Site-Directed Mutations of Conserved Residues of the Rieske Iron–Sulfur Subunit of the Cytochrome bc_1 Complex of *Rhodobacter sphaeroides* Blocking or Impairing Quinol Oxidation†

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ABSTRACT: Site-directed mutations of conserved residues in the domain binding the 2Fe–2S cluster of the Rieske subunit of the ubiquinol:cytochrome c_2 oxidoreductase (bc_1 complex) of *Rhodobacter sphaeroides* have been constructed. The substitution of aspartate for glycine at position 133 in the *Rb. sphaeroides* sequence (mutant FG133D), which mimicked a mutation previously isolated and characterized in yeast by Gatti et al. [Gatti, D. L., Meinhardt, S. W., Ohnishi, T., & Tzagoloff, A. (1989) *J. Mol. Biol. 205*, 421-435], allowed more detailed studies of thermodynamic behavior and the kinetics of the ubiquinol:cytochrome c_2 oxidoreductase on flash activation of the photosynthetic chain. The impaired catalysis in this mutant complex is localized to the quinol oxidizing site. The apparent second-order rate constant for reduction of cytochrome b_H via the quinol oxidizing site is about 20-fold lower than that of the wild-type and correlates with its apparent activation barrier being increased relative to that of the wild-type. Substitutions for the cysteines and a histidine which are conserved in the putative 2Fe–2S binding domain of the Rieske subunit selectively knock out the 2Fe–2S cluster and quinol oxidizing activity, while leaving the cytochromes and other catalytic sites essentially intact. Reversion properties of these strains are consistent with the mutated residues being essential. Membranes of the cytochrome c_1 mutant CQ228stop [Konishi, K., Van Doren, S. R., Kramer, D. M., Crofts, A. R., & Gennis, R. B. (1991) *J. Biol. Chem. 266*, 14270-14276], with the soluble domain of cytochrome c_1 released from the cytoplasmic membrane to the periplasm, retain a crippled complex which contains a relatively unperturbed 2Fe–2S center and cytochrome *b* titrating in the same range as cytochrome b_H, but with a broader *a* band and a peak shifted to the red (λ_{max} at 563 nm). The complex binds antimycin and stigmatellin in the absence of both membrane-bound cytochrome c_1 and any center with the properties of the low-potential cytochrome *b* heme. Hence, the essential architecture of the 2Fe–2S cluster, as reported by EPR spectroscopy and by stigmatellin binding is independent of the cytochrome c_1 subunit.

Enzymes of the ubiquinol:cytochrome *c* oxidoreductase family (cyt[1] bc_1 complex or b_6f complex in chloroplasts) are ubiquitous components of many respiratory and photosynthetic electron transport chains. The electron-transfer reactions through the high-potential iron–sulfur cluster and c_1 heme and through the two lower potential *b* hemes of these complexes drive proton pumping by a Q-cycle mechanisms (Mitchell, 1976; Crofts et al., 1983). Oxidized cytochrome c_1 rapidly oxidizes the Rieske iron–sulfur center. Together the oxidized 2Fe–2S cluster and the low-potential *b* heme oxidize quinol at the quinol oxidizing (Q_Z or Q_O) site where protons are deposited at the positive side of the membrane. The structural role of the Rieske iron–sulfur subunit at this site had not been clear. Mutations conferring resistance to inhibitors of this site such as myxothiazol, mucidin, and stigmatellin have been localized to cytochrome *b* (Daldal et al., 1989; DiRago et al., 1989; Yun et al., 1990) but not to the iron–sulfur subunit (Ljungdahl et al., 1989). Yet some inhibitors of this site have pronounced effects upon the EPR line shape of the Rieske iron–sulfur center (Bowyer et al., 1980; Von Jagow & Ohnishi, 1985), suggesting the proximity of the cluster to the site. Consistent with a location near the Q_Z site, Ohnishi et al. (1989) found that the iron–sulfur EPR signal is weakly relaxed by its cytochrome b_L and c_1 neighbors when paramagnetic and that the cluster may dip into the membrane slightly.

The carboxyl-terminal region of the iron–sulfur subunit contains the strictly conserved sequences CTHLGC followed by CPCHGSxY. In all likelihood these two sequences contain all four of the ligands to the 2Fe–2S cluster. These sequences differ greatly from the CxxxxCxxC...C motif found for the 2Fe–2S clusters of the plant-type ferredoxins. In both cases, however, the ligands are found in a hydrophobic portion of the sequence or on the edge of a hydrophobic region. The Rieske clusters in the cytochrome bc_1 complexes have redox potentials of +250–300 mV, hundreds of millivolts higher than those of ferredoxins. While 2Fe–2S ferredoxins have average *g* values ranging around 1.96, those of the Rieske-

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[1] Abbreviations: b_H, high-potential *b* heme; b_L, low-potential *b* heme; cyt, cytochrome; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; $E_{m,7}$, oxidation–reduction midpoint potential at pH 7.0; ENDOR, electron nuclear double resonance; ESEEM, electron spin echo envelope modulation; EXAFS, X-ray absorption fine structure; G, Gauss; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMS, *N*-methylphenazonium methyl sulfate; *Rb.*, *Rhodobacter*, UHDBT, 5-(*n*-undecyl)-6-hydroxy-4,7-dioxobenzothiazole; UHNQ, 2-(*n*-undecyl)-3-hydroxy-1,4-naphthoquinone; $NaDodSO_4$ or SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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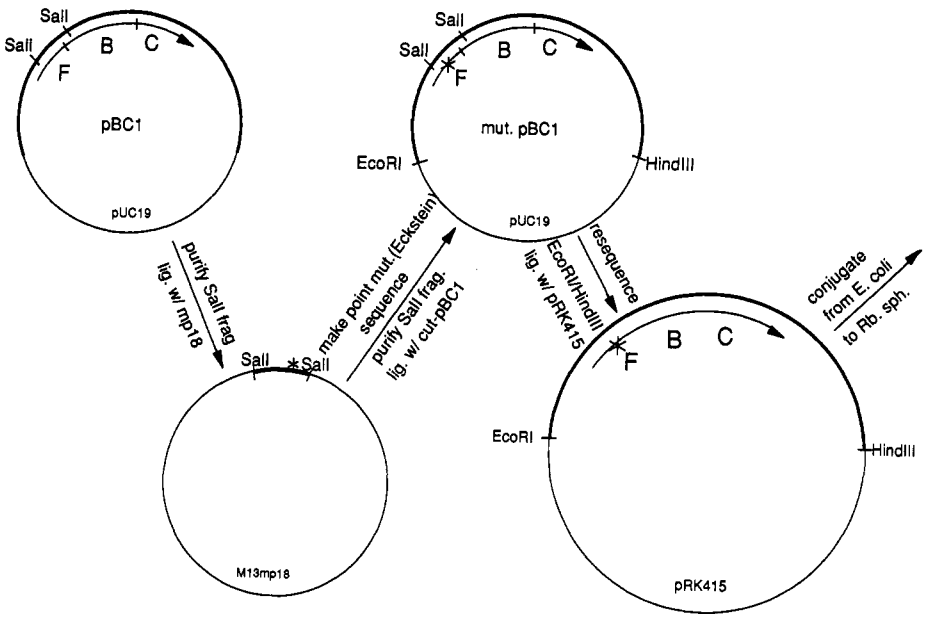

