Tyrosine 147 of Cytochrome *b* Is Required for Efficient Electron Transfer at the Ubihydroquinone Oxidase Site (Q_o) of the Cytochrome bc_1 Complex†

A. Sami Sarıbaş,‡ Huangen Ding,§,|| P. Leslie Dutton,§ and Fevzi Daldal*,‡,§

Department of Biology, Plant Science Institute, Department of Biochemistry and Biophysics, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania 19104

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ABSTRACT: In *Rhodobacter capsulatus*, tyrosine (Y) 147 is a highly conserved residue of the cyt *b* subunit of the bc_1 complex. It is located in the vicinity of residues altered in spontaneous inhibitor resistant mutants that affect the ubihydroquinone oxidase (Q_o) site of this enzyme. In this work, Y147 was substituted with phenylalanine (F), valine (V), serine (S), and alanine (A) using site-directed mutagenesis in an effort to investigate its specific role in the Q_o site. Of the four mutants obtained, Y147S and Y147A exhibited very low ubihydroquinone:cyt *c* reductase activities and were unable to support photosynthetic growth (Ps) while Y147F and Y147V were Ps^+. In all mutants, no changes in the redox midpoint potentials (E_{m7}) of the cyt b_H and cyt b_L, the occupancy of the Q_o site by Q/QH_2, and the flash-induced reverse electron transfer kinetics from Q_i to cyt b_H were observed. On the other hand, rates of electron transfer from Q_o to cyt b_H were mildly reduced (2−3-fold) in Y147F and V but dramatically decreased (about 20-fold) in Y147A and S, localizing the defect to the Q_o site. Thus, Y147A and S are members of a novel class of Q_o site mutants that affect the Q_o site catalysis without perturbing the accessibility or binding of the substrate. Additional insight to the role of Y147 on ubihydroquinone oxidation was gained by analyzing the Ps^+ revertants of these mutants. Two pseudorevertants contained a second mutation [isoleucine (I) or valine (V)] at the highly conserved M154 position, six residues away from Y147. These hydrophobic second-site substitutions restored electron transfer rates from Q_o to cyt b_H to near-wild type levels and conferred resistance to the Q_o site inhibitor, myxothiazol. Considering that M154V mutation alone has no effect on the Q_o site, the compensatory effects of the M154V or M154I substitutions are only needed when position 147 is occupied with a small amino acid side chain such as A or S. These spatial interactions between the positions 147 and 154 suggest that efficient electron transfer at the Q_o site of the bc_1 complex requires either bulky side chains at position 147 or hydrophobic side chains at position 154 when the former position is occupied with small residues. Thus, the overall packing and hydrophobicity of the Q_o site appear crucial for maximum catalytic activity.

Ubiquinone cytochrome *c* oxidoreductase (bc_1 complex,[1] or its plant counterpart b_6f complex) is vital for photosynthetic and respiratory electron transfer pathways in many organisms including bacteria (Dutton, 1986). It catalyzes the oxidation of ubihydroquinone (QH_2) and reduction of cyt *c* and uses the free energy difference between these molecules to establish an electrochemical gradient necessary for ATP production [for recent reviews, see Prince (1990), Knaff (1993), Trumpower and Gennis (1994), Cramer et al. (1994), and Gray and Daldal (1995)].

The simplest structure for a bc_1 complex is found in bacteria, where the corresponding genes are often organized as an operon, denoted as *fbcFBC or petABC* (Figure 1) [for a review, see Gray and Daldal (1995)]. In purple, nonsulfur facultative phototrophs such as *Rhodobacter capsulatus*, the bc_1 complex has three subunits, cyt *b*, cyt c_1, and the FeS protein (Robertson et al., 1993), and is essential for photosynthetic (Ps) growth (Daldal et al., 1987). Cyt *b* is the most highly conserved subunit among various species (Degli Esposti et al., 1993) and contains two *b*-type heme groups, named cyt b_H and cyt b_L after their high and low redox midpoint potentials (E_{m7}) of 50 and −90 mV, respectively (Robertson et al., 1993). These heme groups are located close to two distinct quinone binding sites that catalyze oxidation of QH_2 (Q_o site) and reduction of ubiquinone (Q) (Q_i site). The Q_o site is formed by various amino acid residues from both the cyt *b* and the FeS protein and is located on the positive side of the membrane (Robertson et al., 1986). During QH_2 to Q oxidation at the Q_o site, the first electron reduces cyt c_1 via the [2Fe-2S] cluster of the FeS protein, and the second electron reduces cyt b_H via cyt b_L (Glaser & Crofts, 1984; Robertson & Dutton, 1988). In *R. capsulatus*, cyt c_1 is subsequently reoxidized by the soluble

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* To whom correspondence should be addressed. Phone: (215) 898-4394. Fax: (215) 898-8780. E-mail: fdaldal@mail.sas.upenn.edu.

‡ Department of Biology.

§ Department of Biochemistry and Biophysics.

|| Present address: Department of Molecular and Cellular Toxicology, Harvard School of Public Health, Boston, MA 02115.

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[1] Abbreviations: cyt, cytochrome; bc_1 complex, ubihydroquinone cyt *c* oxidoreductase; QH_2, ubihydroquinone; Q, ubiquinone; Q_o, ubihydroquinone oxidase site; Q_i, ubiquinone reductase site; EPR, electron paramagnetic resonance; cyt b_H, high potential cyt *b;* cyt b_L, low potential cyt *b;* [2Fe-2S], iron−sulfur cluster of the Rieske FeS protein; E_h, ambient redox potential; E_{m7}, equilibrium redox midpoint potential at pH 7.0; Inh^R, inhibitor resistant; Ps, photosynthesis; 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene, DAD) PMS, N-methyldibenzopyrazine methosulfate; PES, *N*-ethyldibenzopyrazine ethosulfate; EDTA, ethylenediaminetetraacetic acid; MOPS, (*N*-morpholino)propanesulfonic acid; DBH, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzohyroquinone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SHE, standard hydrogen electrode.

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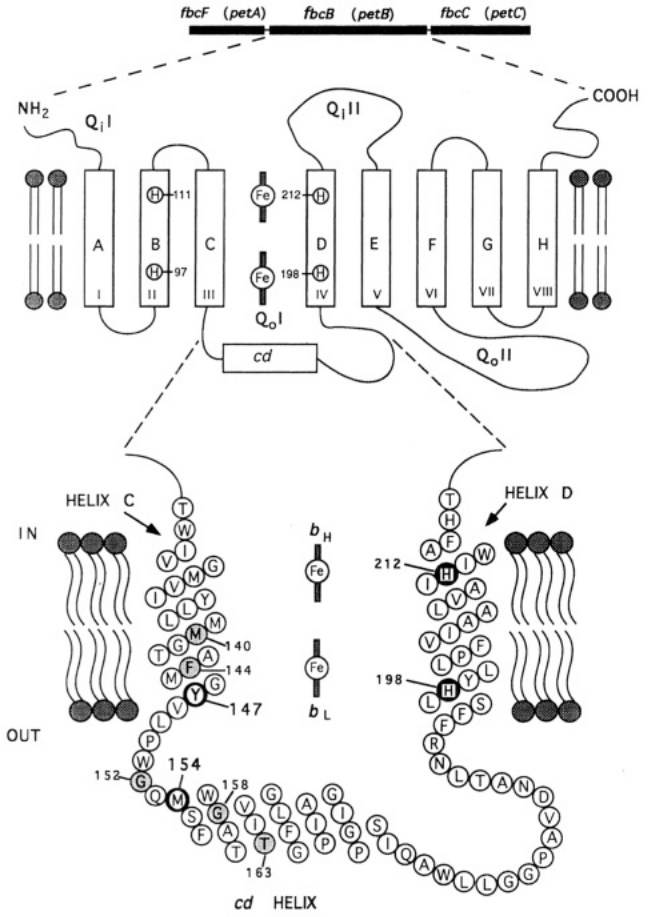

