Mutagenesis of Methionine-183 Drastically Affects the Physicochemical Properties of Cytochrome c_1 of the bc_1 Complex of *Rhodobacter capsulatus*†

Kevin A. Gray, Edgar Davidson, and Fevzi Daldal*

Department of Biology, Plant Science Institute, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6018

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ABSTRACT: Site-directed mutagenesis was used to investigate which of the highly conserved methionine residues (M183 and M205) provides the sixth axial ligand to the heme Fe in the cyt c_1 subunit of the bc_1 complex from the bacterium *Rhodobacter capsulatus*. These residues were changed to leucine (*c*M183L) and valine (*c*M205V). Two additional mutants were constructed, 1 in which a stop codon was inserted at M205 (*c*M205*) and the second in which 127 amino acids were deleted between the signal sequence and the putative C-terminal transmembrane α-helix (*c*Δ*Sfu*I). Only *c*M205V grew photosynthetically, and membranes isolated from this strain catalyzed quinol-dependent reduction of cyt *c* in amounts similar to that in a wild-type strain. Even though *c*M183L could not grow photosynthetically, it contained all the appropriate polypeptides and cofactors of the bc_1 complex, as shown by SDS–PAGE and optical difference spectroscopy of intact membrane particles. Neither of the two deletion mutants contained a stable complex. Flash absorption spectroscopy using chromatophores showed no cytochrome *c* rereduction after oxidation by the reaction center in *c*M183L. The bc_1 complex from each strain was isolated and characterized. Oxidation reduction midpoint potential titrations revealed that cyt c_1 from *c*M183L had a dramatically shifted E_m value (ΔE_m = −390 mV) compared with wild type and *c*M205V. While the optical absorption spectrum of cyt c_1 from *c*M183L suggested that the *c*-type heme was low-spin, nonetheless it was able to react with the exogenous ligand carbon monoxide. The overall data support that M183, and not M205, is the sixth ligand to the heme Fe of cyt c_1 of the bc_1 complex.

The enzyme ubiquinol–cytochrome *c* oxidoreductase (bc_1 complex)[1] is an integral membrane protein involved in cellular energy transduction. Forms of this enzyme are present in a wide variety of organisms. It is involved in both respiratory and photosynthetic electron-transport chains whose ultimate goal is the formation of a proton electrochemical gradient ($\Delta\mu_{H^+}$) which drives such processes as ATP synthesis and active transport [for reviews, see Trumpower (1990), Knaff (1990), Prince (1990), and Cramer and Knaff (1990)]. The prokaryotic enzyme is localized within the cytoplasmic membrane while in eukaryotes it is found embedded in the inner mitochondrial membrane. Chloroplasts contain a functionally analogous enzyme, called the b_6f complex, involved in electron transfer between the two photosystems, in the thylakoid membrane. Even though the number of polypeptides composing the complex tends to vary from source to source (a range of 3–12 subunits), all bc_1 complexes contain a core of 3 proteins which perform the redox chemistry. The prosthetic groups involved in the reactions are two *b*-type cytochromes (cyt b_{560} or b_H and cyt b_{566} or b_L) both contained on the cyt *b* polypeptide, one *c*-type cytochrome (cyt c_1 or *f*), and a [2Fe–2S] cluster on the Rieske FeS protein. In the Gram-negative, facultative phototroph *Rhodobacter capsulatus*, the three genes encoding these subunits are clustered as an operon called *fbc*FBC(*pet*ABC) (Gabellini & Sebald, 1986; Daldal et al., 1987; Davidson & Daldal, 1987a,b) with the 5′ → 3′ order being Rieske FeS (*fbc*F), cyt *b* (*fbc*B), and cyt c_1 (*fbc*C) genes. Subsequently, the genes have also been cloned and sequenced from several other sources [Yun et al., 1990; Thöny-Meyer et al., 1989; Verbist et al., 1989; Majewski & Trebst, 1990; see also Howell (1989) and Hauska et al. (1988) and references cited therein].

In the photosynthetic bacteria, the reaction center provides both the oxidant (ferricytochrome c_2) and reductant (ubihydroquinone) of the bc_1 complex [see for reviews Dutton (1986), Cramer et al. (1987), and Prince (1990)]. Under respiratory growth conditions, the reductant is provided by various dehydrogenases while the oxidant is provided by a cytochrome oxidase. Thermodynamic and kinetic data are not consistent with a linear scheme of electron transfer through the bc_1 complex; thus, the Q cycle was formulated as a possible mechanism of catalysis (Mitchell, 1976). This model envisions a low- and a high-potential chain with the low-potential chain consisting of cyt b_{566} and cyt b_{560} and the high-potential chain consisting of the [2Fe–2S] cluster and cyt c_1. Furthermore, there exist two distinct high-affinity quinone binding sites, termed Q_o (also known as Q_z and Q_p) and Q_i (Q_c and Q_n) for "outside" and "inside" which refer to the orientation of $\Delta\mu_{H^+}$, outside positive and inside negative. The gating of electrons to the high- and low-potential chains occurs at the level of quinol bound to the quinol oxidation (Q_o) site. According to this model, cyt c_1 reduces the soluble cyt c_2 while itself is the oxidant of the [2Fe–2S] cluster.

The mature cyt c_1 from *R. capsulatus* is a protein of 258 amino acids of M_r 28 095 and has been assigned to the class IV *c*-type cytochromes due to it being membrane-bound and part of a complex containing other prosthetic groups (Cusanovich et al., 1987). However, it shares many of the properties of class I cytochromes which are characterized by being low-spin, having a highly electropositive redox midpoint potential (E_m) and ligated to the protein via invariant histidine

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* Author to whom correspondence should be addressed. Phone: (215) 898-4394. FAX: (215) 898-8780.

