cyt b_H reduction rate (s^{-1})[c]
WT	304	509
L136A	118	314
L136Y	115	164
L136G	2	0
L136G/V44F	50	40
L136G/A46T	75	114
V44F	148	216
A46T	203	304
A46V	155	184

[a] Values are mean of several experiments performed at $E_{h7} = 100$ mV. They were obtained by fitting the traces shown in Figure 3 to a single exponential, as described in Materials and Methods. [b] Cyt *c* rereduction kinetics were recorded at 550 nm with a reference at 540 nm. [c] Cyt b_H reduction kinetics were recorded in the presence of 10 μM antimycin at 560 nm with a reference at 570 nm.

on the electron transfer steps internal to the bc_1 complex. Kinetics of cyt *c* re-reduction in the absence of inhibitor, and cyt *b* reduction in the presence of the Q_i site inhibitor antimycin, were monitored in these mutants at an E_h of 100 mV (where the Q_{pool} is partially reduced; Ding et al., 1992) (Table 2 and data not shown). In the case of L136A, the electron transfer rates from Q_o to cyt *c* and to cyt b_H were about 2−3-fold slower than those observed in a wild type strain (118 versus 304 s^{-1} for cyt *c* re-reduction, respectively) but much faster than the rates observed in the Ps$^-$ parents L136D and -G (about 2 s^{-1}; Table 2).

Next, the effects of the L136A and -Y substitutions on UQ/UQH_2 occupancy of the Q_o site, and the ability to sense the redox state of the Q_{pool}, were assessed using EPR spectroscopy. The EPR line shape of the [2Fe-2S] cluster is known to respond to the redox state of the Q_{pool} (Takamiya & Dutton, 1979; De Vries et al., 1982) and to the presence of UQ/UQH_2 or inhibitors at the Q_o site (Ding et al., 1992, 1995a). The data presented in Figure 3A indicate that a wild type strain exhibits g_x signals of 1.782 and 1.799 at 0 mV (i.e., Q pool reduced) and 200 mV (i.e., Q_{pool} oxidized), respectively. The L136A and -Y mutants have broader g_x signals centered at about 1.755 and 1.778, respectively, when

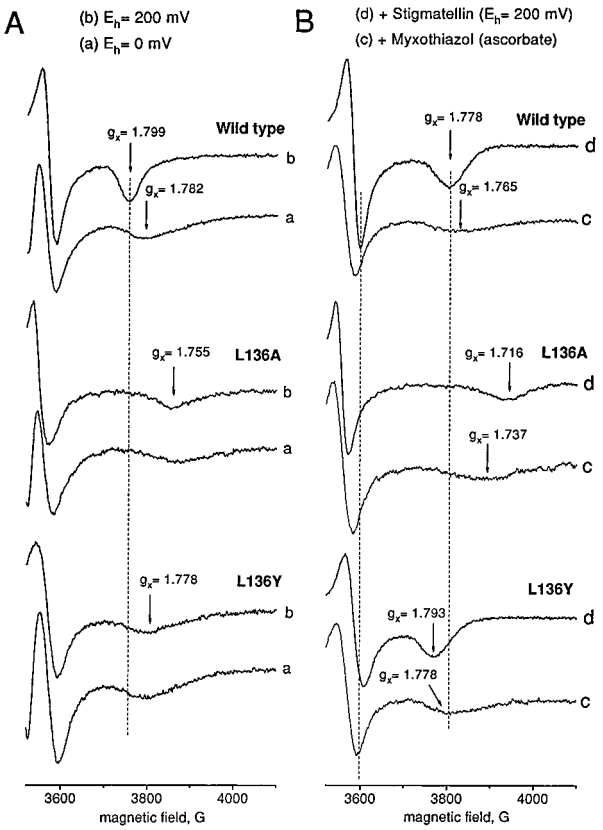

FIGURE 3: EPR spectra of the [2Fe-2S] cluster of the wild type and L136A and -Y same-site revertants of the Rieske Fe-S protein. (A) E_h was poised at 0 mV (a) and 200 mV (b) where the Q_{pool} is completely reduced and oxidized, respectively. For the EPR conditions, see Materials and Methods, and the g_x signal thus recorded was used to monitor the Q_o site occupancy. (B) EPR spectra in the presence of the Q_o site inhibitors 60 μM myxothiazol (c) and 40 μM stigmatellin (d). Vertical dashed lines indicate the wild type g_x and g_y values to be compared with those observed in the others strains. Different scales are used to better depicts the EPR spectra of strains with substoichiometric amounts of the Rieske Fe-S protein, and different E_{m7} values. Thus, the amplitudes of the signals shown do not reflect directly the concentration of the Fe-S protein in these strains. See Table 1 for the amounts of the Fe-S proteins in different mutants determined on the basis of their g_y amplitude.

the Q_{pool} is reduced or oxidized (Figure 3A, traces a and b), suggesting that they do not monitor the Q_{pool} redox state as well as a wild type strain. Further, the flat g_x signal around 1.76 at 0 and 200 mV (Figure 3 and Table 3) indicated that in both mutants the occupancy of the Q_o site has decreased, but not to the level of the parental Ps$^-$ L136 substitutions described in the preceding paper (Liebl et al., 1997).