FIGURE 1: Cyt *b* subunit of *R. capsulatus* bc_1 complex. *fbcB* (*pet B*) is the structural gene encoding cyt *b* for which an eight transmembrane helices model is shown. A to H correspond to helices I to VIII. H97 and H198, and H111 and H212, are the ligands of cyt $b_L$and b_H, respectively. The Q_oI and Q_oII regions involved in QH_2 oxidation and the Q_iI and Q_iII regions involved in Q reduction are indicated. The Q_oI region is shown below in detail, and the residues Y147 and M154, studied in this work, are circled with a thick line. Positions conferring Inh^R (M140, F144, G152, G158, and T163) (Tokito & Daldal, 1993) are shaded.

cyt c_2 or the membrane-anchored cyt c_y, which conveys electrons either to the reaction center in photosynthesis (Jenney & Daldal, 1993; Jenney et al., 1994) or to the cyt *cb* oxidase in respiration (Gray et al., 1994b; Hochkoeppler et al., 1995).

Current structural models describe cyt *b* as an integral membrane protein with eight transmembrane helices (named A−H) and at least one transversal helix (*cd*) (Figure 1). These two-dimensional models are supported by several lines of evidences, including secondary structure analyses (Widger et al., 1984), alkaline phosphatase fusions (Yun et al., 1991a), and overall distribution of inhibitor resistant (Inh^R), or nonfunctional, mutants (Robertson et al., 1986; Howell et al. 1987; di Rago & Colson, 1988; Daldal et al., 1989). Genetic studies conducted in different organisms using specific Q_o site (myxothiazol, stigmatellin, and mucidin) (Daldal et al., 1989; Howell & Gilbert, 1988; di Rago et al., 1990) and Q_i site (antimycin A, diuron and funiculosin) (di Rago & Colson 1988; Howell & Gilbert, 1988; Park & Daldal, 1992; Uhrig et al., 1994; Coppe et al., 1994) Inh^R mutants highlighted two specific portions of cyt *b* (Q_oI and Q_oII) as contributing to the Q_o site of the bc_1 complex (Figure 1). The Q_oI region is located between the transmembrane helices C and D and encompasses the transversal helix *cd*.

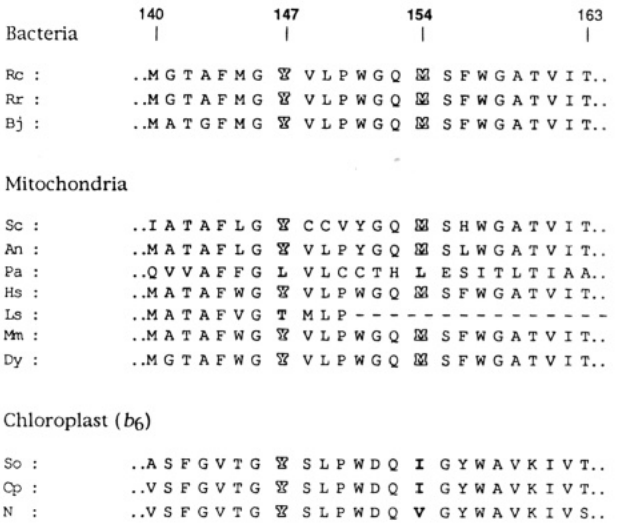

FIGURE 2: Partial amino acid sequence alignment of cyt *b* peptides from various species between position 140 and 163 (*R. capsulatus* numbering) delimiting the Q_oI region (Degli Esposti et al., 1993). Note that Y147 is almost exclusively conserved, except in Pa and Ls, where it is occupied by L and T, respectively. Position 154 is substituted by I or V only in cyt b_6 of chloroplast and is also replaced by L in Pa, and unknown in Ls (M. Degli Esposti, personal communication). Abbreviations are as follows: Rc, *Rhodobacter capsulatus*; Rr, R*hodospirillum rubrum*; Bj, *Bradyrhizobium japonicum*; Sc, S*accharomyces cerevisiae* (yeast); An, *Aspergillus nidulans* (fungi); Pa, *Paramecium aurelle* (cyliate); Hs, *Homo sapiens* (human); Ls, *Lepidophyma smithii* (lepi lizard); M.m, *Mus musculus* (mouse); Dy, *Drosophilia yakuba* (fly); So, *Spinicia oleracea* (spinach); Cp, *Chlorella protothecoides* (green alga); N, *Nostoc* (cyanobacteria).

It is involved in the recognition and binding of Q_o site inhibitors [for reviews, see Gennis et al. (1993) and Gray and Daldal (1995)] and can be labeled with azido-Q derivatives indicating its direct interaction with Q (He et al., 1994). Moreover, site-directed mutagenesis has provided crucial information on the roles of various residues both in the Q_o (Atta-Afaso-Adjei & Daldal, 1991; Tokito & Daldal, 1993) and Q_i (Yun et al., 1992; Hacker et al., 1993; Gray et al., 1994a) sites. A molecular model for the Q_o site has been proposed (Robertson et al., 1990) using the overall data accumulated through the analyses of Inh^R mutants and the similarities between the Q binding sites of the photochemical reaction center and of the bc_1 complex. This model suggests that the helices C, D, and *cd* are in close proximity to each other around cyt b_L, and that the Inh^R conferring residues F144 (on helix C) and G158 (on helix *cd*) are oriented toward the interior of cyt *b* (Tokito & Daldal, 1993) (Figure 1).