[1] Abbreviations: bp, base pair(s); cyt, cytochrome; bc_1 complex, ubiquinol–cytochrome *c* oxidoreductase; E_m, equilibrium oxidation reduction midpoint potential; E_h, ambient potential; DBH, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone; PMS, phenazine methosulfate; kDa, kilodalton(s); FeS, Rieske iron–sulfur protein; [2Fe–2S], Rieske iron–sulfur cluster; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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and methionine residues. Typically the histidine residue is near the amino terminus of the protein while the methionine residue is near the carboxyl terminus. A 21-residue-long signal sequence (Figure 1) is present in *R. capsulatus* cyt c_1 which is cleaved subsequent to translocation across the intracytoplasmic membrane (Gabellini & Sebald, 1986); a hydrophobic stretch near the C-terminus (from M228 to K248) is likely to be the membrane-spanning region that anchors the protein in the membrane as was first indicated by limited proteolysis experiments (Li et al., 1981). More recently, Konishi et al. (1991) have used site-directed mutagenesis on the enzyme from *Rhodobacter sphaeroides* to truncate this region and were able to show the presence of a soluble form of the protein. The consensus heme binding motif (-C-Xaa-Yaa-C-H-) is located close to the amino terminus of cyt c_1; the two cysteines covalently link the heme macrocycle to the protein through thioether linkages formed by condensation of the thiol groups of the cysteine side chains with the porphyrin vinyl groups while the histidine contributes the fifth axial ligand to the Fe via the N3 imidazole nitrogen.

The redox properties of cyt c_1 (for *R. capsulatus*, $E_m \approx 300$ mV) suggest that the Fe axial ligation is probably histidine–methionine. Such a highly positive redox potential is normally associated with cytochromes with methionine acting as the sixth axial ligand, although there are exceptions [e.g., c_{554} from the reaction center of *Rhodopseudomonas viridis* which has a histidine–methionine ligation but an E_m of –60 mV (Dracheva et al., 1986; Alegria & Dutton, 1987; Weyer et al., 1987a,b)]. Data from magnetic circular dichroism (Simpkin et al., 1989) of cyt c_1 are consistent with histidine–methionine ligation although the absorption spectrum of the ferric cytochrome lacks the characteristic 695-nm charge-transfer band which usually signifies methionine in the ligation sphere. A sequence comparison (Figure 1) of several cytochromes c_1 reveals the presence of two highly conserved methionines (M183 and M205, in the numbering of the mature protein from *R. capsulatus*) near the carboxyl-terminal end of the protein. Included in Figure 1 are two sequences of cyt *f* from spinach and pea. Neither methionine is conserved in cyt *f*, where the spectroscopic properties (Rigby et al., 1988; Davis et al., 1988; Simpkin et al., 1989) are consistent with histidine–lysine ligation.

This study combined mutagenesis, biochemical, and biophysical analyses to identify which of these highly conserved methionines (if either) serves as the sixth axial ligand of the heme Fe of cyt c_1. The results indicate that the replacement of M183 with a nonliganding residue resulted in a protein with severely altered thermodynamic properties, and are consistent with M183, not M205, being the ligand.

MATERIALS AND METHODS

Bacterial Strains and Growth. All strains, both *Escherichia coli* and *R. capsulatus*, were grown as described in Daldal et al. (1989). Respiratory (semiaerobic) growth was at 35 °C in MPYE supplemented with appropriate antibiotics (tetracycline or spectinomycin). The photosynthetic apparatus (including the reaction-center and light-harvesting complexes) is induced under conditions of low oxygen tension. The *R. capsulatus* strain pMT0-404/MT-RBC1, overproducing the bc_1 complex (referred to as MT0-404 here), contains a composite plasmid carrying the *fbc* operon (pMT0-404) in trans and complementing a bc_1^- strain (MT-RBC1) in which the chromosomal copy of the *fbc* operon has been deleted (Atta-Asafo-Adjei & Daldal, 1991). The mutant strains are referred to by the nature of the amino acid replacement; that is, *c*M183L and *c*M205V contain the composite plasmids pC:M183L and pC:M205V, respectively, in which the codons corresponding to methionine at position 183 or 205 of *fbc*C have been altered to encode leucine or valine. The strains *c*M205* and *c*Δ*Sfu*I contain plasmids with truncated forms of the *fbc*C gene; the former has a stop codon (TAG) in position 205 while the latter has an internal deletion (corresponding to amino acid residues 32 through 159) in *fbc*C.

Genetic Techniques. Site-directed mutagenesis was performed as described by Atta-Asafo-Adjei and Daldal (1991) except that the uracilated phage M13mp10-BC1*Sma*del6 was used as a template. This phage contains the 2.2-kb *Sma*I/*Eco*RI fragment of the *fbc*FBC operon which is half of *fbc*B and all of *fbc*C (Figure 1). The mutagenic oligonucleotides used were petC-M183, 5′-TGG GCG CGC (NNG/C) CCC CCG CC-3′; and petC-M205, 5′-GTC GAT CAG (NNG/C) GCC CAG GA-3′.

After mutagenesis and screening by nucleotide sequencing, the M183L(TTG), M183R(AGG), M205A(GCG), M205E-(GAG), M205V(GTG), and M205*(TAG) substitutions were retained. For the M183L mutation, the *Sty*I/*Pst*I fragment was exchanged with the *Sty*I/*Hin*dIII fragment of pPET1 after appropriate modifications of the restriction sites, yielding pPET1-M183L. For M205V and M205*, the *Sph*I/*Pst*I fragment was exchanged with the *Sph*I/*Hin*dIII fragment of pPET1, yielding pPET1-M205V and pPET1-M205*, respectively. The *Ssp*I site of pPET1 and the *Hin*dIII site of pRK404 were used after appropriate modification to construct the composite plasmids pC:M183L, pC:M205V, and pC:M205*. The strain *c*Δ*Sfu*I contained the plasmid pKAG1-404 which was constructed by deleting the 380 bp *Sfu*I fragment internal to *fbc*C of pMT0-404, and confirmed by both restriction analysis and sequencing of double-stranded DNA. The plasmids thus obtained were conjugated into the *R. capsulatus* strain MT-RBC1, selecting for tetracycline resistance.

Biochemical Techniques. R. capsulatus cells, suspended in 50 mM MOPS, pH 7.0, containing 100 mM KCl, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM diisopropyl fluorophosphate (DIFP), and DNase, were ruptured by an Aminco French pressure cell at 20 000 psi. Chromatophores were isolated by differential centrifugation as described previously (Atta-Asafo-Adjei & Daldal, 1991). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with an acrylamide concentration of 15%. Samples were incubated at either 37 °C or 65 °C (indicated in the figure legends) for 5 min in the presence of SDS (2%) and dithiothreitol (100 mM) prior to sample loading. Gels were stained either for protein with Coomassie Brilliant Blue R250 or for heme with tetramethylbenzidine (Thomas et al., 1976). Immunoblotting to nitrocellulose filters (0.22-μm pore size) was performed on a Polyblot Transfer System (American Bionetics) apparatus according to the manufacturer's instructions. Monoclonal (Daldal, 1988) and polyclonal (Davidson et al., 1987) antibodies were used to probe the membranes; the second antibody was either peroxidase-conjugated goat anti-mouse (for monoclonals; Cappel, Malvern, PA) or peroxidase-conjugated goat anti-rabbit (for polyclonals; Bio-Rad, Richmond, CA) and stained with 4-chloro-1-naphthol.