Stigmatellin (von Jagow & Ohnishi, 1985) and myxothiazol (von Jagow & Engle, 1981; Meinhardt & Crofts, 1982; De Vries et al., 1983) inhibit completely the Q_o site function of a wild type bc_1 complex and alter the EPR spectra for the [2Fe-2S] cluster with characteristic g_x values of 1.778 and 1.765, respectively (Figure 3B and Table 3). Considering the fact that L136A and -Y were Sti^R and Myx^{HS} *in vivo*, the effects of individual inhibitors on these mutants were monitored by EPR spectroscopy. Addition of stigmatellin to chromatophore membranes of L136A and -Y shifted their g_x signals toward higher and lower magnetic fields compared to that of the wild type, respectively, while their g_y transitions were shifted in the opposite direction, yielding more rhombic (L136A) and less rhombic (L136Y) features for the [2Fe-2S] cluster in these revertants (Figure 3B, traces d; Table

Table 3: EPR Characteristics of the Rieske [2Fe-2S] Cluster in Various L136 Revertants

	g_x[a]			E_{m7} (mV)[b]	
strain	0 mV	200 mV	200 mV with stigmatellin	without stigmatellin	with stigmatellin
WT	1.782	1.799	1.778	312	460
L136G	1.754	1.762	1.725	196	386
L136H	1.753	1.754	1.808	289	≈370
L136A	1.747	1.755	1.716	265	445
L136Y	1.780	1.778	1.793	265	439
L136H/V44L	1.743	1.748	1.807	245	≈440
L136G/V44F	1.77 (broad)	1.76 (broad)	1.74 (broad)	245	377
L136G/A46T	1.756	1.758	1.723	248	413
V44L	1.782	1.798	1.774	381	≈480
V44F	1.789	1.800	1.778	386	486
A46T	1.785	1.801	1.776	385	485

[a] Values were obtained from the analysis of the EPR spectra shown in Figures 4, 6, and 8. The g_x values correspond to the center of the trough at 0 or 200 mV in the absence or presence of the Q_o site inhibitor stigmatellin. Note that these values become less accurate when the signal is broader and smaller in some mutants. [b] The E_{m7} value of the [2Fe-2S] cluster and the effect of stigmatellin are also reported (note that for L136H, L136H/V44L, and V44L these values were determined less accurately than those in the other cases).

3). Note that the g_x trough in L136Y in the presence of stigmatellin is narrowed and shifted toward a low magnetic field (Figure 3B, traces d), similar to what is observed in the wild type strain at 200 mV in the absence of inhibitor (Figure 3A, traces b). The significance of this observation in molecular terms is unclear at this time. Finally, the EPR signal induced by myxothiazol was broad, and in both L136A and -Y less distinct from that seen with a wild type strain, but shifted toward high and low magnetic fields with g_x values of 1.737 and 1.778 in L136A and -Y, respectively compared to 1.765 in wild type (Figure 3B, traces c).

Potentiometric titrations between 0 and 450 mV of the EPR g_y signal of the Fe-S protein in L136A and -Y, in the presence or absence of stigmatellin, are shown in Figure 4, and the E_{m7} values determined are summarized in Table 3. The revertants L136A and -Y had an E_{m7} of 265 mV, about 50 mV lower than that of a wild type strain (approximately 310 mV). Moreover, in these Ps^+ revertants, stigmatellin also raised the E_{m7} values of their Fe-S proteins like a wild type strain (Figure 4 and Table 3).

In summary, the overall data established that the same-site revertants L136A and -Y are the first Sti^R mutations located in the Fe-S protein, and they have unusual but functional Q_o sites that interact differently with stigmatellin.

Phenotypical and Biochemical Characterizations of the Second-Site Revertants of L136 Mutants. The second-site revertants L136H/V44L, L136G/V44F, and L136G/A46T, -V, or -P grew slightly slower with Ps growth (doubling times between 190 and 240 min on MPYE medium) than the same-site revertants L136A and -Y and were all Sti^R and Myx^{HS} *in vivo* (Table 1). SDS−PAGE and immunoblot analyses of the chromatophore membranes of these mutants (Figure 2, lanes 2 and 4) as well as the quantification of the g_y signal of their [2Fe-2S] cluster by EPR spectroscopy (Table 1) indicated that, like their Ps^- parents L136H and -G, they had normal amounts of *b* and *c* type cytochromes but substoichiometric amounts of the Fe-S protein. In addition, the steady state bc_1 complex activity exhibited by these double mutants was very low (5% or less than that seen in a wild type strain) and variable (from 15% for L136H/V44L when the chromatophore membranes were prepared in the presence of sodium ascorbate to minimize oxidation to 0.4% for L136G/V44F when unprotected). Thus, they were very similar to their Ps^- parents with respect to their inhibitor response, oxidative damage of the bc_1 complex, and subunit substoichiometry albeit not with respect to their ability to grow photosynthetically. These findings indicated that the second-site mutations mediated suppression without eliminating the existing defects.