FIGURE 1: Site-directed mutagenesis of the *fbc*F portion of the *Rb. sphaeroides fbc* operon encoding the cytochrome bc_1 complex. A 500 base pair *Sal*I fragment, encoding the carboxyl-terminal half of the Rieske subunit which binds the 2Fe–2S cluster, was subcloned into M13. Oligonucleotide-directed mutagenesis of the single-stranded template was performed in vitro by the method of Nakamaye and Eckstein (1986) and screened by dideoxy sequencing (Sanger et al., 1980). The point mutations, represented by the asterisks, were subcloned back into the *fbc* operon and rechecked there by double-stranded sequencing by the method of Hattori and Sakaki (1985). Each entire mutated *fbc* operon on the 5.6-kilobase *Hind*III/*Eco*RI fragment was subcloned into the broad host range, low copy plasmid pRK415 (Keen et al., 1988). These plasmids were then conjugatively transferred from donor *Escherichia coli* S17-1 (Simon et al., 1983) to recipient *Rb. sphaeroides* BC17 with a chromosomal *fbc* deletion (Yun et al., 1990) by the method of Donohue et al. (1988).

type clusters are close to 1.90, which Blumberg and Peisach (1974) interpreted to suggest that the Rieske clusters have some ligation less electron-donating than sulfur. ENDOR, ESEEM, Mössbauer, and EXAFS studies are consistent with at least one nitrogen being a ligand (Cline et al., 1985; Telser et al., 1987; Fee et al., 1984; Powers et al., 1989). Dioxygenases of *Pseudomonas* have an analogous Rieske 2Fe–2S cluster with EPR spectra remarkably similar to those of the bc_1 complexes, but having lower midpoint potentials of around −110 mV (Geary et al., 1984). In one of these dioxygenases, it has been unambiguously demonstrated through ENDOR studies of uniformly and selectively ^{15}N-labeled samples that this 2Fe–2S center has two distinct through-bond couplings of the histidyl nitrogen to the cluster (Gurbiel et al., 1989). Studying b_6f and bc_1 complexes, Britt et al. (1991) observed by ESEEM two distinct classes of ^{14}N magnetic couplings to the Rieske centers, indicating that they are ligated by two nitrogens, and Gurbiel et al. (1991) reached a similar conclusion from ENDOR studies of the bc_1 complex from *Rb. capsulatus*. Furthermore, the nitrogen coordination environments of the Rieske centers of the bc_1 and dioxygenase complexes are similar, corroborating the conclusion that two histidines bind each cluster. The question of which of the conserved residues in the putative 2Fe–2S binding domain of the bc_1/b_6f Rieske subunits ligate the Rieske center then focuses down to which two of the four conserved cysteines are the other two ligands. To probe the role of this heavily conserved region of the Rieske subunit in the cytochrome bc_1 complex, some site-directed mutations of completely conserved residues of the subunit have been constructed in *Rb. spharoides*. Similar independent studies in yeast (Graham & Trumpower, 1991) and *Rb. capsulatus* (Davidson et al., 1992a,b) have recently been reported. In addition, a substitution of aspartate for the glycine residue in the conserved sequence "CTHLGC", previously reported in yeast by Gatti et al. (1989), has been reproduced in *Rb. sphaeroides* in order to allow the use of flash-induced spectrophotometric methods to follow the turnover of the cytochrome bc_1 complex and to dissect the site of impairment of catalysis. The mutant is shown to have a defective quinol oxidation site (Q_Z site).

We have also investigated the dependence of the iron–sulfur subunit and the integrity of the 2Fe–2S cluster on the presence of cytochrome c_1 in the complex, using a previously characterized site-directed mutant which releases the soluble domain of cytochrome c_1 to the periplasm (Konishi et al., 1991). The effects of loss of the cytochrome c_1 membrane anchor on the stability of the residual components and on the activity of catalytic sites of the mutant complexes are characterized by EPR and optical spectroscopy to measure kinetic and thermodynamic properties and the turnover of partial reactions of the complex.

MATERIALS AND METHODS

Recombinant DNA Techniques. Enzymes were obtained from Bethesda Research Laboratories, Inc. or New England Biolabs, Inc. Cloning techniques were as described by Sambrook et al. (1989). Synthetic oligodeoxynucleotides for mutagenesis and sequencing were synthesized at the Biotechnology Center of the University of Illinos on an Applied Biosystems Model 380A DNA synthesizer. Oligonucleotide-directed mutagenesis was performed using conventional procedures as summarized in Figure 1.

Growth and Fractionation of Rb. sphaeroides Cells. Rb. sphaeroides strains were grown chemoheterotrophically (to avoid selective pressure for second mutations restoring activity to the mutant strains crippled in their cytochrome bc_1 activity) at 32 °C in Sistrom's minimal medium A (Leuking et al., 1978) in the presence of 25 μg/mL kanamycin. Tetracycline was added to 1 μg/mL to maintain the pRK415 derivative plasmids carrying the wild-type and mutant *fbc* operons. To prepare membranes suitable for flash-induced electron transport assay, oxygen tensions in aerobic cultures were decreased by slowing the shaking rate of the flasks to reduce oxygenation

and induce formation of green-pigmented, invaginated cytoplasmic membranes (Kiley & Kaplan, 1988). Chromatophores were prepared from these greened semi-aerobic cultures by the method of Bowyer et al. (1979).

Photosynthetic growth and reversion frequencies were studied by plating aerobic inocula, whose cell numbers were estimated from Klett density using a no. 66 filter (Tai & Kaplan, 1985), on minimal plates mentioned above. The plates were incubated at 30 °C in an anaerobic jar in a hydrogen and carbon dioxide atmosphere generated by a BBL Gas Pack (Becton Dickinson). These plates were illuminated by 60-w bulbs filtered by Carolina Biological far-red 750 filters to minimize photo-oxidation of tetracycline to toxic products (Hasan & Khan, 1986). Photosynthetically competent strains grew up as a lawn, whereas revertants grew up as infrequent, isolated colonies. BC17C (*fbc* deletion complemented with *fbc* on plasmid) was used as a positive control and BC17 (*fbc* deleted) as a negative control.

NaDodSO$_4$–Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out using the Tricine-buffered 10% T, 3% C gels as described by Schagger and Von Jagow (1987). Samples were incubated for 10 min at 45 °C in loading buffer before loading. Prestained low-range molecular weight standards were from BioRad. Protein concentrations were determined using the bicinchoninic acid assay (Pierce Chemical Co.).

Western Blotting. Proteins were electrophoretically transferred to 0.2-μm nitrocellulose (Schleicher and Schuell) using 20% isopropyl alcohol, 25 mM Tris, and 140 mM glycine at pH 8.3. Membranes were blocked for 30 min with 1% BSA in 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% (v/v) Tween 20, incubated for 30 min with anti-*Rb. sphaeroides* Rieske iron–sulfur protein antiserum (a generous gift from R. Niederman), and then incubated for 30 min with goat anti-rabbit IgG (BioRad) conjugated to alkaline phosphatase. Following antibody incubations, membranes were washed three times with 10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% (v/v) Tween 20. Alkaline phosphatase activity was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (ProMega).