Enzyme was purified according to the procedure outlined by Andrews et al. (1990) as modified by Dr. D. Robertson (personal communication) using dodecyl maltoside (Anatrace, Maumee, OH) extraction of chromatophores. In some cases, the second anion-exchange column was omitted, a Pharmacia FPLC MonoQ column was used in its place, and the gel

filtration column was eliminated in all cases. Concentration was performed by adsorption to a small DEAE-Sepharose anion-exchange column and eluted with high salt (500 mM NaCl), followed by dialysis against a low-salt buffer (50 mM Tris-HCl, pH 8, containing 100 mM NaCl and 0.1 mg/mL dodecyl maltoside). If further concentration was necessary, Centricon-30 (MWCO = 30 kDa) miniconcentrators were used. The final purified complex was stored at −80 °C in 50% glycerol.

Cyt c_1 was purified according to Yu and Yu (1991). Briefly, the purified complex (in 0.1 mg/mL dodecyl maltoside) was precipitated with $(NH_4)_2SO_4$ (the enzyme solution was made either 50% or 80% saturated by addition from a saturated solution, pH 7.6) and resuspended in 25 mM Tris-HCl, pH 8 [containing 1 mM DTT, 1 mM EDTA, and 0.25% (w/v) sodium cholate]. The sample was loaded onto a small phenyl-Sepharose (0.5 × 10 cm) column and washed first with the same buffer and then buffer containing 2% sodium cholate. Wild-type cyt c_1 normally eluted with 2% cholate; however, it was noticed that some cyt c_1 from *c*M183L eluted with 0.25% sodium cholate. While it was possible to obtain a pure cyt c_1 fraction for both wild type and *c*M205V, the cytochrome fraction from *c*M183L always contained some Rieske FeS protein though none of the preparations showed any evidence for cyt *b* either by spectroscopic methods or by SDS–PAGE.

Heme was extracted from purified complex (1-mL volume) by the addition of ice-cold HCl/acetone (5-mL volume) and subsequent centrifugation (10 min, 14K rpm, 4 °C). The supernatant containing protoheme was dried under vacuum, and the pellet containing mesoheme was air-dried. Both fractions were then resuspended in 20% pyridine/50 mM NaOH, and their sodium dithionite reduced *minus* potassium ferricyanide oxidized spectra were recorded. The concentrations of protoheme and heme *c* were calculated from the wavelengths and extinction coefficients given in Berry and Trumpower (1987) (heme *c*, $\epsilon_{549-535}$ = 23.97 mM^{-1} cm^{-1}; protoheme, $\epsilon_{556-540}$ = 22.98 mM^{-1} cm^{-1}).

Spectroscopy. For *b*- and *c*-type cytochrome determination in chromatophores, redox difference spectra were measured on a Hitachi U-3210 spectrophotometer. Samples (1 mL, *d* = 1 cm) were oxidized by the addition of a small crystal of potassium ferricyanide, reduced by either solid sodium ascorbate (plus 10 μM phenazine methosulfate, PMS) or sodium dithionite. Difference spectra were calculated by the spectrophotometer from absolute absorption spectra. CO binding experiments were performed on purified intact, or cyt *b*-depleted, complex by bubbling CO through the sample (in a stoppered cuvette) which had been reduced by either sodium ascorbate or sodium dithionite, and reduced + CO *minus* reduced spectra were obtained.

Activity Assays. The quinol cytochrome *c* reductase activity was measured using 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzohydroquinone (DBH) as a substrate as described in Atta-Asafo-Adjei and Daldal (1991).

Flash absorption spectra were measured on a previously described apparatus (Robertson et al., 1986; courtesy of Dr. P. L. Dutton). Chromatophores were suspended (in 50 mM MOPS/100 mM KCl, pH 7.0) to the same concentration of photobleachable reaction center (measured at 605 nm *minus* 540 nm after a saturating flash). Cyt *c* oxidation and reduction were followed at 550 nm *minus* 540 nm after a short (full width at half-height, 8 μs) saturating flash from a xenon lamp. The reaction mixture contained 2 mM ascorbate to poise the potential and 1 μM valinomycin to dissipate the membrane potential.

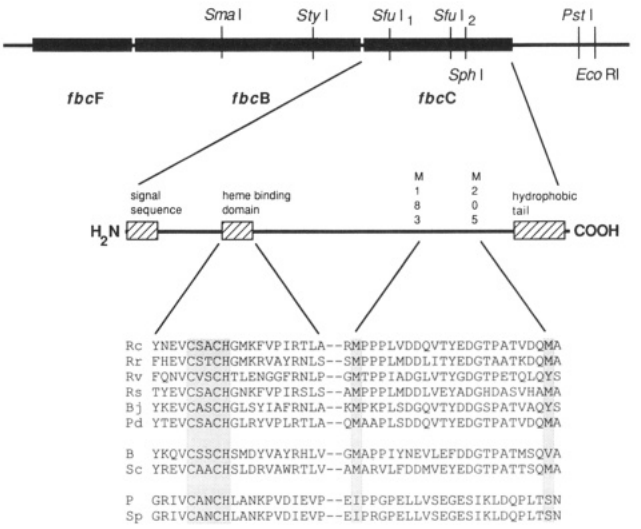

FIGURE 1: Partial restriction map of the *fbc*FBC operon (top), domains of the cyt c_1 polypeptide (middle), and sequence comparison of various cytochromes c_1 (bottom). Rc, *Rhodobacter capsulatus*; Rr, *Rhodospirillum rubrum*; Rv, *Rhodopseudomonas viridis*; Rs, *Rhodobacter sphaeroides*; Bj, *Bradyrhizobium japonicum*; Pd, *Paracoccus denitrificans*; B, beef heart mitochondria; Sc, *Saccharomyces cerevisiae* mitochondria; P, pea chloroplast; Sp, spinach chloroplast. The heme binding domain and the M183 and M205 residues are shaded.