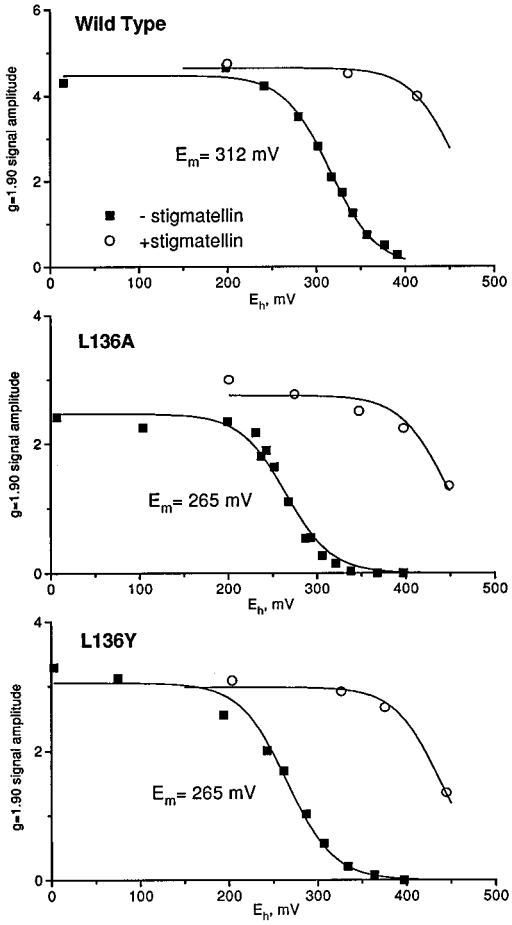

FIGURE 4: Potentiometric dark titrations of the [2Fe-2S] cluster of the Rieske Fe-S protein in the wild type and the same-site revertants L136A and -Y. Chromatophore membranes were resuspended in 100 mM KCl, 50 mM MOPS, and 1 mM EDTA at pH 7.0 in the presence of appropriate mediators (Materials and Methods). The amplitude of the EPR g_y signal recorded between 0 and 450 mV in the presence or absence of the Q_o site inhibitor stigmatellin was fit to an $n = 1$ Nernst equation to deduce the corresponding E_{m7} values.

Biophysical Characterizations of the Second-Site Revertants of L136 Mutants. Flash-induced single-turnover kinet-

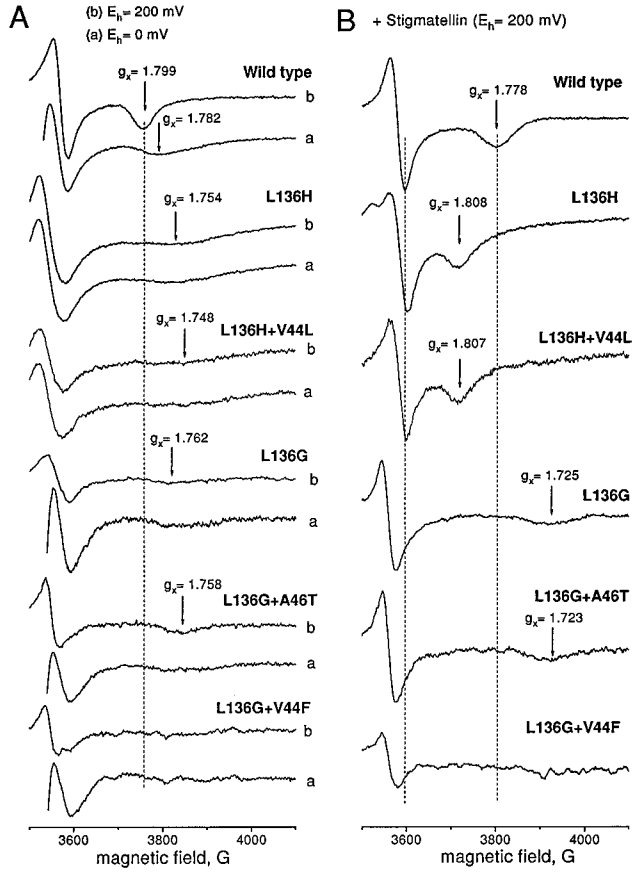

FIGURE 5: EPR spectra of the [2Fe-2S] cluster of the Rieske Fe-S protein in the wild type, the Ps$^-$ mutants L136H and -G, and their second-site revertants L136H/V44L, L136G/A46T, and L136G/V44F of the Rieske Fe-S proteins. (A and B) The experimental conditions used were as in Figure 4, except that only traces obtained in the presence of stigmatellin are shown in panel B.

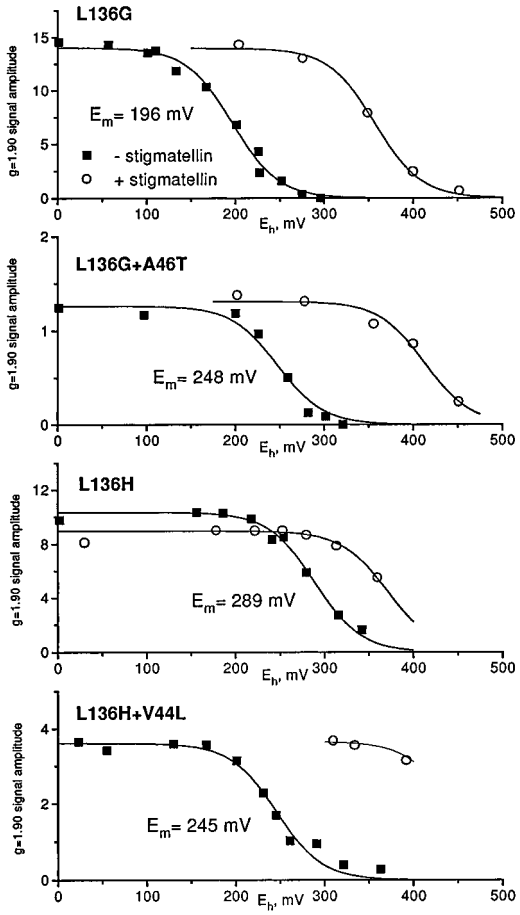

FIGURE 6: Potentiometric dark titrations of the [2Fe-2S] cluster of the Rieske Fe-S protein in the Ps$^-$ mutants L136G and -H and their second-site revertants L136G/A46T and L136H/V44L. Experimental conditions were as in Figure 4.

ics indicated that many of these revertants, like L136G/V44F and L136G/A46T, exhibited about 4−6-fold slower (50−75 s^{-1}) cyt *c* re-reduction and 5−10-fold slower cyt *b* reduction kinetics compared with those of a wild type strain (Table 2). Thus, the second-site mutations were weak suppressors and compensated for the catalytic defects of the Q_o site in L136H or -G only partially.