EPR Spectroscopy. EPR spectra were recorded on a Bruker ESP-300 X-band spectrometer at the University of Illinois Molecular Spectroscopy Lab. Cryogenic temperatures were maintained with an Oxford Instruments liquid helium cryostat and temperature control system. Preliminary spectra were recorded on a Bruker 220D X-band spectrometer at the Illinois ESR Research Center using an Air Products liquid helium cryostat. Membranes were suspended with 25 mM Tris-HCl (pH 7.5) and 10 mM EDTA, reduced with 10 mM ascorbate in the presence of 20 μM PMS, and frozen using a methylcyclohexane/isopentane mixture (1:5, v/v) at liquid nitrogen temperature.

Optical Redox Titration. Optical absorption spectra were collected using a home-built scanning single-beam spectrophotometer (Meinhardt, 1984) interfaced to a Zenith Z-100 microcomputer. Data acquisition and titration fitting software was written by Dr. Edward Berry. Redox potentiometry was performed using the methods of Dutton (1978). Chromatophores were suspended in 50 mM MOPS (pH 7.0) and 100 mM KCl. Nigericin and valinomycin were added to 2 μM each. Mediators present in titrations of cytochromes were the following: 20–50 μM DAD; 20–50 μM *p*-benzoquinone; 10 μM *N,N,N′,N′*-tetramethylphenylenediamine; 10–20 μM 2,5-dimethylbenzoquinone; 10–20 μM 1,2-naphthoquinone; 10–20 μM 1,4-naphthoquinone; 10 μM 1,4-naphthoquinone-2-sulfonate; 10 μM *N*-methylphenazonium methyl sulfate (PMS); 10 μM *N*-ethylphenazonium sulfate, 30 μM duroquinone; 10 μM pyocyanin; 10 μM 2-hydroxy-1,4-naphthoquinone; 10 μM 5-hydroxy-1,4-naphthoquinone. Additionally, 2 μM benzylviologen and 10 μM riboflavin mononucleotide were present for the redox titration of FG133D.

Flash-Induced Electron Transport Assay. A single-beam kinetic spectrophotometer equipped with a xenon flash lamp (10-μs duration at half-maximal intensity, providing 95% saturating, single-turnover actinic light flashes) was interfaced to an IBM PC-AT. Detection of optical changes was through a photodiode and a novel amplifier circuit incorporating a digital sample-and-hold which provided automated base-line correction. The signal was digitized using a Metrabyte DAS 8 data acquisition and control interface board (Metrabyte Corporation, Taunton, MA), and the instrument was controlled by a program written in-house. Flash lamp, timing, and shutter control circuitry were similar to those used in previous instruments (Crofts et al., 1983). Kinetics of redox changes in the reaction center primary donor ($(BChl)_2$ or P870 at 542 nm), cytochrome c_1 (at 552-548 nm), cytochrome c_1 (cytochromes c_1 and c_2 at 551-542 nm), and cytochrome b_H (at 561-569 nm) were measured as described previously (Crofts et al., 1983; Meinhardt & Crofts, 1982b). Studies of the temperature dependence of cytochrome b_H reduction were performed using the protocol of Crofts and Wang (1989).

Materials. Stigmatellin was obtained from Fluka. Other chemicals were obtained from Sigma Chemical Co. or Aldrich Chemical Co. Antiserum containing antibody against the *Rb. sphaeroides* Rieske iron–sulfur protein was a generous gift from Professor R. Niederman, Rutgers University.

RESULTS

Choice of Mutations. Figure 2 shows an alignment of the highly conserved portion of the Rieske iron–sulfur subunit sequences and analogous sequences from subunits of dixoygenases, each ligating a spectroscopically similar "Rieske" 2Fe–2S cluster. A similar alignment has been noted independently by Davidson et al. (1992); however, these authors also added sequences from the spectroscopically distinct ferredoxin subunits of the dioxygenases. The homology with the subunit of the dioxygenases having similar EPR and ENDOR spectra suggests that the two cysteines and two histidines (marked with asterisks in Figure 2) conserved are the ligands to the 2Fe–2S cluster while the other two cysteines are not. Residues in this region which are completely conserved among the cytochrome bc_1 complexes were chosen for mutagenesis. Glycine or serine was chosen to replace cysteine residues to minimize steric and hydrophobicity differences. Cysteine was chosen as a substitution for the histidine in case it might substitute as a ligand to the cluster. The tyrosine substitution for Cys 149 was constructed as a control expected to disrupt the cluster, as it reproduces an equivalent mutation isolated in the yeast complex which knocks out the cluster and activity (Gatti et al., 1989). Likewise, the Gly 133 to Asp substitution was selected as a positive control expected to retain the cluster and some activity as this mutation was also previously isolated in yeast and characterized by Gatti et al. (1989). Electron-transfer reactions of this latter mutation could be characterized in more detail by flash-induced single turnovers once introduced into *Rb. sphaeroides*.

Mutant FG133D with Greatly Slowed Quinol Oxidation via the Q_Z Site. The FG133D mutant strain grows very slowly photosynthetically with a lawn appearing on plates after about 9 days versus about 3 days for the wild-type strain BC17C.

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                                  133
                              129 131  134                 149 151
                                g  c  Ds                    y  s
R.sphaeroides bc1   WLVMWGVCTHLGCVPIGGVSGDFGGWFCPCHGSHYDSAGR
Yeast bc1           WLIMIGICTHLGCVPIGE-AGDFGGWFCPCHGSHYDISGR
Bovine bc1          WVILIGVCTHLGCVPIAN-AGDFGGYYCPCHGSHYDASGR
Spinach b6f         TFGINAVCTHLGCVVPFN-AAE-NKFICPCHGSQYNNQGR
                           * *                   *  *
nap. dioxygenase ndoB FLNVCRHRGKTLVSVEAGNAKGFVCSYHGWGFGSNGD
ben. dioxygenase P3   FLNQCRHRGMRICRADAGNAKAFTCSYHGWAYDTAGN
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FIGURE 2: Alignment of the 2Fe–2S cluster ligating regions of Rieske iron–sulfur subunits of cytochrome bc_1 and b_6f complexes and of dioxygenase subunits containing an analogous 2Fe–2S cluster, showing point mutations constructed. Numbered letters above the sequences represent substitutions for the corresponding *Rb. sphaeroides* residues. Lower case letters represent the mutations for which no cytochrome bc_1-dependent growth, quinol oxidation, or iron–sulfur cluster is detected, while the upper case letter represents the mutation for which an iron–sulfur cluster and activity were retained. Positions in the *Rb. sphaeroides* sequence are numbered from fMet. Bold-faced letters represent residues fully conserved among the cytochrome bc_1 and b_6f complexes. Residues among these also conserved with the dioxygenases of *Pseudomonas* are bold-faced in the dioxygenase sequences and marked with asterisks. Sequences are taken from the following: *Rb. sphaeroides*, Yun et al. (1990); yeast, Beckmann et al. (1987); bovine, Schagger et al. (1987); spinach chloroplasts, Steppuhn et al. (1987); naphthalene dioxygenase *ndoB* of *Pseudomonas putida* NCIB9816, Kurkela et al. (1988); benzene dioxygenase open reading frame product P3 of *Pseudomonas putida* 136R-3, Irie et al. (1987).