Oxidant-induced reduction of cyt *b* was measured using purified complexes by monitoring changes at 560 nm. The enzyme (1 μM in cyt c_1) was in 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl and 0.1 mg/mL dodecyl maltoside. Antimycin A (dissolved in DMSO) was added to a final concentration of 5 μM, and DBH (20 mM in DMSO) was added during the measurement to a final concentration of 75 μM. After a base line had been reestablished (about 1 min), potassium ferricyanide (10 mM) was added to a final concentration of 50 μM.

Redox Titrations. Chemical oxidation–reduction midpoint potential titrations of purified complexes were performed according to Dutton (1978). All potentials are given versus the standard hydrogen electrode (SHE). Redox mediators used were anthraquinonedisulfonate (50 μM), pyocyanine (40 μM), duroquinone (100 μM), 2-hydroxynaphthoquinone (30 μM), benzoquinone (50 μM), phenazine ethosulfate (40 μM), phenazine (40 μM), 1,2-naphthoquinone (50 μM), phenazine methosulfate (40 μM), and 2,3,5,6-tetramethyl-*p*-phenylenediamine (140 μM). If the titration was performed in the α-band region (500–580 nm), small aliquots of potassium ferricyanide (made fresh in buffer) were added to completely oxidize the sample, and then reduced with the addition of small volumes of a sodium dithionite solution (made fresh in buffer) to give approximately 10-mV increments. Titrations in the Sorét region (400–480 nm) omitted the use of ferricyanide; thus, the titration started with cyt c_1 already mostly reduced (E_h typically 290 mV), and only dithionite was used in a reductive titration.

RESULTS

Choice of Mutants. Two methionine residues, M183 and M205, are highly conserved near the carboxyl-terminal end of cyt c_1 (Figure 1). Of these, M183 is absolutely conserved in all known cyt c_1 sequences and has been proposed to be the sixth axial ligand to the heme Fe in this subunit [see Gabellini (1987), Hauska et al. (1988), Davis et al. (1988), and Simpkin et al. (1989)]. In contrast to M183, M205 is not totally conserved; in *Bradyrhizobium japonicum* and *Rhodo-*

Table I: Various Properties of the Strains Used in This Study

strain	photosynthetic growth[a]	reversion frequency	DBH cyt c reductase act. (% wild-type act.[c])
MT0-404	+	na[b]	100
MT-RBC1	–		1.3
cM183L	–	$<10^{-10}$	1.6
cM205V	+	na	83.5
cM205*	–	10^{-6}	0.88
cΔ*Sfu*I	–		nd[d]

[a] (+) and (–) indicate the ability to grow photosynthetically on MPYE plates at 35 °C. [b] na, not applicable. [c] Wild-type activity in this particular instance was 2813 nmol of cyt c reduced min^{-1} mg^{-1} membrane protein. [d] nd, not determined.

pseudomonas viridis, a tyrosine residue occupies this position, and in beef heart mitochondria, a valine is present. While it is possible for tyrosine to ligate a metal, it would be a weak-field ligand (Moore & Pettigrew, 1991); however, valine cannot be a ligand. During the initial screening after mutagenesis using mixed oligonucleotides, two (leucine and arginine) and four (alanine, valine, glutamate, and a stop codon) substitutions were obtained at positions M183 and M205, respectively. Of these, cM183L and cM205V were chosen for detailed studies related to their possible liganding roles. The aliphatic side chains of leucine and valine cannot serve as ligands to a metal ion, and would not be expected to change the polarity of the heme pocket.

The mutant cM205* was retained to define its effect on the assembly of the complex since it should yield a truncated cyt c_1 without its putative membrane-spanning region (Figure 1). The mutation cΔ*Sfu*I was constructed as a complement to cM205*. The deletion of the *Sfu*I fragment, encompassing the heme binding domain, removed most of the soluble portion of cyt c_1 while retaining its signal sequence and its putative transmembrane anchor. This internal deletion keeps the reading frame intact, and would be expected to result in a protein devoid of heme if secretion and heme incorporation are uncoupled processes. The mutant product would be shortened by 127 amino acids from residue 32 to 159, making the polypeptide 131 amino acids long of approximate M_r 14K.

Growth Properties of the Mutants. A functional bc_1 complex is necessary for photosynthetic growth of *R. capsulatus* (Daldal et al., 1987), thereby providing a simple phenotypic test for any deleterious bc_1 mutation. Of the mutants analyzed in this study, only cM205V is capable of photoheterotrophic growth (Table I). No differences between this strain and the wild-type overproducer MT0-404 were observed when growth was tested on agar plates, or in liquid culture (MYPE medium) under photosynthetic conditions (doubling times of approximately 140 min). The three other mutants, cM183L, cM205*, and cΔ*Sfu*I, are unable to sustain photoheterotrophic growth. Of these, cM205* had a reversion frequency of approximately 10^{-6} while cM183L cells reverted with a frequency of less than 10^{-10} (Table I) and cΔ*Sfu*I did not revert. All strains grow by respiration; however, cM183L, cM205*, and cΔ*Sfu*I grow slower, similar to MT-RBC1 (a bc_1^- strain), via the cyt bc_1-independent quinol oxidase pathway (La Monica & Marrs, 1976; Daldal et al., 1987).

Cytochrome c Reductase Activity of the Mutants. The bc_1 complex catalyzes the oxidation of quinol and the reduction of ferricyt c. There are two methods of choice to measure the catalytic activity of the enzyme; one is a chemical steady-state turnover assay in which the overall rate of cyt c reduction by quinol is measured without regard to the individual intracomplex electron-transfer reactions. Representative

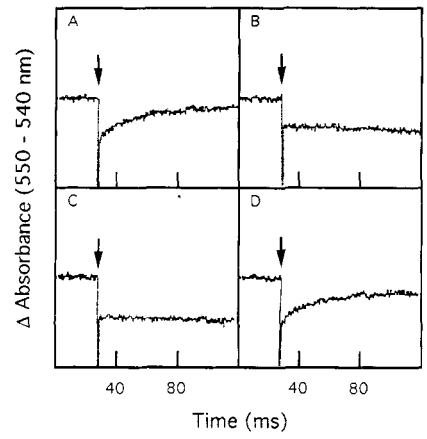

FIGURE 2: Postflash kinetics of cytochrome c oxidation and reduction in chromatophores. (A) MT0-404 (wild-type bc_1 overproducer strain); (B) MT-RBC1 (bc_1^- strain); (C) cM183L; (D) cM205V. The arrows indicate the flash point. Assay conditions are given under Materials and Methods.