A general feature of the Q_o-site mutants studied previously is a decreased Q/QH_2 occupancy, as revealed by the EPR features of the [2Fe-2S] cluster (Robertson et al., 1990; Ding et al., 1992, 1995a). Increased myxothiazol resistance (Myx^R) is often correlated with a decreased substrate binding, yielding mutants with partially occupied Q_o sites and inactive or less active bc_1 complexes (Robertson et al., 1990; Ding et al., 1992). Thus, to define cyt *b* residues essential for Q_o site catalysis, it was important to search for mutations that affect the Q_o site reactions without hindering Q/QH_2 occupancy. In the Q_oI region of cyt *b* several highly conserved residues, including G146, Y147, W151 and Q153, are present (Figure 2) (Degli-Esposti et al., 1993). Of these Y147 is located on the same face of helix C as the residues M140 and F144, previously shown to influence the Q_o site functions

(Robertson et al., 1990; Ding et al., 1992). Further, its substitution with a cysteine in *Chlamydomonas reinhartii* (position 132 in this organism) confers Myx^R (Bennoun et al., 1992). These observations led us to examine the role of Y147 in Q_o site catalysis by substituting it with several amino acids. The mutants obtained and their compensatory second-site revertants at position M154 were analyzed using flash-induced transient kinetics, EPR spectroscopy, and equilibrium redox titrations. The overall data presented in this study demonstrate that Y147A and S substitutions define a new class of cyt *b* mutations that abolish efficient electron transfer during the initial steps of QH_2 oxidation without affecting the substrate occupancy of the Q_o site.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Genetic Methods. Wild type and mutant *R. capsulatus* strains were grown in MPYE, RCVB, or "mixed medium" [1 volume Medium A (Sistrom, 1960) + 1 volume MPYE]. *Escherichia coli* strains were grown in LB medium, supplemented with appropriate antibiotics as described previously (Gray et al., 1992).

Site-directed mutagenesis was carried out as in Atta-Asafo-Adjei and Daldal (1991) using phage M13-73R2BC1 as a template, and the mixed oligonucleotides petB-Y147, 5′-GGCAGCAC((**G(T/G)(A/C)**)GCCCATGAA-3′, and petB-M154V, 5′-CCAGAACGACA**C**CTGGCCCC-3′, to substitute Y147 with F, V, A, S, and M154 with V, respectively. After mutagenesis and sequencing, the approximately 450 bp *Sma*I−*Eco*RI fragment carrying the desired mutation was cloned from the replicative form of the corresponding phage M13 to plasmid pMTS1 containing a wild type copy of the *fbcFBC* operon (Gray et al., 1994a). The plasmids pMTS1-bY147F, V, S, A, and pMTS1-bM154V thus obtained were conjugated into *R. capsulatus* strain MT-RBC1 from which the entire *fbcFBC* operon has been deleted (Atta-Asafo-Adjei & Daldal, 1991). The mutations were then reconfirmed by sequencing the plasmid DNA isolated from these transconjugants.

Spontaneous Ps^+ revertants of the Ps^- mutants pMTS1-bY147A and pMTS1-bY147S were isolated on MPYE plates containing kanamycin using chemoheterotrophically grown cultures. One Ps^+ colony was retained from each of the eight different cultures and purified twice under the same growth conditions, and its plasmid DNA was isolated and sequenced. The entire Q_oI region [between the nucleotides 1660 to 1830, see Davidson and Daldal (1987)] of five independent pMTS1-bY147A and three independent pMTS1-bY147S revertants was analyzed in the same way.

Biochemical Methods. Chromatophore membranes were prepared in 50 mM MOPS (pH 7.0) or glycine (pH 9.0) buffer containing 100 mM KCl and 1 mM PMSF using a French Press as described before (Atta-Asafo-Adjei & Daldal, 1991), except that they were washed three times with the same buffer. Bacteriochlorophyll concentration was measured spectroscopically using an ϵ_{775} of 75 mM^{-1} cm^{-1}, and the protein amounts were determined according to Lowry et al. (1951).

Redox titrations to determine the E_{m7} of cyt b_L and cyt b_H and flash-activated time-resolved kinetics to monitor Q_o and Q_i site redox reactions were performed as before (Gray et al., 1994a) and described in detail in the preceding paper (Ding et al., 1995a). An amount of chromatophores containing 50 μM bacteriochlorophyll were used in the presence of the redox mediators as described in Dutton (1978). Similarly, flash (8 μs actinic light pulse)-induced cyt b_H and cyt *c* single turnover kinetics were measured using a dual-wavelength spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania). An amount of chromatophore containing 0.20 μM bacterial reaction center was used in the presence of 3 μM valinomycin, 2.5 μM *N*-methyldibenzopyrazine methosulfate (PMS), 2.5 μM *N*-ethyldibenzopyrazine ethosulfate (PES), 6 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (diaminodurene, DAD), 10 μM 2-hydroxy-1,4-naphthoquinone, and 10 μM $FeCl_3$-EDTA. Reaction center concentration was determined by measuring the optical absorption difference between 605 and 540 nm after four flashes at E_h = 370 mV and using an extinction coefficient of 29.8 mM^{-1} cm^{-1}, and the bc_1 complex inhibitors [antimycin (10 μM) and myxothiazol (5 μM)] were added as needed. Cyt *b* reduction and cyt *c* re-reduction were monitored at 560 *minus* 570 nm and 550 *minus* 540 nm, respectively, and the single-turnover rates were calculated as in Gray et al. (1994a). The Q_o site response to the Q_{pool} redox state was monitored by cyt *c* reduction kinetics at E_h values ranging from 220 to 50 mV. The single-turnover rates deduced from these measurements were fit to an n = 2 Nernst equation to determine the Q_o site midpoint potential (Robertson et al., 1990). The oxidation of QH_2 at the Q_i site was monitored at alkaline pH in the presence of myxothiazol as described (Glaser et al., 1984; Robertson et al., 1984).

EPR spectroscopy was used to monitor the Q/QH_2 occupancy to the Q_o site at different E_h values (400 to −300 mV) via their known interactions with the [2Fe-2S] cluster of the FeS protein (Robertson et al., 1990; Ding et al., 1992, 1995). The measurements were carried out using a Bruker model ESP-300E, equipped with a helium cryostat under the conditions described previously (Ding et al., 1992, 1995a).

Chemicals. PMS, PES, and antimycin A were purchased from Sigma (St. Louis, MO), and myxothiazol and restriction enzymes were from Boehringer-Mannheim Biochemicals. 2,3,5,6-Tetramethyl-*p*-phenylenediamine (diaminodurene) and 2-hydroxy-1,4-naphthoquinone were purchased from Aldrich, and stigmatellin was from Fluka. All other chemicals were reagent grade and purchased from commercial sources.

RESULTS

Phenotypic Characterization of the Mutations at Position Y147 of Cyt b. Photosynthetic growth ability of *R. capsulatus* being an excellent test for the presence of an active bc_1 complex (Daldal et al., 1987), the effect of the Y147F, V, S, and A mutations on Ps growth was determined. The Y147F and V mutations were Ps^+ (doubling times on MPYE medium of 180 and 200 min, respectively, versus 150 min for a wild type strain), but the Y147S and A were Ps^- with reversion frequencies of approximately 3×10^{-8} and 10^{-7}, respectively (Table 1). Of the Ps^+ mutants Y147F was as Myx^S as a wild type strain (Ps growth completely inhibited in the presence of 5×10^{-6} M myxothiazol). However, Y147V exhibited Myx^R since it was able to grow under the previous conditions, but not at higher concentrations (5×10^{-5} M) of this inhibitor. Thus, elimination of the aromatic