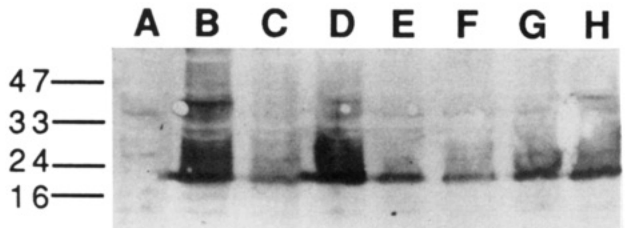

FIGURE 3: Western blotting of strains with point mutations in the Rieske iron–sulfur protein. The strains were grown to similar densities in the early stationary phase and harvested. In order to avoid the endogenous proteolysis which occurs when membranes are prepared, solubilized cells were run on the gel. Cell pellets from 75 μL worth of each culture were solubilized in loading buffer and run on a 10% T, 3% C Tricine gel, using the nomenclature of Schagger and Von Jagow (1987). Blotting was performed as described in Materials and Methods. Molecular weight standards are marked at left. The lanes were loaded as follows: A, BC17 (*fbc* operon deleted); B, BC17C (wild-type, complemented strain); C, FC129G; D, FH131C; E, FC134S; F, FC149Y; G, FC151S; H, FG133D. The arrow indicates the position of the Rieske protein.

Fast-growing photosynthetic colonies arise at a high frequency of about 10^{-7}. Immunoblotting reveals wild-type levels of the Rieske subunit in FG133D cells (Figure 3). EPR spectra of ascorbate-reduced FG133D membranes are quite similar to those of the wild-type BC17C but for a slight upfield shift of the broad $g_x = 1.77$ transition (Figure 4) similar to that reported for the equivalent mutation in yeast (Gatti et al., 1989). In the wild-type, $g_x = 1.77$ implies that the quinone pool is mostly reduced under these conditions. The EPR signal amplitude normalized to the cytochrome b_H concentration for FG133D is about 70% of that of the wild-type, which is similar to the ratio from the measurements of Gatti et al. (1989) using the equivalent mutation in yeast. While the mutant membranes are as fully sensitive to myxothiazol or stigmatellin as the wild-type by kinetic assay (not shown), the shifts of the EPR spectrum from saturating stigmatellin concentrations are not as dramatic as those of the wild-type (Figure 4). The narrowing of the g_y transition induced by stigmatellin is as great as in the wild-type but is not shifted upfield as much. The g_x transition is not narrowed as much as in the wild-type. The redox potentials of the *b*-type cytochromes were not changed in fresh preparations, although in aged preparations the potentials were lowered. The redox potential of cytochrome c_1 was not significantly affected. Cytochrome c_1 in the mutant is rapidly oxidized with a half-time perturbed little compared to that of the wild-type (Table II). Antimycin induces a red shift of the b_H heme spectrum of FG133D, as it does in the wild-type (Table II and Figure 5). Antimycin also induces an oxidation of the cytochrome b_{150} component as it does in the wild-type (not shown).

FG133D exhibits a striking slowing of turnover of quinol oxidation at the Q_Z site, as measured by kinetics induced by single-turnover flashes. At 26 °C, the second-order rate constant for reduction of cytochrome b_H in the presence of antimycin is approximately 20-fold slower in FG133D than in the control BC17C strain, judging from the ratio of initial velocities (Figure 6). The rate of re-education of *c* cytochromes is likewise slowed in FG133D (Figure 7). Quinol oxidation is blocked by both stigmatellin and myxothiazol, as in the wild-type. When stigmatellin is added either alone or after addition of myxothiazol, the extent of the fast phase of oxidation of *c* cytochrome is greater than the maximal extent in the absence of inhibitor (Figure 7) or the extent in the presence of myxothiazol (which was similar), in both the wild-type and FG133D. This reveals a fast re-reduction of cytochrome c_1 by the iron–sulfur center, which stigmatellin blocks but which myxothiazol does not, in both the wild-type and FG133D (see Meinhardt and Crofts (1982a)). Arrhenius plots of the temperature dependence of cytochrome b_H reduction with the quinone pool about half-reduced showed a somewhat steeper temperature dependence of FG133D relative to wild-type BC17C (Figure 8); this increased activation barrier was greater in aged preparations, which also showed a slower rate and a lower potential for cytochrome b_L (not shown). BC17C and FG133D (cytochrome bc_1 complex encoded by low copy plasmid) chromatophores were prepared from semi-aerobically grown cultures. The slopes of the lines drawn for the BC17C and FG133D samples represent, respectively, apparent activation barriers of 29 and 37 kJ mol^{-1}.

Mutations Eliminating the Rieske 2Fe–2S Cluster and Quinol Oxidation via the Q_Z Site. Mutants FC129G, FH131C, FC134S, FC149Y, and FC151S fail to grown photosynthetically. Strains FC129G, FC134S, and FC151S revert at rates of almost 10^{-10} back to the original cysteine residue among those revertants sequenced. No revertants of FH13A (a two base pair change) and FC149Y can be isolated at rates of 10^{-11} or higher. While strains FH131C and FC151S have near-wild-type levels of immunodetectable Rieske subunit, the levels in FC129G, FC134S, and FC149Y are substantially depleted (Figure 3). None of these strains appear to contain ascorbate-reducible Rieske iron–sulfur signals detectable by EPR. FC149Y lacks optically detectable cytochromes and was not characterized further. Optical redox