EPR spectroscopy indicated that the [2Fe-2S] clusters in the L136H/V44L, L136G/A46T, L136G/A46V, and L136G/V44F mutants (Figure 5A, traces a and b; data not shown) exhibited a very broad g_x signal centered around 1.76 (typical for an almost empty Q_o site; Ding et al., 1992), and did not respond to the Q_{pool} redox state. In addition, various second-site revertants responded differently to stigmatellin (Figure 5B). For example, in L136H/V44L, the g_x signal specific to this inhibitor ($g = 1.807$) was identical to that seen in its Ps$^-$ parent L136H and shifted toward the lower magnetic field in comparison with that seen in a wild type strain [Figure 5B and Table 3; see also Liebl et al. (1997)]. On the other hand, L136G/A46T showed very broad g_x signals (*g* values around 1.72) shifted toward the high magnetic field again like their Ps$^-$ parent L136G (Figure 5B and Table 3). Thus, clearly the nature of the mutation at position 136 (and not that at position 44 or 46) governs the type of g_x signal induced by stigmatellin. This finding was consistent with the fact that the second-site mutations mediated suppression without eliminating the defects imposed by the L136G or -H mutations to the Q_o site. The E_{m7} values of the [2Fe-2S] cluster of the Fe-S protein in the Ps$^+$ double mutants L136G/V44F and L136G/A46T were around 245 mV, a value much higher than that (196 mV) of the Ps$^-$ parent L136G but lower than that (312 mV) of a wild type Fe-S protein (Figure 6 and Table 3). On the other hand, in L136H/V44L, this value was 245 mV, about 40 mV lower than that of its respective Ps$^-$ parent L136H (Liebl et al., 1997). Why these two mutants behaved differently in this respect is unclear at this time.

In summary, the Ps$^+$ double mutants L136G/V44F and L136G/A46T or -V analyzed in this work had substoichiometric amounts of the Fe-S protein subunit in their bc_1 complexes, almost empty Q_o sites, and modified responses to stigmatellin. This further confirmed that the second-site mutations found in the amino-terminal part of the Rieske Fe-S protein subunit do not overcome the Q_o site defects by eliminating the impairments inflicted by the initial mutations, but rather by attenuating them via additional contributions from the amino-terminal part of this subunit to the Q_o site.

Characterization of the Suppressor Mutations Located in the Amino-Terminal Region of the Rieske Fe-S Protein. To better define the molecular nature of the suppression, the mutations at position 44 or 46 were segregated from the original mutations at position 136 by appropriate constructions as described in Materials and Methods. The single mutants V44L and -F and A46T and -V thus obtained were Ps$^+$ (doubling times of 181−195 min in MPYE medium) and Sti^S Myx^S like the wild type strain pMTS1/MT-RBC1

(Table 1). This finding indicated that the inhibitor phenotype of the second-site revertants was unrelated to the compensatory substitutions at position 44 or 46. Therefore, this feature must be an intrinsic property of the mutations at position 136.

Optical difference and EPR spectroscopy, SDS−PAGE, and immunoblotting analyses indicated that the single mutants contained wild type levels of the bc_1 complex (Table 1 and Figure 2). Further, their steady state bc_1 complex activity was between 70 and 100% of that of the wild type strain, with the exception of the V44F (Table 1), while their cyt *c* re-reduction rates measured by single-turnover kinetics, including those of V44F, were at most 2-fold lower (148−203 s^{-1}) than that (304 s^{-1}) of the wild type strain (Table 2). Why the steady state turnover of the bc_1 complex in V44F was about 20-fold lower than that of the wild type (Table 1) while its single-turnover rates were only lowered by 2-fold (Table 2) is unknown. Whether this observation reflects the inability of this mutant to use efficiently DBH_2 instead of the endogenous UQ_{10} remains to be determined.

EPR spectroscopy indicated that the suppressor mutations located in the amino-terminal end of the Fe-S protein were like wild type with respect to their Q_o site occupancy, response to stigmatellin, and ability to monitor the Q_{pool} redox state (Table 3 and data not shown). However, while the V44L and -F and A46T and -V substitutions did not affect UQH_2 oxidation when position 136 is occupied with leucine, unexpectedly, they raised drastically the E_{m7} values of the [2Fe-2S] cluster by about 70 mV, higher than that of the wild type Rieske Fe-S protein (Figure 7). Note that, since the Rieske Fe-S protein is thought to be the immediate electron donor to cyt c_1 and that its E_{m7} value is about 325 mV (Robertson et al., 1993), this thermodynamically unfavorable situation (i.e., E_{m7} of the Rieske Fe-S protein being higher than that of cyt c_1) apparently does not affect appreciably the overall turnover rate of the bc_1 complex in the suppressor mutants, with the exception of the V44F mutant mentioned above.