Table 1: Initial Characterization of Y147 Mutants

strains	phenotype[a]	reversion frequency	Ps doubling time (min)	DBH activity[a]	E_{m7} (mV)[b]		
					b_L	b_H	b_{150}
pMTS1(wt)	Ps^+/Myx^S	na	150	100[c]	−126	71	173
pBY147F	Ps^+/Myx^S	na	180	43	−100	66	172
pBY147V[d]	Ps^+/Myx^R	na	200	23	−108	71	190
pBY147S	Ps^-	3×10^{-8}	nd	14	−110	68	160
pBY147A	Ps^-	1×10^{-7}	nd	13	−132	63	172
MT-RBC1	Ps^-	na	nd	<1	na	na	na

[a] Ps^+ and Ps^- refer to photosynthetically competent and incompetent, respectively; Myx^R and Myx^S to myxothiazol resistance and sensitive respectively; nd, not determined; na, not applicable. [b] Determined as described in Materials and Methods. [c] DBH, cyt *c* reductase activity was measured as nmol of horse cyt *c* reduced $min^{-1} \cdot mg^{-1}$ membrane proteins using an ϵ_{550} of 20 $mM^{-1} \cdot cm^{-1}$ for cyt *c* and indicated as a percent of the activity that of the wt strain pMTS1, which had, in this instance, an activity of 4222 nmol of cyt *c* reduced $min^{-1} \cdot mg^{-1}$ membrane proteins. [d] This strain is slightly resistant to 5×10^{-6} M myxothiazol in MPYE plates.

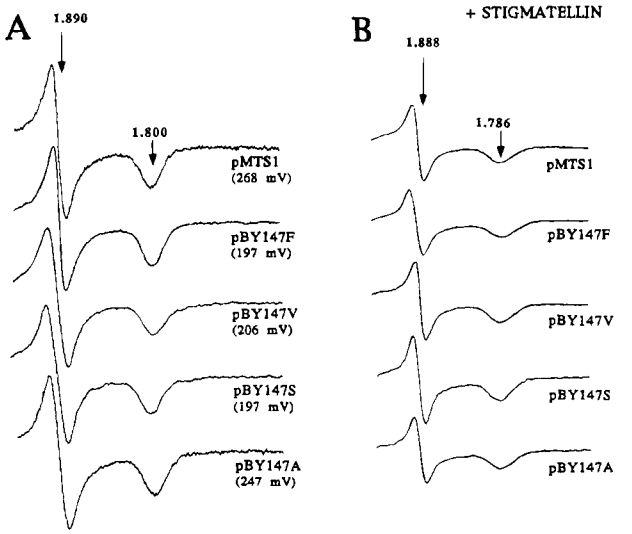

FIGURE 3: Response of the [2Fe-2S] cluster of the FeS protein EPR Signal to Q_o site Occupancy without inhibitor (A) and in the presence of stigmatellin (B) poised at an E_h value between 100 and 200 mV in various samples. In panel A and B the *g* values are $g_y = 1.890$, $g_x = 1.800$ and $g_y = 1.888$, $g_x = 1.786$, respectively.

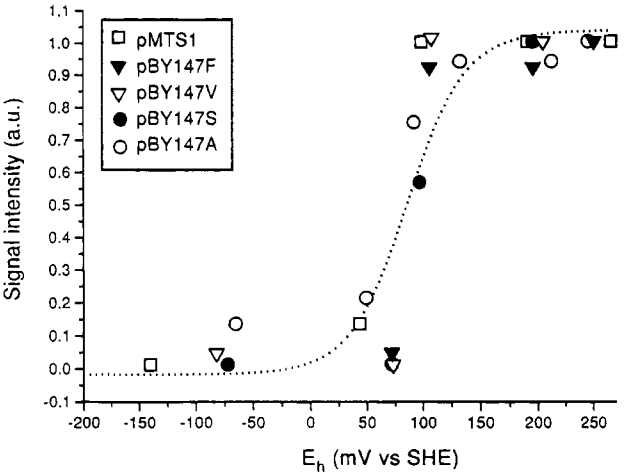

FIGURE 4: Redox titration of Q/QH_2 at the Q_o site, monitoring the g_x signal of the [2Fe-2S] cluster of the FeS protein. For each mutant, data points were collected between −150 and 250 mV and were fit separately to an $n = 2$ Nernst equation to determine the E_{m7} value of the Q/QH_2 couple in the Q_o site. The fit shown here in dotted line respresents that obtained using the data points available for all mutants and yielded an E_{m7} value for the Q/QH_2 couple of approximately 90 mV.

ring structure at position 147 increases Myx^R, as previously seen with various substitutions at position 144 (Tokito & Daldal, 1993).

The Ps^- mutants, Y147A and S, were analyzed by optical absorption difference (dithionite *minus* ascorbate and dithionite *minus* ferricyanide) spectroscopy, SDS−PAGE, and immunoblotting. Chromatophore membranes of these mutants contained all three subunits of the bc_1 complex in amounts similar to their parental strain (pMTS1/MT-RBC1, overproducing the bc_1 complex, data not shown). Thus, they probably contained an assembled but poorly active bc_1 complex. This finding was confirmed by measuring the steady-state DBH:cyt *c* reductase activity of the mutant bc_1 complexes (Atta-Asafo-Adjei & Daldal, 1991). In the Y147F, V, S, and A mutants this activity was approximately 43, 23, 14, and 13% of a wild type overproducer strain, respectively, in agreement with their Ps phenotypes (Table 1).

Biochemical and Biophysical Characterization of Y147 Mutants. Potentiometric titrations of the heme groups were performed to determine the effect of the Y147F, V, S, and A substitutions (Table 1). In all mutants, the E_{m7} values of cyt b_L and cyt b_H were virtually identical to those of a wild type bc_1 complex. Thus, these mutations did not perturb significantly the heme groups of cyt *b*.

EPR spectroscopy of chromatophore membranes has previously shown that the EPR line shape of the [2Fe-2S] cluster responds to the redox state of the Q_{pool} (Takamiya & Dutton, 1979; de Vries et al., 1982). More recent studies have indicated that various line widths and positions of this signal reflect varying degrees of occupancy of the Q_o site by Q/QH_2 or specific inhibitors (Ding et al., 1992, 1995a). Thus, effects of different Y147 substitutions on the occupancy of the Q_o site were probed using EPR spectroscopy (Figure 3). In these mutants, the EPR spectra of the [2Fe-2S] cluster in the absence (Figure 3A) and in the presence of stigmatellin (Figure 3B) were identical to those seen in a wild type strain (Ding et al., 1992). Therefore, these mutations modify neither the occupancy of the Q_o site nor the binding of stigmatellin. To further analyze the possible effects of these mutations on the redox properties of Q/QH_2 in the Q_o site, the amplitude of the g_x signal was titrated between 250 and −150 mV (Figure 4). The E_{m7} values determined for the parental strain (76 mV) and the Y147F, V, S, and A mutations (91, 91, 97, and 78 mV, respectively) were not significantly different from the previously reported value for a wild type strain (Ding et al., 1992). In addition, the E_{m7} values of the FeS protein in these mutants were determined by titrating the g_y signal between 100 and 400 mV (data not shown). In all cases these values were also