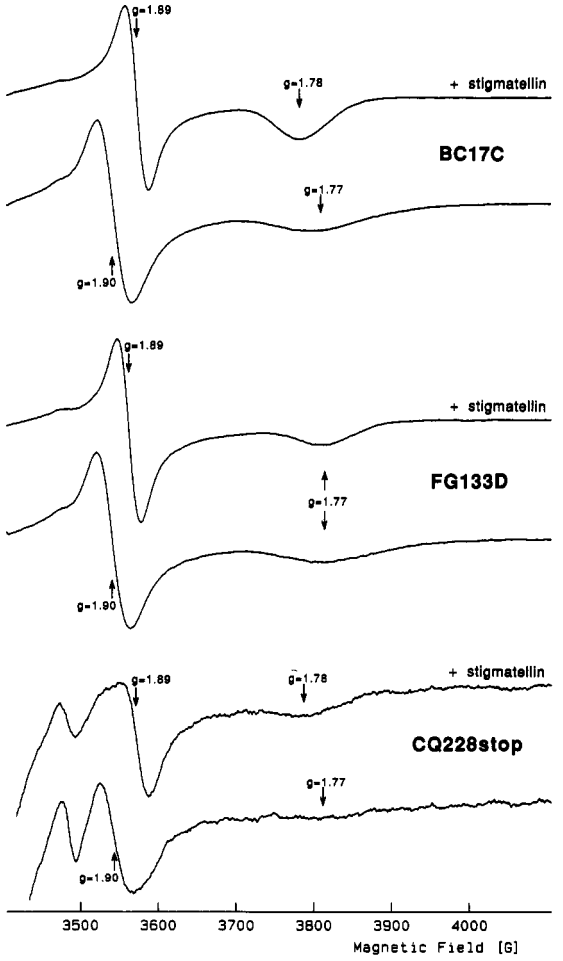

FIGURE 4: Stigmatellin-induced changes in the EPR spectra of strains retaining an iron–sulfur cluster. To membranes suspended in 10 mM Tris (pH 7.5) were added ascorbate to 10 mM, PMS to 20 μM, sodium azide to 5 mM, ethylenediaminetetraacetic acid to 10 mM, and either methanol to 2% or stigmatellin dissolved in methanol to 2%. Cytochrome b_H concentrations were estimated by using an $\epsilon_{561\text{-}569}$ of 25 mM^{-1} (Wang, 1990). Stigmatellin concentrations in methanol were estimated using an ϵ_{267} of 65.4 mM^{-1} (Thierbach et al., 1984). For the wild-type BC17C samples, the cytochrome b_H concentration was estimated at 140 μM, and stigmatellin was at 280 μM where added. For FG133D, the cytochrome b_H concentration was estimated to be 110 μM and the stigmatellin concentration 280 μM. For CQ228stop, the cytochrome b_H concentration was at most 15 μM (as it is only a small proportion of the b cytochrome in this strain), and the stigmatellin concentration was ~20 μM where added. The CQ228stop samples are on a scale of about an order of magnitude more sensitive than the others. Single scans were used for the BC17C samples, averages of two scans for the FG133D samples, and averages of twelve with the cavity background subtracted for the CQ228stop samples. The conditions used were as follows: power, 20 mW; modulation amplitude; 16 G; temperature, 24 K; frequency, 9.44 GHz; modulation frequency, 100 kHz; sweep time, 170 s; time constant, 330 ms.

titrations reveal minor shifts of $E_{m,7}$ values of cytochrome b_L downward about 20 or 30 mV and of cytochrome c_1 upward about 25 mV (Table I). Shifts of the peaks of the cytochrome α bands are not observed.

The cytochrome c_2 reductase and quinone reductase (Q_C) sites appear to be scarcely perturbed. The rates of cytochrome c_1 oxidation are similar to those of wild-type as the half-times are typically around 200 μs (Table II). The degrees of the antimycin-induced red shifts of the cytochrome b_H spectra are near those of wild-type, each having a trough at about 557.5 nm and a peak at about 562.5 nm with the peak-to-trough absorbance difference ranging around 16–17% of the unshifted 561 minus 569 nm absorbance difference (Table II and Figure 5). However, flash-induced electron-transfer studies show no evidence for quinol oxidation via the Q_Z site of each of FC129G, FH131C, FC134S, and FC151S. These mutant membranes behave as if fully inhibited by stigmatellin

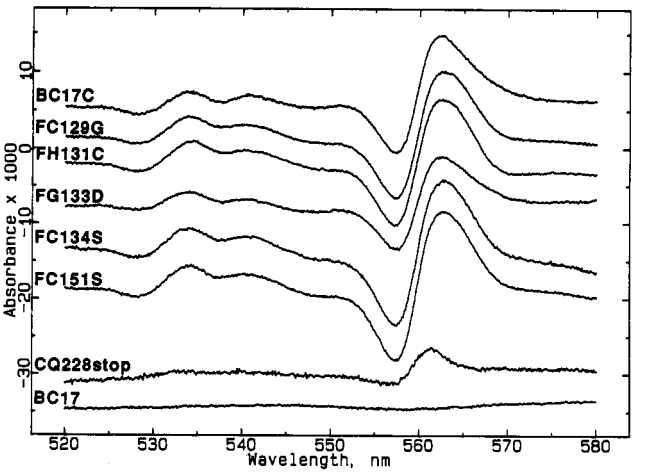

FIGURE 5: Antimycin-induced red shift of cytochrome b_H spectra of the mutant membranes. Membranes were suspended to at least 2 mg of protein mL^{-1} in 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.0) and 100 mM KCl. PMS was added to 6 μM. Excess solid sodium dithionite was added under an argon atmosphere. After about 8 min, when reduction was essentially complete, four scans at 5 nm s^{-1} were averaged. Antimycin was then added to 15 μM and four more scans were averaged. The former spectrum was subtracted from the latter spectrum and normalized to the change which occurred for a 100 mAU difference between 561 and 569 nm (about four μM cytochrome b_H). The BC17 sample was included as a control, since it lacks any cytochrome bc_1 complex because the *fbc* operon is deleted from the chromosome.

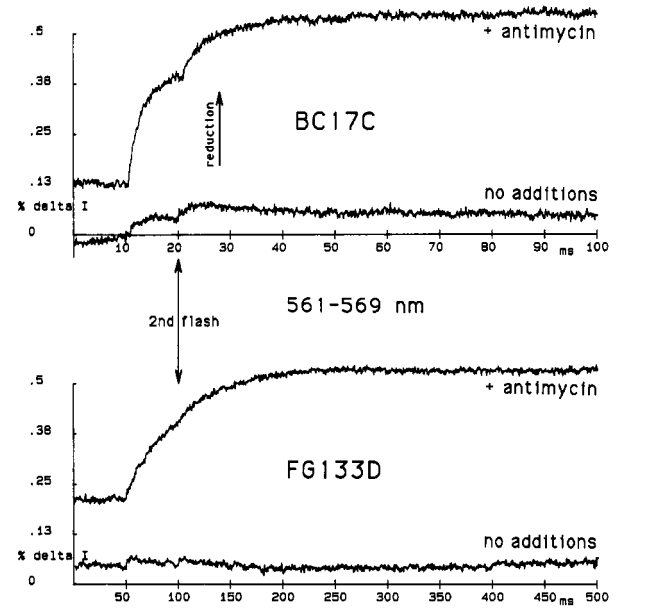

FIGURE 6: Flash-induced cytochrome b_H reduction. Chromatophores of semi-aerobically grown wild-type BC17C or mutant FG133D chromatophores were suspended in 50 mM MOPS (pH 7.0), 100 mM KCl with 1 mM ascorbate, 1 μM PMS, 10 μM 1,4-naphthoquinone, 10 μM 2,3,5-trimethylbenzoquinone, 2 mM sodium azide, 2 μM nigericin, and 2 μM valinomycin. The membranes were poised at an ambient redox potential (E_h ~90 mV at 26 °C) with the quinone pool about half-reduced. The reaction center concentration was about 0.24 μM for BC17C and about 0.18 μM for FG133D. Antimycin was added to 10 μM where indicated. Cytochrome b_H was monitored by the difference between the changes at 561 and 569 nm, an average of four each. Upward change represents reduction. 0.5% ΔI is equivalent to a 2.17 mAU increase. Two actinic flashes were delivered 10 ms apart for BC17C and 50 ms apart for FG133D. Note the 5-fold longer time scale for FG133D.