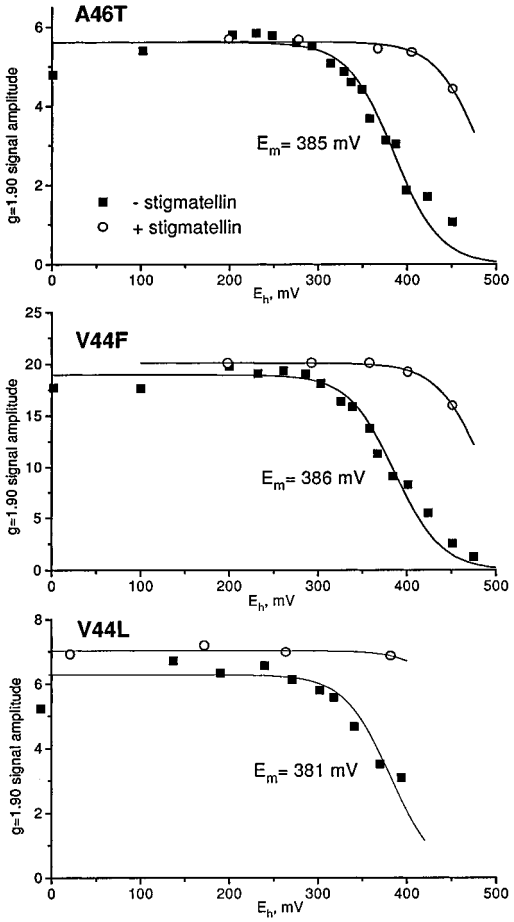

FIGURE 7: Potentiometric dark titrations of the [2Fe-2S] cluster of the Rieske Fe-S protein in the suppressor mutants A46T, V44F, and V44L. Experimental conditions were as in Figure 4.

DISCUSSION

In the present work, structural and functional interactions between the amino acid residues at positions 44−46 and 136 of the Fe-S subunit of the bc_1 complex were studied using genetic, biochemical, and biophysical approaches. Mutagenesis of position 136 of this protein described in the preceding paper (Liebl et al., 1997) established that L136R, -H, -D, and -G mutants were Ps^- mutants due to the impairment of the Q_o site of the bc_1 complex. Here, Ps^+ revertants of these mutants were characterized to define the role of the Fe-S protein in Q_o site catalysis, and yielded new insights into substrate and inhibitor binding, and the modulation of the physicochemical properties of the [2Fe-2S] cluster. In particular, this is the first report that describes the isolation and characterization of Sti^R mutants located in the Rieske Fe-S protein and that reveals functional involvement of the amino-terminal portion of this subunit with respect to the Q_o site of the bc_1 complex.

Analysis of a large number of independent Ps^+ revertants of the Ps^- L136R, -H, -D, and -G mutants revealed that the Ps growth ability, and hence the bc_1 complex activity, was restored in two ways. The first route involved the same-site revertants at position 136 (L136A and -Y), and the second one used second-site revertants which contained an additional mutation in the amino-terminal part of the Fe-S protein (L136H/V44L, L136G/V44F, and L136G/A46T, -V, or -P). The nature of the mutations encountered at position 136, along with the codon usage and the theoretically possible substitutions, suggested that the amino acid side chains that yield a functional Q_o site are restricted. Among the revertants, no charged residues were found at positions 44−46 or 136, suggesting that a charge at these positions may be deleterious. In addition, while the Ps^+ revertants L136A and -Y which required two base pair changes were readily obtained, no L136S, -C, or -P substitutions which could have emerged because of a single base change were encountered. This observation underlines the highly hydrophobic nature of the amino acid side chain required at this position of the Fe-S protein for a functional Q_o site (Liebl et al., 1997).

In contrast to the same-site revertants, the second-site revertants of the L136H and -G mutants were in general weaker suppressors as indicated by their poor Ps growth, low and labile bc_1 complex activity, and substoichiometric amounts of the Rieske Fe-S protein with respect to the other subunits of the bc_1 complex (Figure 2 and Table 1). The molecular mechanism underlying this substoichiometry is unclear, but increased oxygen sensitivity of the [2Fe-2S] cluster because of a decreased E_{m7} or enhanced proteolytic degradation induced by structural changes is plausible (Davidson et al., 1992; Graham et al., 1993). In any event, these observations reconfirmed our earlier finding that, in

the absence of the Rieske Fe-S protein, cytochromes *b* and c_1 subunits form a stable bc_1 subcomplex which can be purified and reactivated upon reconstitution with the Rieske Fe-S subunit *in vitro* (M. Valkova-Valchanova et al., in preparation).

Among the second-site suppressors of the L136 mutants, V44F was unusual. While the V44L and A46T and -V mutants had no drastic effect on Ps growth and bc_1 complex activity, V44F exhibited a surprisingly low steady state bc_1 complex activity (Table 1), but quasi-wild type single-turnover kinetics and Q_o site occupancy (Table 2). This interesting riddle may be related to the structural differences between the hydroquinone molecules oxidized at the Q_o site under steady state (DBH_2) and single-turnover (UQH_2) assay conditions. The synthetic substrate DBH_2 has a shorter hydrophobic tail than the endogenous ubihydroquinone UQ_{10} used during single-turnover kinetics. In any event, if indeed V44F is a suppressor mutation improving hydroquinone binding at the Q_o site, then this position apparently far away (about 30 Å) from position 136 on the structure of the soluble bovine Rieske Fe-S protein (Iwata et al., 1996) must somehow affect the UQ/UQH_2 catalysis at the Q_o site.

The study of the intragenic revertants of the Rieske Fe-S protein reported here provided information on three crucial issues related to the structure and function of the Q_o site of the bc_1 complex.