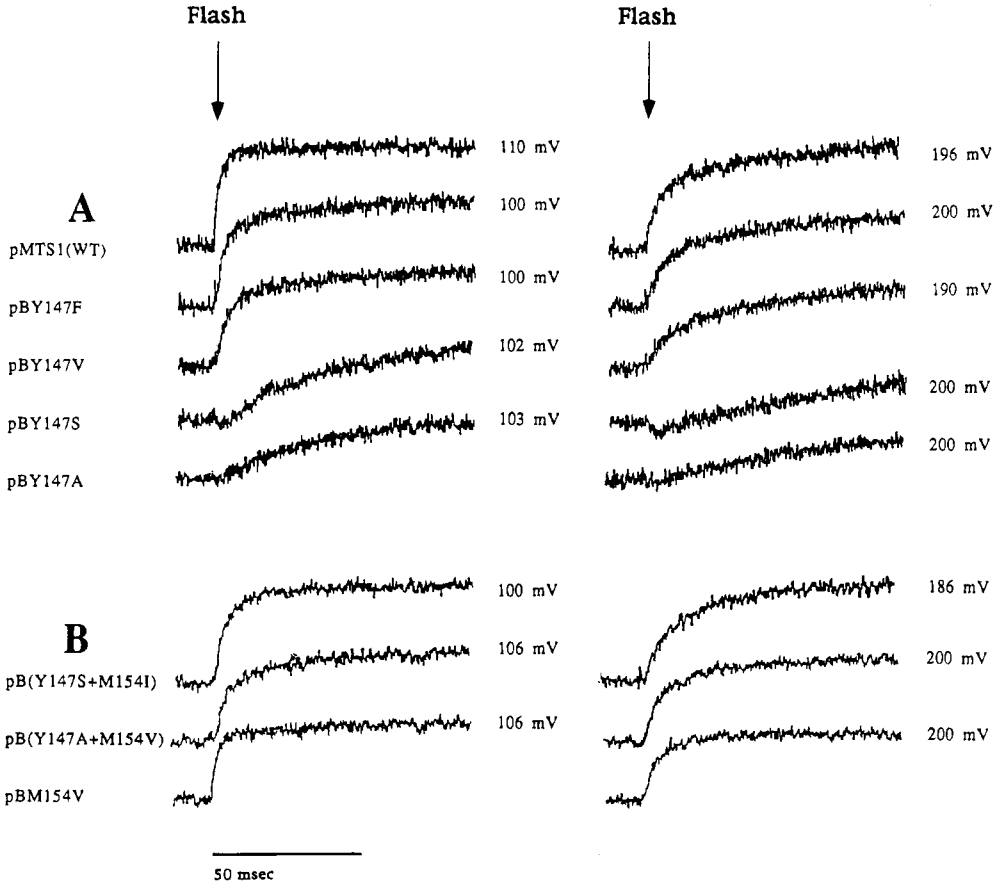

FIGURE 5: Cyt b_H reduction kinetics of Y147 mutants (A) and their revertants (B). The redox potentials were poised at either 100 to 110 mV (left panel) or 186 to 200 mV (right panel). The flash kinetics were carried out using chromatophore membranes suspended in an assay buffer containing 50 mM MOPS (pH 7.0), 100 mM KCl, appropriate mediators (see Materials and Methods), 3 μM valinomycin, and 10 μM antimycin A. In each case, an amount of chromatophore containing 0.20 μM reaction center (determined as described in Materials and Methods) was used. Difference spectra were taken at 560 and 570 nm after a short (8 μs) flash of light. The rates calculated from these traces are shown in Table 2.

Table 2: Single-Turnover Kinetics of Various Y147 Mutants and Their Revertants

	Q_{pool} half reduced				Q_{pool} oxidized					
	$Q_o \rightarrow$ cyt b_H[a]		cyt c rereduction[a]		$Q_o \rightarrow$ cyt b_H[a]		cyt c rereduction[a]		$Q_i \rightarrow$ cyt b_H[b]	
strains	s^{-1}	(mV)	s^{-1}	(mV)	s^{-1}	(mV)	s^{-1}	(mV)	s^{-1}	(mV)
pMTS1(wt)	553	(110)[c]	338	(106)	171	(196)	24	(215)	237	(117)
pBY147F	233	(100)	108	(100)	127	(200)	11	(200)	218	(120)
pBY147V	200	(100)	87	(100)	80	(200)	11	(210)	193	(120)
pBY147S	25	(102)	14	(102)	8	(200)	2	(200)	84	(120)
pBY147A	31	(103)	20	(103)	13	(190)	3	(202)	194	(110)
pBM154V	475	(106)	181	(108)	176	(200)	25	(195)	nd	
pB(Y147A+M154V)	210	(106)	98	(106)	76	(186)	12	(180)	nd	
pB(Y147S+M154I)	240	(100)	117	(103)	80	(194)	14	(196)	nd	

[a] These values were determined by fitting the traces (shown in Figures 4 and 5 and not shown, as described in Materials and Methods. [b] These experiments were carried out at pH 9.0 (where Q_{pool} is oxidized above 100 mV), and the values were determined by fitting the traces shown in Figure 7 to an exponential equation as described in Materials and Methods. [c] Numbers in parentheses indicate the ambient potential E_h (mV) at which the indicated electron transfer rate was measured. nd, not determined; wt, wild type.

similar to that of a wild type cluster [see, for example, Ding et al. (1992)]. The overall data indicated that position 147 of cyt b affect neither the occupancy of the Q_o site nor the thermodynamic properties of the [2Fe-2S] cluster. Therefore, unlike the G158D and F144L mutations studied earlier (Robertson, 1990; Ding et al., 1992), Y147S and A do not perturb the function of the bc_1 complex by decreasing the accessibility or binding of Q/QH_2 to the Q_o site.

Flash-induced single-turnover transient kinetics were used to probe the effects of the Y147S and A mutations on the internal electron transfer steps of the bc_1 complex. Cyt b_H reduction kinetics were measured in the absence and presence of the Q_i site inhibitor antimycin (Figure 5A and Table 2). In Y147F and V, at an E_h of around 100 mV where the Q_{pool} is partially reduced (E_{m7} of the Q_{pool} is approximately 90 mV), the electron transfer rates from Q_o to cyt b_H were about 2−3-fold slower (233 and 200 s^{-1}, respectively) than that of a wild type strain (533 s^{-1}). On the other hand, in Y147A and S this rate was decreased by almost 20-fold (approximately 31 and 25 s^{-1}, respectively) (Figure 4A, left panel, and Table 2). The Q_o to cyt b_H electron transfer was also measured in all mutants at an E_h of around 60 mV where