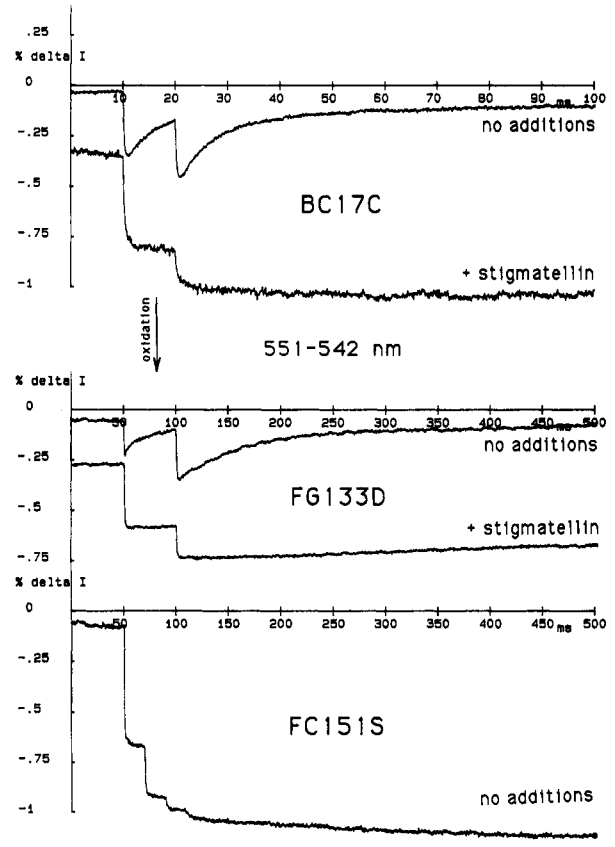

FIGURE 7: Flash-induced cytochrome c_1 plus c_2 absorbance changes in wild-type BC17C, FG133D, and FC151S. Conditions were as described for Figure 7 for the BC17C and FG133D samples. About 0.31 μM reaction center was present in the FC151S sample. FC151S had the following mediators present instead: 10 μM each of 2,5-dimethylbenzoquinone, 1,2-naphthoquinone, 1,4-naphthoquinone, and DAD and 20 μM 2,3,5-trimethylbenzoquinone and 1,4-naphthoquinone-2-sulfonate. The ambient redox potential was poised at 90–100 mV, except for the FG133D sample with stigmatellin at about 30 mV. Two actinic flashes were delivered to the BC17C and FG133D samples. Four flashes were delivered 20 ms apart to the FC151S sample (averaged only twice). The traces are the difference between the changes at 551 nm minus the changes at 542 nm. Downward changes represent oxidation. Negative 1.0% ΔI is equivalent to a 4.36 mAU decrease. Stigmatellin was added to BC17C to 1.5 μM and to FG133D to 1.0 μM where indicated.

or UHDBT, lacking *b* or *c* cytochrome reduction via the Q_Z site. An example of this behavior manifested by flash-induced oxidation of cytochrome c_1 and c_2 (note the large extent upon the first flash) and lack of subsequent re-reduction is shown for FC151S in Figure 7. Addition of Q_Z site inhibitors myxothiazol or UHNQ has no effect upon these kinetics.

Membranes of the CQ228stop Mutant which Releases Cytochrome c_1 in a Soluble Form to the Periplasm. Konishi et al. (1991) recently reported the construction of a mutation truncating cytochrome c_1 just before its carboxyl-terminal transmembrane anchor and the purification of the resulting soluble, heme-binding domain from this strain. This mutant strain reverts at high frequencies of about 10^{-7} (K. Konishi and S. R. Van Doren, unpublished observations). The authors reported an $E_{m,7}$ value of about +25 mV for the cytochrome *b* remaining in the membrane (Table I). A spectrum of this *b* cytochrome resolved by redox titration is shown in the lower panel of Figure 9. Note the red shift and significant broadening of this spectrum relative to the spectrum from a similar redox cut for the wild-type Ga (chromosomally encoded bc_1 complex) in the upper panel. Also note the absence of a split α peak at low potential in the CQ228stop mutant membrane (Figure 9, lower panel), which is characteristic of cytochrome b_L (upper panel). The concentration of the *b* cytochrome present in the mutant is, on a protein basis, at most 25% of the cytochrome b_H concentration found in the BC17C strain (wild-type, plasmid-encoded bc_1 complex) grown under similar conditions. The BC17 strain, which lacks the *fbc* operon encoding the bc_1 complex, contains a low concentration of *b*-type cytochrome with similar characteristics, which is presumably unrelated to the bc_1 complex (Yun et al., 1990). Addition of antimycin

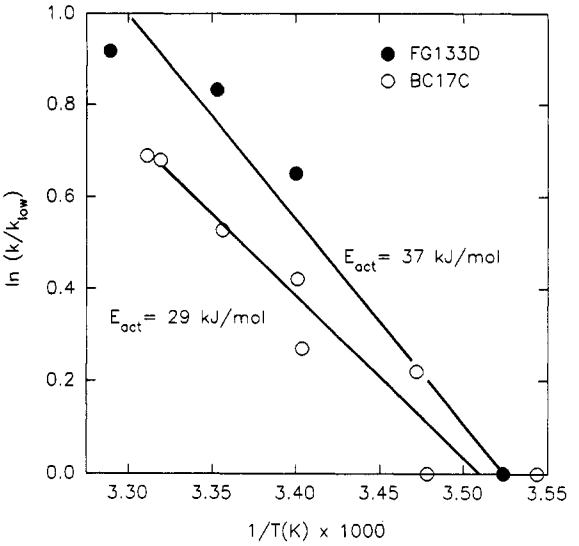

FIGURE 8: Arrhenius plot of the temperature dependence of cytochrome b_H reduction in BC17C and FG133D chromatophores. The conditions used were those of Figure 7, but the temperature was varied in the range 9–31 °C. The temperature was adjusted and maintained using a thermostated Neslab Endocal RTE-4 water bath and monitored by dipping a thermometer into the membrane suspension in the cuvette. The redox potential was poised with the quinone pool nearly half-reduced (90–95 mV at 25 °C) and stepped down by about a millivolt per increase in temperature by a degree as described by Crofts and Wang (1989). Averages of eight 25-ms sweeps were used for BC17C and averages of eight 250-ms sweeps were used for FG133D. The BC17C points are pooled from measurements of two different samples. The ratio of second-order rate constants is estimated by taking the ratio of the initial velocity to the initial velocity of the lowest temperature measurement of the experiment. Apparent activation barriers estimated from the slopes of the lines shown are listed.

Table I: Redox Titration of Cytochrome Subunits

strain	$E_{m,7}$ of cytochrome component (mV)			
	cyt b_L	cyt b_H	cyt b_{150}[a]	cyt c_1
Ga[e]	−97	+49	+151	+262
FC129G	−129	+54	+154	+291
FH131C	−118	+52	+137	+291
FG133D	−92	+55	+150	+254[b]
FC134S	−134	+47	+137	+283
FC149Y[c]				
FC151S	−124	+39	+155	+285
CQ228stop[d]		+25	+135	+220

[a] Cytochrome b_{150} is a form of cytochrome b_H titrating at $E_{m,7} \sim 150$ mV. Addition of antimycin induces the oxidation of a large fraction of this component, which is then indistinguishable from the cyt b_H titrating at $E_{m,7} \sim 50$ mV (Meinardt & Crofts, 1984). [b] A low-potential cytochrome c_1 component developed during long-term storage, possibly due to the formation of a disulfide bridge between otherwise free cysteines in cytochrome c_1 as suggested by Konishi et al. (1991). $E_{m,7}$ values of *b*-cytochromes were also lower in older samples. [c] The cytochromes of the complex could not be detected spectrophotometrically in this mutant. [d] These values were recently reported by Konishi et al. (1991). See text for discussion of the cyt b_H component. The potential of cytochrome c_1 shown is that of the soluble form released to the periplasm. [e] Components of the bc_1 complex in strain BC17C were similar to those in the Ga strain.