(a) UQ/UQH_2 Occupancy of the Q_o Site and Inhibitor Response. Until this work, all Sti^R mutants of the bc_1 complex have been located in cyt *b* [for a review, see Brasseur et al. (1996)], and it was not clear why despite extensive searches (Graham et al., 1993) they have never been observed in the Rieske Fe-S protein known to interact with this inhibitor. The data obtained here reveal that two base pair changes are required at position 136 of *R. capsulatus* Rieske Fe-S protein to convert the wild type leucine residue to a functional Ps^+ Sti^R substitution like A or Y. Thus, the earlier lack of Sti^R mutations located in this subunit may simply reflect this lower probability.

Initially, whether the mutations at position 136 constituted the molecular basis of Sti^R was unclear since the L136R, -H, -D, and -G mutants had no functional bc_1 complexes (Liebl et al., 1997). This work revealed that the same-site and second-site revertants confer Sti^R and Myx^{HS} (Table 1) and that the segregated suppressor mutations at the amino-terminal end of the Rieske Fe-S protein (V44L and -F and A46T and -V) are like wild type in response to Q_o site inhibitors. Therefore, the substitutions at position 136 must be responsible for these inhibitor phenotypes. In addition, the finding that the EPR spectra of the second-site revertants (e.g., L136H/V44L) are very similar to those of their parents (e.g., L136H) further confirms that the effects are due to the mutations located at position 136 in the vicinity of the [2Fe-2S] cluster and not to those present at the amino-terminal end of this subunit.

A comparison with respect to the Q_o site UQ/UQH_2 occupancy of the same-site and second-site revertants with the single suppressor mutations located at positions 44 and 46 indicates that the mutations at position 136 decrease UQ/UQH_2 occupancy. The three-dimensional structure of the Rieske Fe-S protein indicated that L136 sticks out of the protein (Iwata et al., 1996), and this work demonstrated that this residue closely interacts with both stigmatellin and UQ/UQH_2. The details of these interactions are unknown, but they could be similar to those described by Lancaster and Michel (1996) for the L subunit His190-Leu189 residues at the Q_B site of the reaction center. Resolution of the structure of the reaction center crystallized in the presence of stigmatellin revealed that His190, which is ligated to the iron in the reaction center, also binds UQH_2 and stigmatellin via a double hydrogen bond from the δ-nitrogen atom of its imidazole ring to the oxygen atoms of the chromone ring of stigmatellin (Lancaster & Michel, 1996). In addition, the immediately adjacent residue Leu189 is in van der Waals contact with the hydrophobic tails of stigmatellin and UQ/UQH_2. These interactions were mediated by similar planar orientations of the UQH_2 and chromone rings, and no water molecule was present in the Q_B site occupied by stigmatellin unlike when it was occupied by UQH_2. Link and Iwata (1996) have noted this close analogy between the UQ/UQH_2 and stigmatellin binding to the Q_B site of the reaction center. They have proposed, in conjunction with the structural analogy between the Q_o site of the bc_1 complex and the Q_B site of the reaction center described earlier (Robertson et al., 1990), that L136 of the Fe-S protein, which is adjacent to the cluster ligand H135 (*R. capsulatus* numbering), may also be involved in binding stigmatellin to the Q_o site. Accordingly, stigmatellin would be coordinated to the H135 ligand via a double H bond, and be in van der Waals contact with L136 as in the reaction center. Furthermore, these authors also noticed that, at the Q_B site, Ser223 of the L subunit of the reaction center provides a hydrogen bond to stigmatellin and UQH_2 via its hydroxyl group. Interestingly, a highly conserved serine residue (S158 in *R. capsulatus* Fe-S protein box II) is also part of the Q_o site (Figure 1), and its replacement with leucine leads to the absence of the [2Fe-2S] cluster and the Rieske Fe-S apoprotein in yeast (Gatti et al., 1989). Obviously, in light of these observations, the role played by S158 needs to be analyzed in detail to define its function in the Q_o site of the bc_1 complex.

(b) Modulation of the E_{m7} Value of the [2Fe-2S] Cluster of the Fe-S Protein. This work revealed that it is possible to modulate the E_{m7} value of the [2Fe-2S] cluster of the Fe-S protein by about 70 mV in both directions without hindering drastically the activity of the bc_1 complex. While the Ps^- substitutions at position 136 decreased it by about 100 mV (e.g., 196 mV in L136G) (Liebl et al., 1997), this value was raised to between 245 and 265 mV in the same-site (L136A and -Y) and the second-site revertants (L136G/V44F and L136G/A46T). Consistent with this increase, the E_{m7} values observed with the V44F and -L and A46T single mutants were around 385 mV, about 70 mV higher than that of the wild type protein (312 mV). Thus, the increase seen in the double mutants is apparently due to the additive effects of the different mutations in the amino- and carboxyl-terminal regions of the Rieske Fe-S protein. How the amino-terminal part of the Fe-S protein increases the E_{m7} of the [2Fe-2S] cluster is for the moment unclear, and the available structure of the soluble bovine Rieske Fe-S protein does not encompass this region located about 30 Å from the [2Fe-2S] cluster. The three-dimensional structure of the entire bc_1 complex, currently in progress (C.-A. Yu, personal communication; E. Berry personal communication), should shed light on this issue.