DBH cyt c reductase activities of the different strains are shown in Table I. While chromatophores isolated from cM205V had essentially wild-type activity (83.5%), those from a culture of cM205* and cM183L cells had barely detectable levels of cyt c reductase activity (0.88% and 1.6% activity of the wild type, respectively). This residual activity represents the nonenzymatic reduction of cyt c since a comparable rate (1.3%) of cyt c reduction is also seen in the cyt bc_1 deletion strain (MT-RBC1).

The second method, which can only be performed using chromatophores, is a flash-induced single-turnover assay. In this method, one can monitor the kinetics of oxidation/reduction of individual components within the enzyme following a short flash which initiates electron transfer at the reaction center. In chromatophores, under conditions where the higher potential chain (Rieske [2Fe–2S] cluster, cyt c_1, and cyt c_2) would be expected to be reduced (E_h approximately 150 mV), the flash-induced cyt c oxidation is rapid in all strains, with a time constant outside of the resolution of the instrument (Figure 2). However, cyt c is re-reduced only in the wild-type overproducing strain (Figure 2A) and in cM205V (Figure 2D), with no apparent differences in the rate. No re-reduction of cyt c is observed in cM183L (Figure 2C), similar to the situation in the bc_1^- strain MT-RBC1 (Figure 2B). Therefore, both of these assays clearly indicate that of the mutants only cM205V has an active bc_1 complex.

SDS–PAGE and Redox Difference Spectra of the Mutants. The presence of the various protein subunits of the bc_1 complex in membrane fractions of the mutants was assayed using SDS–PAGE and Western blot analyses, and these results are shown in Figure 3. The strain MT0-404 overproduces the gene products of the *fbc* operon (Atta-Asafo-Adjei & Daldal, 1991) and allows direct observation of the individual subunits, cyt b (44 kDa), cyt c_1 (30 kDa), and the Rieske FeS protein (22 kDa), on a Coomassie-stained gel of total membrane protein (Figure 3, top). Both cM205V (lane 4) and cM183L (lane 3) appear to contain all three apoproteins; however, it is not possible using this technique to determine whether the apoproteins are present at low amounts or absent in cM205* (lane 5) and cΔ*Sfu*I (lane 6). The band assigned to cyt c_1 is also detectable by a heme stain, confirming the presence of a covalently bound heme (Figure 3, middle) in both cM205V (lane 4) and cM183L (lane 3) but the absence in both cM205* (lane 5) and cΔ*Sfu*I (lane 6). There are also other c-type cytochromes that stain in the same molecular mass range, as can be seen in MT-RBC1 which does not contain any cyt c_1

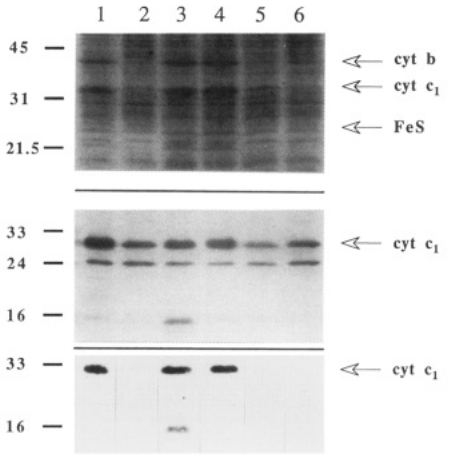

FIGURE 3: SDS–PAGE and Western blot analyses of chromatophores. A 15% acrylamide gel was used and stained for proteins with Coomassie Blue (top, 30 μg of total membrane protein) and for heme with tetramethylbenzidine (middle, 20 μg of protein) and immunoprobed (bottom, 30 μg of protein) using the anti-cyt c_1 monoclonal antibodies D1 and D3. Samples were heated at 37 °C for 5 min prior to being loaded. Lane 1, MT0-404; lane 2, MT-RBC1; lane 3, *c*M183L; lane 4, *c*M205V; lane 5, *c*M205*; lane 6, *cΔSfu*I. The cyt *b*, cyt c_1, and Rieske FeS subunits of the bc_1 complex are indicated, and the standards were from Bio-Rad.

(lane 2). An additional heme-staining band (M_r 16K) is apparent in *c*M183L, which is not seen in either of the other two strains containing a bc_1 complex. Monoclonal antibodies (designated D1 and D3) directed against cyt c_1 reacted specifically with the band at 30 kDa in the wild-type, *c*M205V, and *c*M183L samples (Figure 3, bottom). These antibodies also recognized the lower molecular weight heme-staining band in *c*M183L, suggesting that it is a proteolytic fragment of cyt c_1. Another monoclonal antibody (designated FD22) recognized both the intact 30-kDa protein and a third, non-heme-staining band (approximately 12 kDa) in *c*M183L (data not shown). No reaction was observed with chromatophores from either *c*M205* or *cΔSfu*I using either monoclonal or polyclonal (not shown) antibodies, suggesting that neither of these strains incorporate a truncated cyt c_1 in the membrane.