Table II: Assay of Catalytic Sites

strain	QH_2 oxidation via Q_Z site (%)[a]	cyt c_1 oxidation[b] approx. $t_{1/2}$ (μs)	antimycin-induced red shift of cyt b_H[d] trough, peak (nm)
BC17C (WT)	100	210	557.5, 562.5
FC129G	0	320	557.5, 562.5
FH131C	0	220	557.5, 562.5
FG133D	5	240	557.5, 562.5
FC134S	0	210	557.5, 562.5
FC151S	0	150	557.5, 562.5
CQ228stop	not detmd	400[c]	557.5, 561.3

[a] See Figures 6 and 7; WT rate taken as 100%. [b] Measured using the 552–548 nm wavelength pair, averaging 8–16 sweeps of 10 ms, with membranes poised at E_h ~100 mV in the presence of stigmatellin or UHNQ to block re-reduction of cytochrome c_1. [c] Estimated from *in vivo* assay of the soluble form of cytochrome c_1; follows 100-μs lag (Konishi et al., 1991). [d] See Figure 6.

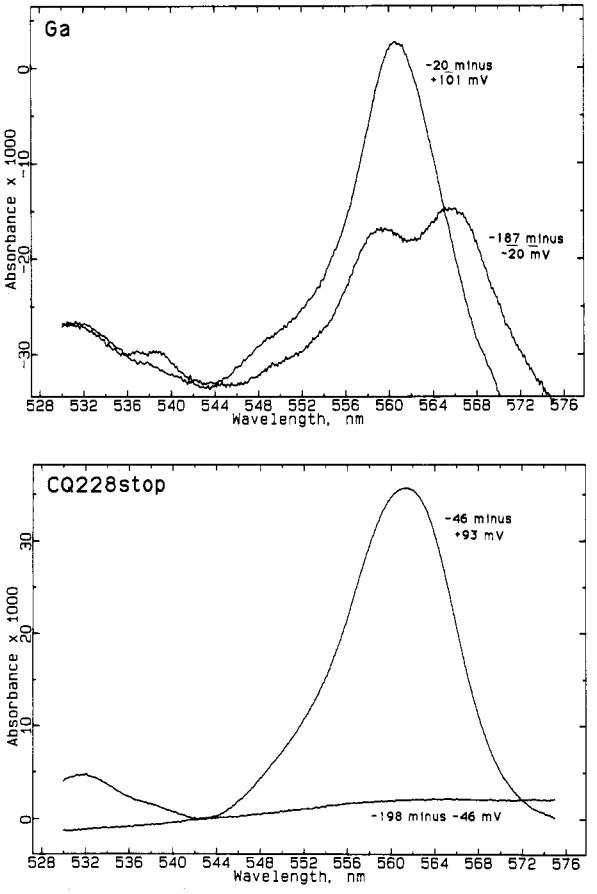

FIGURE 9: Redox-resolved spectra of *b* cytochromes in wild-type Ga and CQ228stop membranes. Redox titrations were performed as described in Materials and Methods. In the Ga (wild-type) chromatophores, the spectrum of cytochrome b_H was resolved by the difference between spectra taken at –20 and +101 mV, and the spectrum of cytochrome b_L was resolved by the difference between cuts at –187 and –20 mV. Spectra for chromatophores of CQ228stop are plotted for comparable differences between spectra taken at –46 and +93 mV and at –198 and –46 mV. The absorbance of the high-potential *b* cytochromes were used to normalize the spectra to similar scales.

to membranes of the CQ228stop mutant, in contrast to similar experiments with BC17, induced a modest red shift centered at about 559.5 nm (Table II and Figure 5), but did not shown the shift to the blue seen in the native complex when myxothiazol is added (not shown). The antimycin-induced red shift of cyt b_H is diagnostic of the antimycin binding site of the bc_1 complex and is consistent with a contribution of cytochrome b_H to the *b* cytochrome spectrum. Overlapping spectra of cyt b_H from the bc_1 complex and higher potential *b* hemes from other enzymes probably contribute to the apparent broadening of the peak of cytochrome *b* seen in the redox cut between 93 and –46 mV in the mutant. If a modified cytochrome b_L were present, it could also contribute to the broadening of the spectrum and the lowering of the apparent midpoint potential. However, it is evident that no significant component with the spectrum or midpoint potential of native cyt b_L is present. The relatively small difference in absorbance upon the addition of antimycin, when normalized to the content of high-potential *b* cytochrome, probably reflects the low ratio of cytochrome b_H to *b*-type cytochrome which is not associated with the bc_1 complex.

The membranes also retain a low concentration of the Rieske subunit with its characteristic EPR spectrum, including the $g_y = 1.90$ and $g_x = 1.77$ features (Figure 4). Most significantly, as indicated by the shifts of the above transitions, respectively, to $g = 1.89$ and $g = 1.78$ on addition of stigmatellin, these membranes have a stigmatellin binding site and presumably elements of the Q_Z catalytic site, although they lack a normal cytochrome b_L heme. Quantification of the bc_1 complex from either the Rieske g_y band or the antimycin-induced shift gives similar amounts (6.7 and 8.3%, respectively, of the changes seen in BC17C).

DISCUSSION

The activity of FG133D confirms previous observations from yeast that mutations of conserved residues near the probable ligands can retain activity (Ljungdahl et al., 1989; Gatti et al., 1989). It might be expected then that mutation of conserved cysteine not involved in ligating the iron–sulfur cluster could also yield complexes that retain the cluster and some quinol-oxidizing activity. Results for the limited set of mutation reported here, as well as similar experiments in *Rb. capsulatus* by Davidson et al. (1992a,b), and the preliminary mutagenesis of the same residues in yeast (L. A. Graham and B. L. Trumpower, personal communication) fail to yield such hypothetically active mutants which might identify residues not essential for iron ligation. Davidson et al. (1992a) reported a small EPR signal of an altered Rieske center in a *Rb. capsulatus* strain with the fourth conserved cysteine replaced by a serine and suggested that this cysteine was not a ligand, although the modified complex in this mutant was inactive. In contrast, we observe no such center, to a threshold of 1% of the wild-type concentration, in an equivalent mutant, FC151S, in *Rb. sphaeroides*. Graham and Trumpower (1991) also reported the absence of the Rieske 2Fe–2S cluster in the equivalent mutant is yeast. The homology with the Rieske-containing dioxygenase subunits (Figure 2) and spectroscopic identification of two histidine ligands (Gurbiel et al., 1989, 1991; Britt et al., 1991) suggest that Cys 129, His 131, Cys 149, and His 152 are probably the ligands. The extremely low reversion frequencies of substitutions for these four residues of less than 10^{-11} are consistent with these residues being essential for activity. The similarly low reversion rate back to the wild-type cysteine for the substitutions at positions 134 and 151 suggests that the cysteines at these two positions are also essential. Since Cys 134 and Cys 151 may not be ligands, yet are still apparently essential, they could conceivably form a necessary disulfide bridge as they lie on the oxidizing periplasmic side of the bacterial membrane.