In any event, since some of the Ps^- mutants have higher (e.g., $E_{m7} = 289$ mV in L136H) and other Ps^+ mutants lower (e.g., $E_{m7} = 210$ mV in T134G) E_{m7} values for their Rieske

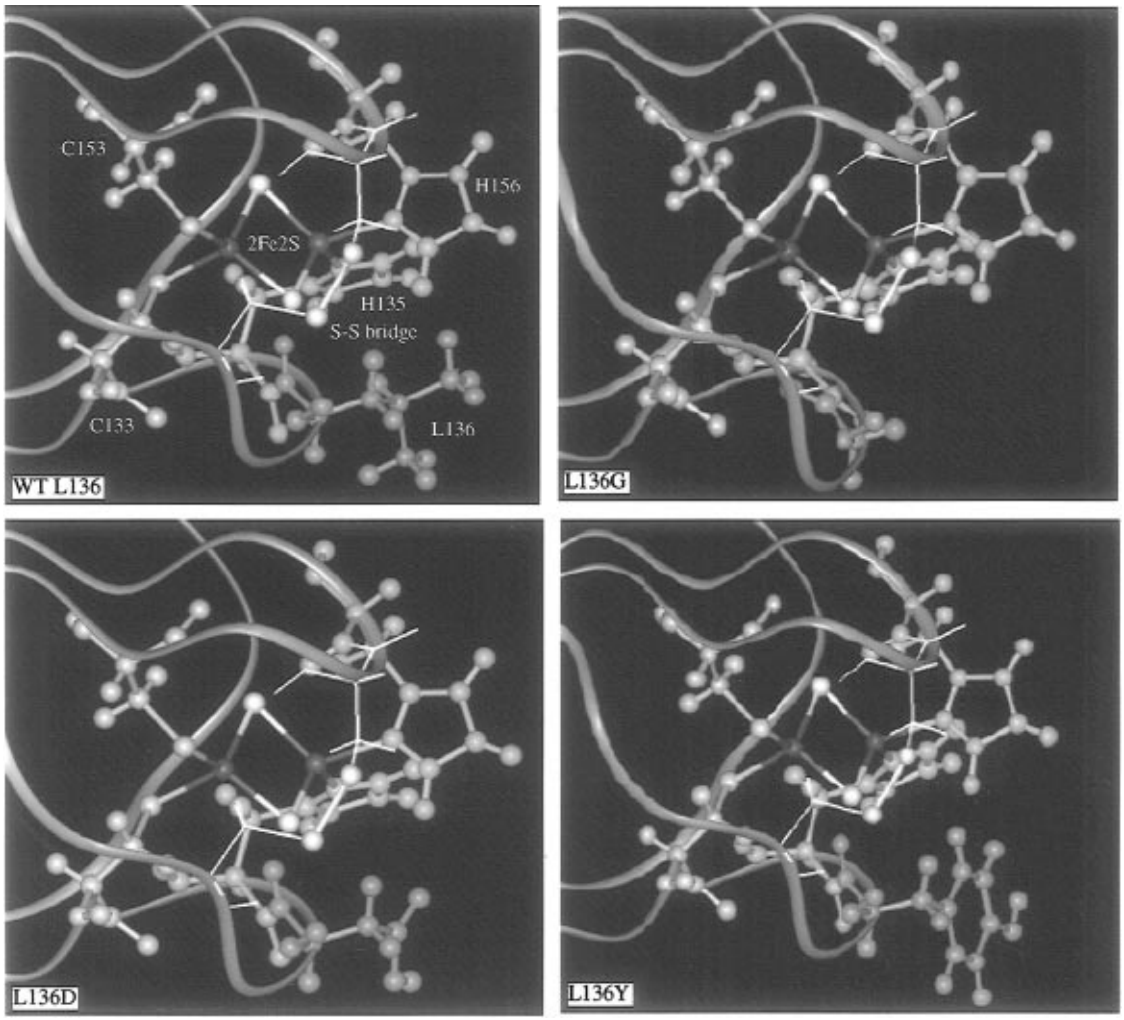

FIGURE 8: Model of the [2Fe-2S] cluster binding region of the Fe-S protein in the wild type, the Ps^- mutants L136G and -D, and the same-site revertant L136Y. The figure is intended to depicts the three-dimensional conformation of the amino acid residue at position 136 using the atomic coordinates of the bovine soluble Rieske Fe-S protein (Iwata et al., 1996). 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 (*R. capsulatus* numbering) are almost perpendicular to each other and are shown in blue. The Cys133 and Cys153 ligands are in pink, and the Cys138 and Cys155 residues forming a disulfide bridge are yellow. The green residue highlights position 136 (142 in the bovine Fe-S protein) where the mutations are displayed.

Fe-S protein, the molecular basis of the functional defect in the Ps^- mutants cannot be attributed only to this feature. Considering that in these mutants the UQ/UQH_2 occupancy of the Q_o site is also perturbed, it is likely that UQ/UQH_2 binding is equally important. However, a decreased UQ/UQH_2 occupancy of the Q_o site does not always modify the E_{m7} of the Rieske Fe-S protein since many cyt *b* mutants with an empty Q_o site have unchanged E_{m7} values for their [2Fe-2S] clusters, and are functional (Ding et al., 1992, 1995a). Moreover, the EPR characteristics of the bovine soluble Rieske Fe-S protein are also similar to those of an empty Q_o site, yet its E_{m7} value is very close to that of the [2Fe-2S] cluster when it is associated with the bc_1 complex (Link et al., 1996a).