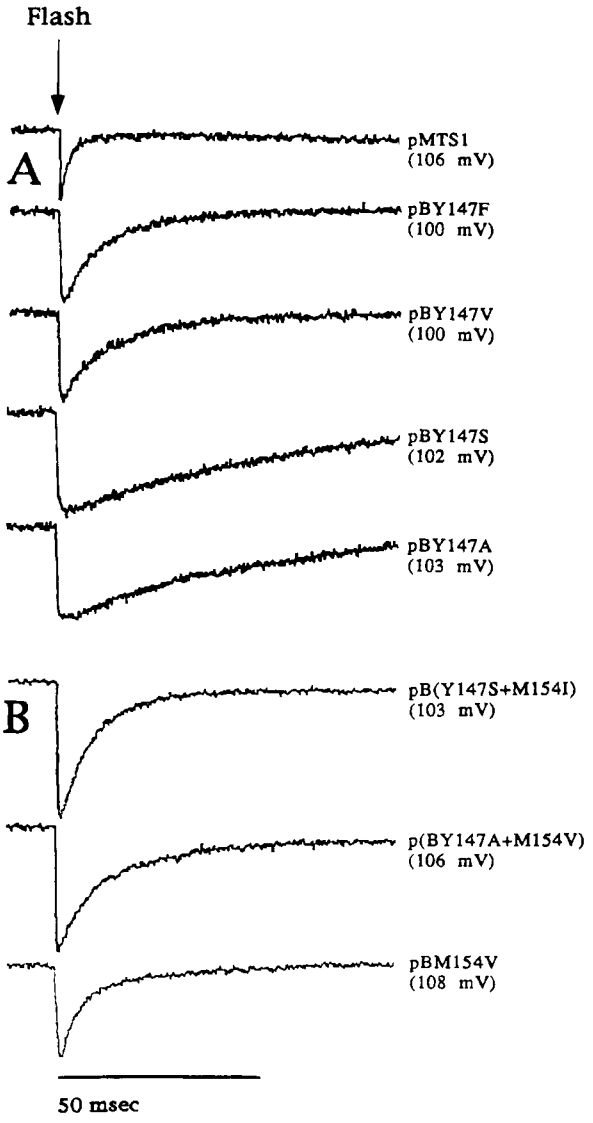

FIGURE 6: Cyt *c* re-reduction kinetics of Y147 mutants (A) and their revertants (B). The potentials were poised at 100 to 108 mV or at 200 to 215 mV (not shown). Experimental conditions were as described in the legend to Figure 2, except that no antimycin A was used. Difference spectra were taken at 550 and 540 nm after a short (8 μs) flash of light, and the rates calculated from these traces are shown in Table 2.

the Q_{pool} is more reduced than oxidized (data not shown), and around 200 mV where the Q_{pool} is fully oxidized (Figure 5A, right panel, and Table 2). At these E_h values, the electron transfer rate becomes slower [approximately 170 s^{-1} in a wild type strain; see, for example, Gray et al. (1994a)]. As expected, under these conditions the Y147F, V, S, and A mutants also exhibited slower rates of approximately 127, 80, 8, and 13 s^{-1}, respectively.

The rate of the slow phase of cyt *c* reduction, which is attributed to the second turnover at the Q_o site (i.e., the second QH_2 oxidation at this site) (Matsuura et al., 1983; Crofts et al., 1983), was also monitored at an E_h of around 100 mV (Figure 6A and Table 2). The cyt *c* reduction rate is approximately 338 s^{-1} for a wild type bc_1 complex and was approximately 108, 87, 14, and 20 s^{-1} for Y147F, V, S, and A, respectively (Table 2). Similar data obtained at an E_h of around 200 mV revealed that these rates were approximately 24, 11, and 11 s^{-1} for the wild type and the Ps^+ mutants Y147F and V, respectively. However, they were

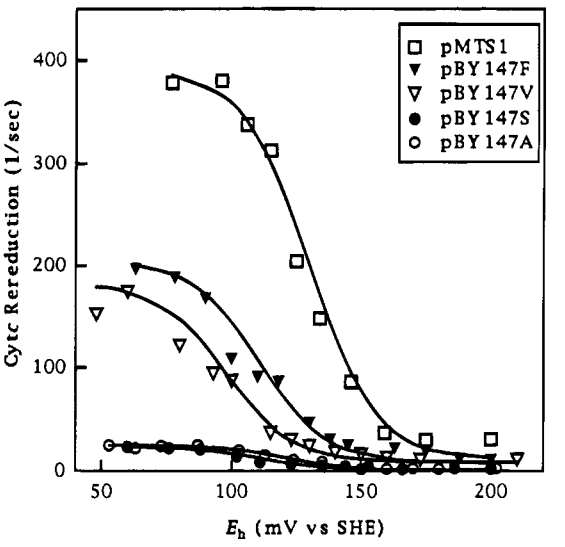

FIGURE 7: Q_o site response to varying QH_2/Q ratio in the Q_{pool} monitored by cyt *c* re-reduction kinetics. Cyt *c* kinetics were measured in mutant and wild type chromatophores (in 50 mM MOPS, pH 7.0, and 100 mM KCl with mediators) at redox potentials (E_h) between 220 and 50 mV. A single-exponential equation was used to obtain the cyt *c* re-reduction rates at various E_h values, which were then fit to a $n = 2$ Nernst equation to determine their half-maximal rates.

undetectable for the Ps^- mutants Y147A and S at the time scales used (data not shown). Cyt *c* reduction kinetics were monitored at different E_h values (from 200 to 50 mV) (Figure 7). The E_{m7} value thus defined was approximately 129 mV for a wild type strain, which is similar to that measured earlier (Robertson et al., 1990), and 111, 98, 110, and 122 mV for Y147F, V, S, and A substitutions, respectively. In summary, the overall data obtained by flash-induced single-turnover transient kinetics demonstrated that only the Y147A and S substitutions slowed down cyt b_H and cyt *c* reduction rates drastically, hence localizing their perturbing effects to the Q_o site reactions.

Whether the Y147 substitutions had any effect on the Q_i site reactions was then checked by measuring the nonphysiological electron transfer rate from Q_i to cyt b_H at pH 9.0 in the presence of Myx, as described earlier (Glaser et al., 1984; Robertson et al., 1984). As shown in Figure 8, the rates of electron transfer from Q_i to cyt b_H were almost identical in the Y147F, V, and A mutants (approximately 214, 193, and 194 s^{-1}) and similar to that of a wild type strain [approximately 227 s^{-1}; see, for example, Gray et al. (1994a)]. Only in Y147S, this rate was 2−3-fold slower (approximately 83 s^{-1}) (Figure 8 and Table 2). The data indicated that, possibly with the exception of Y147S, the effects of these mutations are largely confined to the Q_o site only.