While SDS–PAGE detects the presence of the individual apoproteins and *c*-type hemes, it offers no information on the disposition of the various prosthetic groups. A more accurate measure of the heme-containing subunits is redox optical difference spectroscopy. Figure 4A shows the ascorbate/PMS-reduced *minus* ferricyanide-oxidized difference spectra of chromatophores isolated from each strain. Ascorbate, having an $E^{\circ\prime}$ of 58 mV (Loach, 1975), only reduces the higher potential components. In the wild-type strain, this difference spectrum has a λ_{max} near 550 nm which reflects the total cyt *c* content, including cyt c_2 (E_m = +358 mV; Meyer & Cusanovich, 1989), cyt c_1, and other higher potential cytochromes which may be present in the membrane. In the bc_1^- strain (MT-RBC1), a less intense peak at 550 nm is observed—the decrease in intensity is due at least partly to the absence of cyt c_1. The difference spectrum of membranes from *c*M205V is indistinguishable from wild type while those of *c*M183L, *c*M205*, and *cΔSfu*I are more similar to MT-RBC1 in that the absorption difference is small, consistent with a lack of ascorbate-reducible cyt c_1. Sodium dithionite (with an $E^{\circ\prime}$ of −527 mV; Loach, 1975) reduces the lower potential components of the bc_1 complex; the peak observed near 560 nm in the dithionite-reduced *minus* ascorbate/PMS-reduced difference spectra (Figure 4B) represents *b* cytochromes in the membrane. This peak has a much lower intensity in the spectrum of the deletion strain due to the loss of cytochromes b_{560} (b_H) and b_{566} (b_L). Once again, the difference spectra of MT0-404 and *c*M205V are essentially indistinguishable. The dithionite *minus* ascorbate/PMS difference spectrum of chromatophores of *c*M183L confirms the presence of the *b*-type cytochromes in the membrane; however, there is a distinct shoulder present at approximately 551 nm in this latter spectrum (indicated by the arrow) that is not present in that of the wild type or *c*M205V. The position of this shoulder suggests that it represents a *c*-type cytochrome, presumably the mutant cyt c_1. *c*M205* and *cΔSfu*I have intermediate signals at 560 nm, significantly less than wild type but more than the deletion strain, suggesting the presence of a small amount of cyt *b* in these samples. EPR spectra (not shown) of membrane fractions confirmed the presence of an intact [2Fe–2S] cluster in both *c*M183L and *c*M205V.

Analyses of Purified Complexes. The results presented above obtained using chromatophores suggested that while cyt c_1 of *c*M205V appeared very similar to the wild-type strain there were distinct changes in the physicochemical properties of that from *c*M183L. These studies of chromatophores were necessary since it is not uncommon that the properties of a membrane-bound protein may change when it is extracted from the lipid environment by detergents, thus relating the results obtained using exclusively detergent-dispersed purified complexes to in situ properties may be complicated. However, in order to fully characterize the mutant complexes, it was necessary to purify the enzyme complex, in particular to remove cyt c_2, cyt c', other membrane-attached *c*-type cytochromes, and cytochrome oxidase(s) which are present in chromatophores and whose properties overlap those of cyt c_1. The final preparations exhibited no cytochrome oxidase activity, and SDS–PAGE (Figure 5) revealed the presence of all subunits of the enzyme with only minor contaminating bands. The optical absorbance spectra of the purified complexes are also shown in Figure 5; ascorbate had been added to 1 mM prior to the measurement so that the ambient potential was approximately 150 mV, a potential at which cyt c_1 should be reduced while both cytochromes *b* should be oxidized. This is apparent in the spectra of the wild-type and *c*M205V complexes (Figure 5A,C) which show prominent peaks at 551.5 and 523 nm, the α- and β-peaks of cyt c_1. On the other hand, the spectrum of the *c*M183L complex (Figure 5B) shows no 551.5- and 523-nm peaks. Furthermore, the maximum in the Sorét region is different for the wild-type, *c*M205V (both at 416 nm), and *c*M183L (410 nm) complexes. Each panel in Figure 5 also shows the absorption spectrum after the addition of the strong reductant sodium dithionite. Under these conditions, the spectra of the wild-type and *c*M205V complexes show distinct, well-separated α- and β-bands of both cyt c_1 (552 nm) and cytochromes b_L and b_H (~560 nm). On the other hand, only a slight asymmetry on the blue side of the cyt *b* α-peak is evident in the spectrum of the *c*M183L complex. These data could indicate that the cyt c_1 subunit had lost the heme moiety during purification; however, pyridine hemochromogen difference spectra of the acid/acetone-extracted samples verified the presence of both protoheme and *c*-type heme in all three complexes with comparable ratios of 1.7:1 (protoheme:heme *c* ratio).

Oxidant-induced reduction of cyt *b* was one of the properties of the bc_1 complex that led to the proposal of the Q-cycle mechanism (Mitchell, 1976). Upon the addition of an oxidant (in this case ferricyanide, Figure 6), quinol (at Q_o) donates an electron to the oxidized FeS, and the semiquinone thus formed reduces cyt $b_{566(560)}$, and in the presence of the Q_i-site

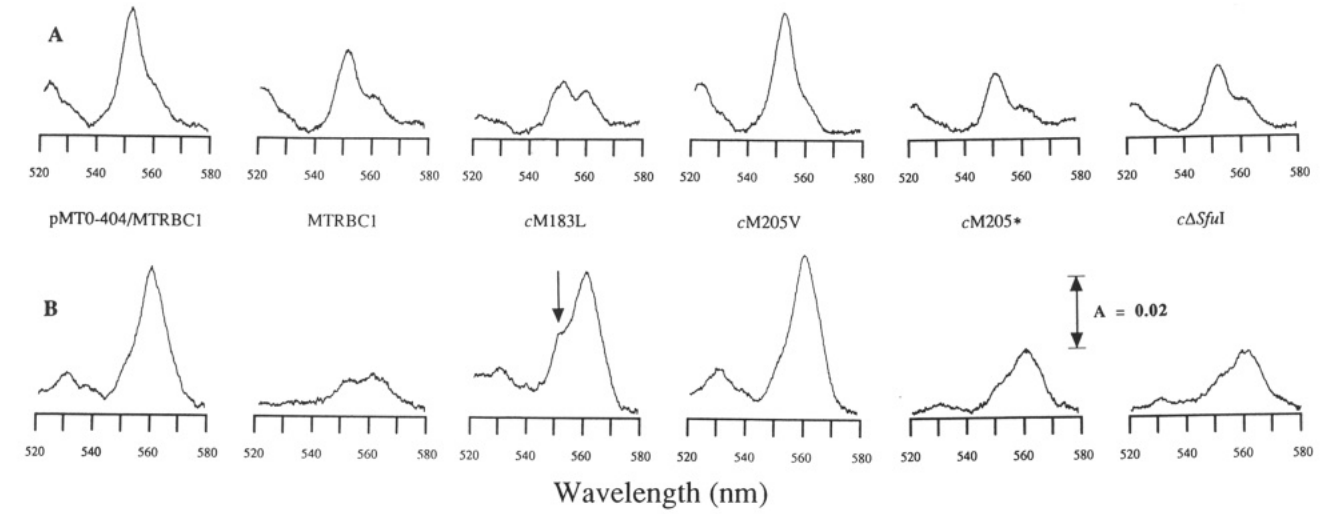

FIGURE 4: Reduced *minus* oxidized difference spectra of various strains. Chromatophores (800 μg/mL) were suspended in 50 mM MOPS (pH 7.0) containing 100 mM KCl. The spectra in (A) were obtained by subtracting the ferricyanide-oxidized spectrum from the ascorbate/PMS-reduced spectrum. The spectra in (B) were obtained by subtracting the ascorbate/PMS-reduced spectrum from the dithionite-reduced spectrum. The arrow indicates the shoulder visible only in the *c*M183L mutant.