Rationalization of how both the E_{m7} value of an Fe-S protein and its g_x signal are modified simultaneously by a single mutation involves several parameters. At least four additional parameters, the electrostatic potential around the cluster, the hydrogen bond network to the redox active groups, the changes in local dielectric constants due to solvent accessibility and fixed water molecules, and the conformational constraint around a metal center, have been proposed to modulate the E_{m7} values of proteins containing redox-active prosthetic groups (Moore et al., 1986). First, note that the fourth cysteine ligand of the Fe of *Desulfovibrio vulgaris* rubredoxin (Adman et al., 1991) corresponds to L136 of the Fe-S protein of the bc_1 complex when the metal clusters are superimposed, underlining the critical role of this position for the geometry of the Fe cluster (Iwata et al., 1996). In addition, the backbone amide group of L136 is H-bonded to the inorganic sulfur-2 of the [2Fe-2S] cluster and the H-bond network around the cluster has been proposed to be an important contributor to the high E_{m7} value of the Rieske cluster (Iwata et al., 1996). Mutations at position 136 could distort the backbone of the Rieske Fe-S protein, thereby affecting this H bond, and modify drastically the redox properties of the cluster. It has been described recently that, in the *R. sphaeroides* reaction center, the E_{m7} value of the bacteriochlorophyll dimer can be modulated by more than 300 mV with mutations affecting the hydrogen bonding between the histidine residues and the Bchl dimer (Lin et al., 1994). In this case, the removal of a H bond decreases the E_{m7} value whereas the addition of a new H bond increases it in an additive manner, yielding mutants with altered electron transfer properties (Mattioli et al., 1995). Therefore, if this finding is extrapolated to our study, the lower E_{m7} of

the mutants could be attributed to the removal of H bonds between the backbone of L136 and the inorganic sulfur of the [2Fe-2S] cluster.

On the other hand, an alternate hypothesis for rationalization of the lower E_{m7} of the [2Fe-2S] cluster in our mutants could be a change of the solvation of the cluster environment, especially when the amino acid side chain at position 136 becomes small and more polar, like L136G. The revertants encountered in this work at position 136 (L136A and -Y), 44 (V44F and -L), or 46 (A46V, -T, and -P) which increase the size or hydrophobicity of the side chain at their respective positions to ultimately decrease the solvent accessibility of the cluster environment are compatible with this idea. Note in particular that an additional methyl group is sufficient to restore Q_o site function, as seen with the L136G (Ps^-) and L136A (Ps^+) mutants.

Finally, Langen et al. (1992) reported that H bonding from amide groups to Fe-S clusters and the presence of water in the neighborhood of the cluster are important for the reported differences in the E_{m7} values of the Fe-S proteins. Thus, several mechanisms could be involved in modulating the E_{m7} values of the Rieske Fe-S proteins in the mutants described here. Figure 8 depicts the cluster region of the Rieske Fe-S protein, with position 136 occupied with various side chains. Our molecular modeling trials based on the structure of the bovine soluble Fe-S protein suggested that different substitutions at position 136 can modify both the H bond network around the [2Fe-2S] cluster and its solvent accessibility, with the most drastic effect being seen in the case of the L136G mutation, which yields a Ps^- mutant with the lowest [2Fe-2S] cluster E_{m7} value (Liebl et al., 1997).

(c) The Amino-Terminal Portion of the Fe-S Protein Is Important for the Structure and Function of the Q_o Site. Alignment of the amino acid sequences of Fe-S proteins from phylogenetically diverse species reveals that, while L136 is totally conserved in all bc_1/b_6f complexes, the amino-terminal portions of the Fe-S proteins are more divergent than their carboxyl-terminal portions (Graham et al., 1993; Gray & Daldal, 1995; Sone et al., 1996; Atteia & Franzen, 1996). Nonetheless, note that V44 and A46 are well conserved among mitochondrial and bacterial species. Interestingly, in *Rhodopseudomonas viridis*, these positions are naturally occupied with leucine and proline, respectively [see Gray and Daldal (1995)], which have been encountered among the second-site revertants (V44L and A46P) analyzed here. The amino acid sequence stretch encompassing positions 44−46 corresponds exactly to the thermolysin cleavage site used to obtain the soluble Fe-S protein in the bovine bc_1 complex (Link et al., 1996a) and is also the cleavage site for a soluble periplasmic form of the *R. capsulatus* Rieske Fe-S protein, which was found very recently in revertants of a nonassembly cyt *b* mutant in *R. capsulatus* (A. S. Saribas et al., in preparation). These findings indicate that this stretch of the Rieske Fe-S protein located after the transmembrane helix is on the membrane surface and could confer some mobility to the [2Fe-2S] cluster domain in the Q_o site of the bc_1 complex. If such a mobility is linked to the oxidized and reduced state of the Rieske Fe-S protein, this movement may prevent its further reduction by the second electron from the semiquinone formed at the Q_o site, and then it may provide structural support at atomic resolution to the Q cycle mechanism of Mitchell (1975). It may also provide an experimental basis for the apparent differences in the distances between the three redox centers (Fe-S, b_L, and c_1) determined using different crystal forms of the bc_1 complex (C.-A. Yu, personal communication; and E. Berry, personal communication).

In summary, the work presented here has provided the first evidence that position 136 of the Rieske Fe-S protein is critical in binding UQH_2/UQ and stigmatellin and that the amino-terminal part of this subunit is critical for the structure and function of the Q_o site of the bc_1 complex. While the inhibitor and the hydroquinone binding pockets overlap around position 136, they clearly do not extend to amino-terminal positions 44 and 46. Yet all of these positions contribute to modulating the E_{m7} of the [2Fe-2S] cluster of the Rieske Fe-S protein. Finally, the discovery of compensatory mutations far from position 136 now reveals that the amino- and the carboxyl-terminal regions of the Rieske Fe-S protein contribute together to the Q_o site of the overall bc_1 complex. To further define in detail the interactions between these two distant regions, a higher-resolution structure of the intact Rieske Fe-S protein in the bc_1 complex, which is now in progress, will be essential.

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