The ratio of the extent of cyt *c* oxidation to the extent of reaction center rereduction (c_{ox}/P_{rered}) induced by a flash in the presence of inhibitors is a useful measure to monitor the different steps of electron transfer at the Q_o site (Robertson et al., 1986). In a wild type strain addition of myxothiazol inhibits electron transfer from the Q_o site to the [2Fe-2S] cluster and to cyt b_L while it still allows that between the [2Fe-2S] cluster and cyt c_1 and leads to an increased c_{ox}/P_{rered} ratio (Figure 9, pMTS1). On the other hand, stigmatellin inhibits electron transfer from the [2Fe-2S] cluster to cyt c_1 and reveals the maximum extent of cyt *c* oxidation. In a mutant lacking cyt b_H, such as H212Y (Yun et al.,

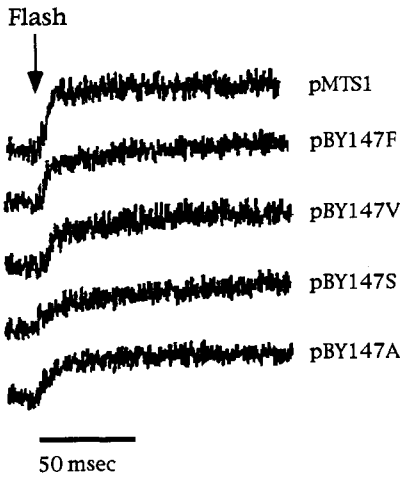

FIGURE 8: Reverse reaction kinetics of wild type (pMTS1) and Y147 mutants. Wild type and mutant chromatophores containing 0.2 μM reaction center were resuspended in 50 mM glycine (pH 9.0) and 100 mM KCl with appropriate mediators (see Materials and Methods), 3 μM valinomycin and 5 or 20 μM myxothiazol. The redox potentials were poised at 110−120 mV where Q_{pool} is fully oxidized at pH 9.0. Difference spectra were taken at 560 and 570 nm after a short (8 μs) flash of light, and the rate of electron transfer from Q_i to cyt b_H was calculated using a single-exponential equation, and these rates are shown in Table 2.

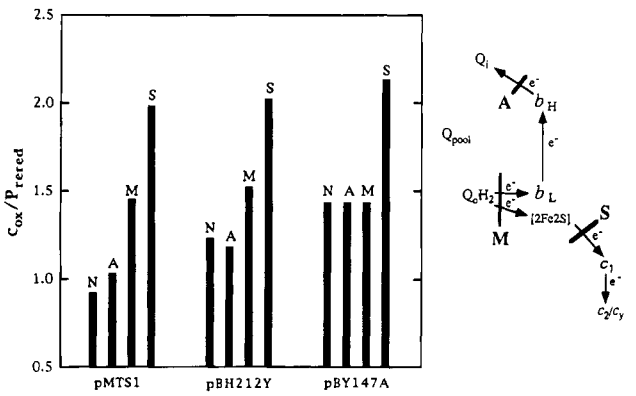

FIGURE 9: Ratio of the extent of cyt c oxidation (c_{ox}) to that of the reaction center re-reduction (P_{rered}) in the presence of different inhibitors in a wild type strain (pMTS1), in a mutant lacking cyt b_H (H212Y), and in Y147A. Experimental conditions were as in Figure 4 (E_h = 200 mV), and antimycin A (10 μM), myxothiazol (5 μM), and stigmatellin (5 μM) were added as indicated. The extent of both cyt c oxidation (absorbance difference at 550−540 nm) and P re-reduction (absorbance difference at 605−540 nm) were measured 20 ms after a single flash (8 μs) of light, and the ratio c_{ox}/P_{rered} was plotted in each case. N, A, M, and S correspond to no inhibitor, antimycin, myxothiazol, and stigmatellin, respectively. Electron transfer pathways internal to the bc_1 complex, and the point of impact of the inhibitors used are shown next to the bar graph.

1991b; Daldal et al., unpublished results), c_{ox}/P_{rered} ratio does not increase in response to antimycin unlike that in a wild type strain, but it increases when myxothiazol or stigmatellin is added (Figure 9). This indicates that while no electron transfer takes place from Q_o to cyt b_H in this mutant, electron transfer from Q_o to the [2Fe-2S] cluster and to cyt c_1 does. When tested in a similar way, Y147A responded to neither myxothiazol nor antimycin, but only to stigmatellin (Figure 9). Thus, in this latter mutant electron transfer from the [2Fe-2S] cluster to cyt c_1 occurs while that from the Q_o site to the [2Fe-2S] cluster and to cyt b_L is blocked. In summary, the overall data clearly established that while position 147 of cyt b does not interfere with the occupancy of the Q_o site, the nature of the amino acid side chain at this position is important for optimal QH_2 oxidation.

Revertants of the Ps⁻ mutants Y147A and S. The Ps⁻ strains Y147A and S reverted to Ps⁺ with an apparent frequency of approximately $10^{-7}-10^{-8}$. Eight independent Ps⁺ revertants were obtained as described in Materials and Methods, and their Q_oI regions were sequenced in order to gain further insight into the role of Y147 at the Q_o site (Table 1). The data obtained indicated that all of the five Ps⁺ revertants derived from Y147A (GCC) contained an additional mutation at position 154 of cyt b, converting methionine (M, ATG) to either valine (V, GTG) (in three cases) or an isoleucine (I, ATA) (in two cases). Similarly, of the three Ps⁺ revertants derived from Y147S (TGC) one contained the mutation M154I while the two remainder were back revertants to Y (TAC). This limited analysis indicated that all the Ps⁺ revertants contained at least a second mutation at the Q_oI region. The most frequent suppressor, M154V, thus recognized was reconstructed as a single mutation using site-directed mutagenesis to assess its role independently of the Y147 substitutions. Analysis of M154V indicated that it is Ps⁺ and, like Y147V, slightly Myx^R (i.e., exhibiting slow growth on MPYE plates containing 5×10^{-6} M myxothiazol), while the double mutants Y147A+M154V, Y147A+M154I, and Y147S+M154I were highly Myx^R ($>2.5 \times 10^{-5}$ M). These phenotypic analyses indicated that the second site suppressors, M154V and I, improved the Ps growth abilities of Y147A and S and also conferred Myx^R.

Biochemical and Biophysical Properties of the Suppressor Mutations. Analyses similar to those done with the Y147 mutants were repeated with the Y147A+M154V and Y147S+M154I double, and M154V single, mutants to determine the extent of their compensatory effects on the Q_o site reactions. In M154V, a cyt b_H reduction rate of 475 s^{-1} (at 106 mV) or 176 s^{-1} (at 200 mV) and a cyt c re-reduction rate of 181 s^{-1} (at 108 mV) were obtained (Table 2 and Figure 5B). Furthermore, in this mutant, no significant effect was observed either on the E_{m7} value of the half-maximal rate of cyt c reduction or on the occupancy of the Q_o site. Thus, the effects of M154 substitution on the Q_o site reactions were negligible or at best similar to those seen with the Ps⁺ mutants Y147F and V (Table 2 and Figure 5B). In Y147A+M154V and Y147S+M154I electron transfer rates from Q_o to cyt b_H were approximately 210 s^{-1} (at 106 mV) [or 76 s^{-1} (at 186 mV)] and 240 s^{-1} (at 100 mV) [or 80 s^{-1} (at 194 mV)], respectively (Table 2). Similarly, cyt c rereduction rates observed with these revertants were approximately 98 s^{-1} (at 106 mV) and 117 s^{-1} (at 103 mV) (Table 2). These values are about 10-fold higher than those observed with Y147A and S and comparable to those observed with the Y147F, V, or M154V mutants. Therefore, while M154V alone does not perturb significantly the Q_o site reactions, it increases significantly the electron transfer rates decreased by the Y147A and S mutations.