While the substitutions for the conserved histidine and cysteines selectively remove the 2Fe–2S cluster and abolish the Q_Z or Q_O site catalysis necessary for turnover of the enzyme, the remainder of the complex can remain apparently largely unperturbed. This observation seems to be independent of

whether the Rieske subunit remains at high concentration, e.g., mutants FH131C and FC151S, or is depleted, e.g., mutants FC129G and FC134S. Although the midpoint potentials of the cyt b_L and c_1 neighbors may be modestly affected, cyt b_H, antimycin binding and cyt c_1 oxidation appear unperturbed (Tables I and II). The failure of FC149Y to assemble the bc_1 complex cytochromes (Table I) can be rationalized in terms of the introduction of a bulky aromatic side chain into this sensitive region. With the exception of FG133D, in all mutants which assemble, the mutation introduced a side chain of comparable or smaller volume. We might speculate that Gly 133 is located in an external loop, so that the volume change on substitution of aspartate has little consequence, but that Cys 149 occupies a restricted volume and that the bulk of the tyrosine side chain prevents the formation of bonds necessary for the stability of the protein. Davidson et al. (1992b), on the basis of deletion mutants for each of the subunits, proposed that the cytochrome *b* and c_1 subunits can form a subcomplex independent of the presence of the Rieske subunit. Our own observation of functional catalytic sites and normal cytochromes in mutants with the Rieske subunit absent or modified, supports this suggestion.

The CQ228stop mutant in which the cytochrome c_1 is released to the soluble phase may be the first example of a mutation to lose the spectrum characteristic of the low-potential *b* heme while the high-potential *b* heme remains. Perhaps the cytochrome c_1 subunit is needed for stabilization of the b_L heme pocket. The high reversion frequency of this mutant of around 10^{-7} may mean that any of a number of residues can replace the stop codon to restore the carboxyl-terminal anchor, which in turn restores assembly and function of the complex. The cytochrome b_H and Rieske iron–sulfur center (Table I) remaining at low concentration in the mutant membranes still display spectral shifts which reveal antimycin and stigmatellin binding in the apparent absence of cyt b_L. Van Doren et al. (1991) recently observed that the Rieske iron–sulfur subunit assembles in the *Rb. sphaeroides* membrane in the absence of the cytochrome *b* and c_1 subunits and does not exhibit a stigmatellin-induced shift of its EPR spectrum. Brandt and Von Jagow (U. Brandt and G. Von Jagow, personal communication) find that extraction of the Rieske subunit from the purified complex decreases the affinity of stigmatellin for the complex by over 5 orders of magnitude. Thus, it appears that both the cytochrome *b* polypeptide and the Rieske subunit are necessary for the binding of stigmatellin.

In the FG133D mutant reproduced in this work, a high rate of reversion of about 10^{-7} from very slow to rapid photosynthetic growth, suggests that second site mutations occur. Sequencing of revertants isolated might localize compensatory mutations elsewhere in the Rieske or cytochrome *b* subunits, which could be informative for modeling neighboring regions in the structure. The presence of an antimycin-induced red shift of cyt b_H and the intact cytochrome c_1 oxidation kinetics suggest that the distal catalytic sites are somewhat intact.

In contrast, the behavior and properties of the Q_Z site are markedly altered. The effect of the redox state of the quinone pool upon the EPR line shape of the Rieske center (broad, upfield g_x when reduced and narrow, downfield g_x when oxidized) has been described earlier by Siedow et al. (1978) and Matsuura et al. (1983). When the quinone pool is reduced, the upfield g_x feature of the EPR spectrum of FG133D appears to be shifted upfield and broadened slightly relative to the wild-type, just as Gatti et al. (1989) observed for the corresponding mutation in yeast. These authors also found that, when the quinone pool was oxidized, the g_x feature of their G133D spectrum did not shift downfield and narrow at all, as it does for the wild-type. Robertson et al. (1990) interpreted the spectra of this yeast mutant as evidence that the mutant complex has a much lowered affinity for quinone and quinol. They cited their experience with extraction of the quinone pool, causing a significant upfield shift and broadening of the g_x transition, and their experience with cytochrome *b* mutants severely impaired at the Q_Z site having much broadened and upfield-shifted g_x transitions when the quinone pool is oxidized (cf. Ding et al. (1992)). The EPR spectrum of the Rieske center of FG133D is altered less dramatically by stigmatellin than is the spectrum of the wild-type, although the FG133D complex is fully inhibited by stoichiometric quantities of stigmatellin. The spectrum of FG133D mutant may be generally less responsive to quinone and inhibitors of the Q_Z site.

The kinetic difference in single-turnover *c* cytochrome changes between myxothiazol inhibition and stigmatellin inhibition (greater oxidation upon the first flash, Figure 7) reveals that the Rieske center of FG133D rapidly reduces cytochrome c_1 as in the wild-type. Because the oxidation of cytochrome c_1 and the Rieske iron–sulfur center proceeds normally in FG133D, the impairment is localized to the electron transfer from quinol to the Rieske center and cytochrome b_L at the Q_Z site.

The substantial impairment of the Q_Z site is manifested in the 20-fold slower rate of reduction of cytochrome b_H in the presence of antimycin (Figure 6) and in the slower rate of re-reduction of cytochrome c_1 and c_2 in the absence of inhibitors (Figure 7). Crofts and Wang (1989) identified the rate-determining step as the oxidation of bound quinol by the Rieske 2Fe–2S cluster and speculated that the transient semiquinone species formed at the site on oxidation of QH_2 could be an intermediate near the peak of the apparent activation barrier. The approximately 20-fold slowing of cytochrome b_H reduction at 26 °C could be explained by the increase in the activation barrier by $\sim$8 kJ mol^{-1} over the value measured for the control (BC17C) strain. In terms of the above hypothesis, the transient semiquinone would be further destabilized in FG133D, and this is consistent with a decreased midpoint of the Rieske 2Fe–2S cluster (down 100 mV in the yeast mutant; Gatti et al., 1989). Although the overall driving force (for oxidation of QH_2 by $(BChl)_2^+$) would be the same in the mutant strain, a lowered midpoint of the 2Fe–2S center would increase the $\Delta G°$ for formation of the semiquinone at the Q_Z site, and this would certainly contributed to an increased activation free energy. However, there is a significant uncertainty in the slopes of the Arrhenius plots of Figure 8, and the 29 kJ mol^{-1} we measured in strain BC17C is less than the value of 32 kJ mol^{-1} reported by Crofts and Wang (1989) for the wild-type (Ga) strain (cytochrome bc_1 encoded by a single chromosomal copy). Furthermore, similar experiments with stored preparations showed a variability suggesting that other factors might be important, so that these values should be treated as preliminary. An alternative explanation for the slowed rates might be found in a lowered occupancy of the site by QH_2, either through a lower affinity of quinol and quinone for the Q_Z site, as suggested by Robertson et al. (1990), or by a change in the relative affinities of quinol and quinone.

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