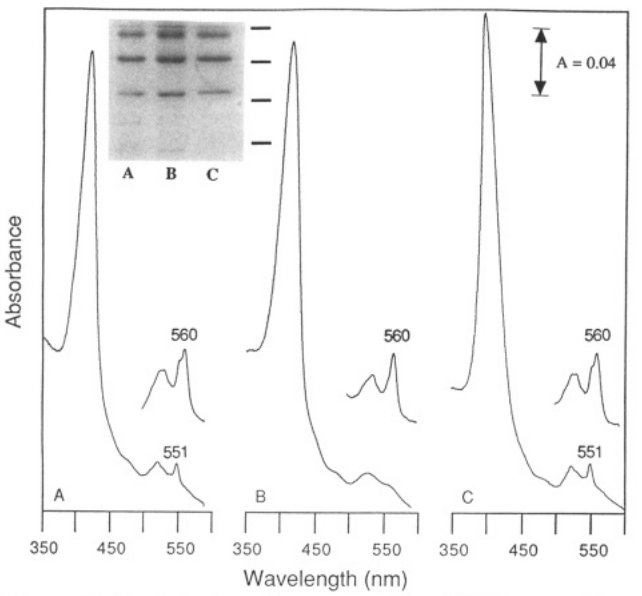

FIGURE 5: Optical absorption spectra of purified bc_1 complexes isolated from the various strains. The complex was in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mg/mL dodecyl maltoside. Ascorbate was added to 1 mM; solid sodium dithionite (upper enlarged spectra in the α-band region) was added to obtain the fully reduced complex. (A) Wild type; (B) *c*M183L; (C) *c*M205V. Inset: SDS–PAGE of purified complexes from the three strains. The electrophoresis conditions were as in Figure 3 except that the samples (5 μg of protein) were heated at 65 °C for 5 min prior to being loaded in the presence of 100 mM DTT. The indicated standards of 45, 31, 21.5, and 14.4 kDa were from Bio-Rad.

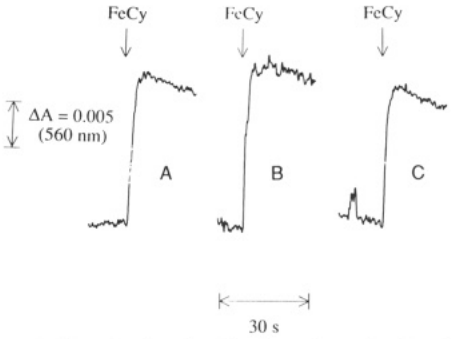

FIGURE 6: Oxidant-induced reduction of cyt *b*. Cyt *b* reduction (ΔA_{560}) was monitored after the addition of the quinol analog DBH to a final concentration of 75 μM and potassium ferricyanide (FeCy) to a final concentration of 50 μM in purified bc_1 complex from (A) wild type, (B) *c*M183L, and (C) *c*M205V. The concentration of the complex was 1 μM in c_1 in each case, and the enzyme was solubilized in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl, 0.1 mg/mL dodecyl maltoside, and 5 μM antimycin A.

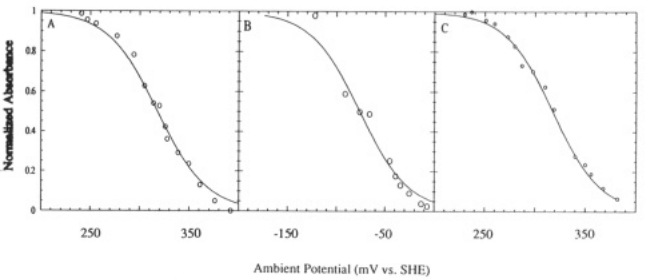

FIGURE 7: Redox titrations of various cytochromes c_1. Reductive titrations of purified complexes were performed as described under Materials and Methods. The data are fit to a one-component, n = 1 Nernst curve. (A) Wild type monitored at 550–540 nm; (B) *c*M183L monitored at 418 nm; (C) *c*M205V monitored at 550–540 nm as described in the text.

inhibitor, antimycin A, the reoxidation of cyt *b* via Q_i is inhibited. This assay provides a test for both the reactions $Q_oH_2 \rightarrow FeS^{ox}$ and $Q_oH^\bullet \rightarrow b_{566} \leftrightarrow b_{560}$. As shown in Figure 6, purified *c*M183L bc_1 complex supports oxidant-induced reduction in a manner similar to that seen in the wild-type and *c*M205V complexes, indicating that the absence of flash-induced cyt *c* rereduction in *c*M183L chromatophores is not due to a lesion proximal to FeS but that the quinol oxidation site is functionally intact. As expected, this ferricyanide-induced reduction of cyt *b* was inhibited by the Q_o-site inhibitor myxothiazol (not shown).

Redox Titrations of the Cytochromes. The nature and number of out of plane ligands play major roles in modulating the oxidation reduction midpoint potential of the Fe(II)/Fe(III) couple (Cusanovich et al., 1987; Moore & Pettigrew, 1990). Chemical reductions either with ascorbate or with dithionite presented above indicated that cyt c_1 from *c*M183L in the membrane has a midpoint potential (E_m) dramatically lower than wild-type cyt c_1. Redox titrations of cyt c_1 from wild-type and *c*M205V-purified complexes (Figure 7A,C) reveal an E_m value of about +318 mV. The titrations shown in Figure 7 were performed in the α-band region (A_{551}–A_{540}) and yielded slightly higher E_m values than those for the couple