DISCUSSION

In this work, the structural and functional role of Y147 in cyt b of the bc_1 complex was studied using various approaches. Four different substitutions, F, V, S, and A were introduced using site-directed mutagenesis at this highly conserved position of cyt b (Figure 2). These substitutions were chosen to probe the role of the aromatic ring, the hydroxyl group, the hydrophobic nature, and the size of the

amino acid side chain at this position. None of these mutations affected the assembly of the bc_1 complex although they perturbed, to varying degrees, its function. This was manifested by the Ps^+ growth impairment, the decrease of DBH:cyt *c* reductase activity and the rates of internal electron transfer reactions in the bc_1 complex. In the Y147 mutants the E_{m7} values of the cyt b_L and cyt b_H as well as their EPR spectra (data not shown) were similar to those of a wild type strain. Thus, these substitutions do not interact with the heme groups or their environment. Further, in Y147F, V, and A the rates of electron transfer from Q_i to cyt b_H were similar to that of wild type, indicating that the impairing effects are confined to the Q_o site. The most important finding was that none of the Y147 mutants affected the occupancy of the Q_o site, while they had drastic effects on electron transfer rates from Q_o to cyt b_H. Moreover, c_{ox}/P_{rered} ratio in Y147A indicated that transfer of the first electron from QH_2 to the [2Fe-2S] cluster, and that of the second electron to cyt b_L does not occur. These findings clearly show that the kinetic defects observed in Y147A and S are independent of the occupancy of the Q_o site. This situation is unlike the previously studied Q_o site-Inh^R mutants, such as the F144X and G158X (X indicates any amino acid) mutations where impaired QH_2 oxidation was generally correlated with a decreased Q/QH_2 occupancy of the Q_o site (Robertson et al., 1990; Ding et al., 1992, 1995a). Therefore, the Y147 mutations are unique, and they define a novel class which may have other members. A similar situation, i.e., fully occupied Q_o site but impaired catalytic activity, was also observed with the M140R mutant (Tokito & Daldal, 1993; Ding et al., 1995b).

Genetic analyses of the selected Ps^+ second site revertants of Y147S and A were useful in further clarifying the role of position 147. Among eight revertants analyzed six had substitutions at position 154 to either I or V, which are by themselves silent mutations. The relatively high frequency with which these second mutations were observed suggests that a spatial relationship must exist between the positions 147 and 154 of cyt *b* (Figure 10). It appears that the impairments inflicted on the Q_o site by substituting Y147 (141 $Å^3$) with smaller amino acid side chains (A and S of 67 and 73 $Å^3$, respectively) are compensated by substituting M154 (124$Å^3$) with V and I, which are more hydrophobic but of similar sizes (105 and 124 $Å^3$, respectively).

Finally, it is worth noting that while none of the single mutations confers a high degree of Myx^R, the suppressors Y147A+M154V and Y147S+M154I are highly Myx^R indicating that suppression has taken place at the expense of a partial modification of the Q_o site. Thus, the binding domains of myxothiazol and of Q/QH_2 in the Q_o site do not superimpose, since the double mutants are highly Myx^R and yet have a fully occupied Q_o site. A comparison of the mitochondrial and bacterial cyt *b* sequences with chloroplast cyt *b* indicated that Myx^R are mediated by specific substitutions of defined positions of the Q_oI region (Daldal et al., 1989; Degli-Esposti et al., 1993). Apparently, the bc_1 complex becomes Myx^R when position 144 or 158 is occupied by V or A, respectively, which are naturally present in cyt b_6 at these positions (Tokito & Daldal, 1993). The Myx^R observed here with specific cyt b_6-like substitutions at position 154 of cyt *b* is consistent with this view.

In summary, the interchangability of specific side chains without affecting efficient electron transfer during QH_2 oxidation illustrates the plasticity of the protein backbone in forming the Q_o site. It also reveals a strict requirement for a tight packing, as well as a high degree of hydrophobicity, possibly to limit the accessibility of H_2O to this site. It is conceivable that these properties are necessary for establishing efficient electron transfer paths from QH_2 to the [2Fe-2S] cluster and to cyt b_L. In this respect, Y147 of cyt *b* is reminiscent of position Y162 of the L-subunit of the photosynthetic reaction center from *Rhodobacter sphaeroides* (Farchaus et al., 1993; Wachtveitl et al., 1993). Based on the impairing effect of the Y162G and L substitutions on electron transfer from cyt c_2 to the reaction center and the structural location of Y162, it was proposed that this residue facilitates electron transfer for re-reduction of the photooxidized bacteriochlorophyll dimer. The three-dimensional structure of bc_1 complex is not yet available, although X-ray diffracting crystals have been obtained in several cases (Berry et al., 1992; Yu & Yu, 1992; Kawamoto et al., 1994). Therefore, a more detailed definition of the role of Y147 in the Q_o site beyond the effects described here will await the availability of the structure. Nonetheless, it should be emphasized that this study describes the first example of a new class of Q_o site mutation that affects specifically the electron or proton transfer mechanisms at the Q_o site of the bc_1 complex, without interfering with the accessibility or binding of Q/QH_2. Further analyses of other residues with similar properties such as M140 or G152 should yield additional important information about the functional mechanism of the Q_o site of the bc_1 complex.

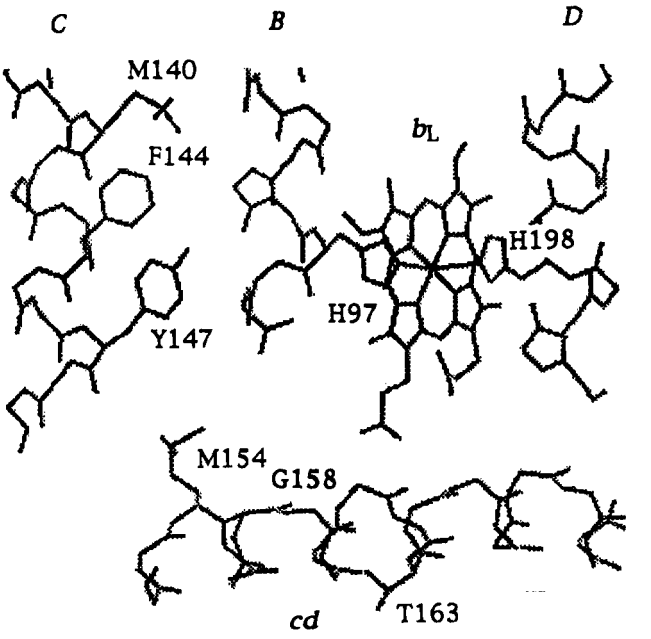

FIGURE 10: A model for the Q_oI region of cyt *b*. Only parts of the helices B, C, *cd*, and D are shown along with the cyt b_L heme. The orientation of these helices in respect to each other and their relative distances are arbitrary. This figure is only to illustrate the relative topology of the side chains of various residues that are thought to contribute to the Q_o site of cyt *b* and are relevant to this work.

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