of the *R. sphaeroides* cytochrome in the purified complex (+285 mV; Gabellini et al., 1982) and other purified preparations of *R. capsulatus* complex (+290 mV; D. Robertson et al., unpublished results). The reductive titrations were repeated in the Soret region (400–450 nm) due to much higher extinction coefficients. A titration of the wild-type complex showed that cyt b_{560} (with an E_m of about +50 mV) has a Soret peak of the reduced protein at 429 nm while cyt b_{566} (with an E_m of about −120 mV) has a Soret peak of the reduced protein at 431 nm (data not shown). This made it possible to titrate the two independently in this region, as well as to observe any components due to cyt c_1 at lower potentials. A shoulder at approximately 420 nm, which was not present in wild type or *c*M205V, titrated at potentials less than 0 mV in the *c*M183L sample and was attributed to the Soret transition of the mutant cyt c_1. In fact, purified reduced cyt c_1 (see below, Figure 8) has a Soret transition at 418 nm. Redox difference spectra were deconvoluted using the program PeakFit (Jandel Scientific), and a plot (Figure 7B) of the amplitudes (A_{418}) obtained from the fits revealed an $n = 1$ Nernst curve with a midpoint of −74 mV. If this component specific to the *c*M183L complex corresponds to the mutant cyt c_1, then it has an E_m some 390 mV more electronegative than the wild-type protein. The finding that in the *c*M183L mutant the E_m of cyt c_1 is much lower than that of the Rieske [2Fe–2S] cluster would explain all of the observed growth and kinetic defects of this mutant.

Ligand Binding. One of the purposes of this study was to assign the sixth axial ligand to the heme Fe of cyt c_1. If this ligand is removed, one would expect the ligation sphere to be pentacoordinate. It is highly probable that the sixth position would not be vacant but occupied by a water molecule or a side chain of another amino acid in the vicinity depending upon the solvent exposure of the heme pocket and the flexibility of the protein. If the position is empty, an exogenous ligand such as carbon monoxide, azide, or cyanide may bind to the heme Fe (Wood, 1984); binding may also occur if a labile protein ligand is present. Upon binding, the heme becomes hexacoordinate and either low-spin or high-spin, depending on the ligand field strength of the small molecule. Binding can be easily observed by optical difference absorption spectroscopy since the strong-field ligand CO binds preferentially to ferrous hemes (Cotton & Wilkinson, 1988). Thus, by specifically reducing either cyt c_1 (with ascorbate) or cyt *b* (with dithionite; thus, both b_{566} and b_{560} are reduced), it can be ascertained to which heme the CO is binding.

Using purified complexes, no CO binding was observed for ascorbate-reduced wild-type bc_1 complex; however, further reduction by dithionite resulted in spectral perturbations indicating that CO cannot bind to cyt c_1 but can bind to the *b* cytochromes (not shown). Previously, results from EPR spectroscopy indicated that purified samples of chloroplast b_6f (Nitschke & Hauska, 1987) and bacterial bc_1 complexes (Salerno et al., 1986; McCurley et al., 1990) do contain some high-spin cytochrome which was assigned to partial unfolding of the cyt *b* polypeptide and may then bind CO. Unfortunately, in the case of the *c*M183L complex, this made it difficult to distinguish binding of CO to cyt *b* from binding to the mutant cyt c_1. To overcome this complication, the complexes were depleted of cyt *b*, as described by Yu and Yu (1991), and the CO binding experiments were repeated. Figure 8 shows the optical absorption spectra of the dithionite-reduced cyt *b*-depleted cytochromes c_1 from wild type, *c*M205V, and *c*M183L. All three preparations lack spectral features around 560 nm due to cyt *b*, and SDS–PAGE analysis showed no

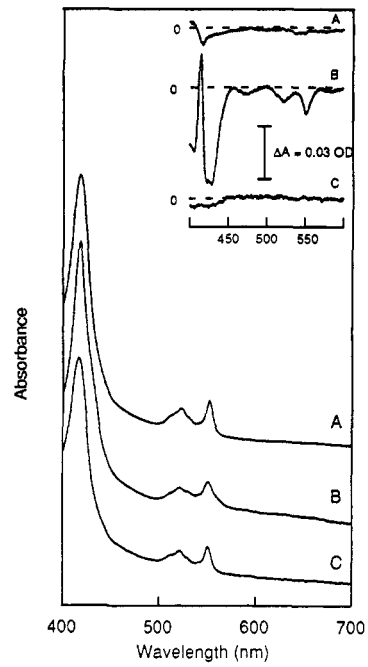

FIGURE 8: Dithionite-reduced absorption spectrum of various cytochromes c_1. (A) Wild type; (B) *c*M183L; (C) *c*M205V. Cytochrome was solubilized in 50 mM Tris-HCl (pH 8.0) containing 1 mM DTT, 1 mM EDTA, and 2% sodium cholate, except for spectrum B where 0.25% cholate was used, as described under Materials and Methods. Inset: CO difference spectra of cyt *b*-depleted complexes. Samples were reduced with sodium dithionite prior to bubbling with CO, and the spectra shown are those obtained from the subtraction of dithionite + CO *minus* dithionite for the wild type (A), *c*M183L (B), and *c*M205V (C).

band near 44 kDa, although certain fractions contained some Rieske FeS protein (not shown). Therefore, any binding of CO that may be observed with these preparations is not due to contaminating cyt *b*. The results of the CO binding experiments are shown in the inset of Figure 8. The cyt *b*-depleted wild-type (panel A) and *c*M205V (panel C) complexes did not bind CO, consistent with the results from intact complex. On the other hand, large spectral perturbations caused by CO binding are observed for *c*M183L (panel B); positive peak positions are 402 and 500 nm while negative peaks are at 422, 428 (this peak is split), 474, 522, and 551.2 nm.

It is interesting that all three cyt c_1 absorption spectra (Figure 8) are very similar in that the Soret and α and β transitions occur at the same energies although the ratios of the amplitudes vary. The ΔA_{418}:ΔA_{550} ratio is 11.2 and 12.9 for wild type and *c*M205V, respectively, but that for *c*M183L is 20.7, indicating either a decrease in the probability of an electronic transition in the α-band or an increase of that of the Soret band. The former is more likely since the spectrum of totally reduced wild-type bc_1 complex has a distinct feature at 550 nm from cyt c_1 while that of *c*M183L shows only a slight shoulder at the same wavelength (see Figure 5 for purified complexes and Figure 4 for chromatophores).

DISCUSSION

This study considers two different aspects of cyt c_1 structure and function. A minor theme is related to the role of the cyt c_1 subunit in the in vivo stability of the bc_1 complex as a whole. Davidson et al. (1992) have shown that if the C-terminal portion of the *fbc*C gene is deleted (the *R. capsulatus* mutant designated pMT4-404/MT-RBC1) the other two components of the complex, namely, cyt *b* and the Rieske FeS protein, are

highly unstable in the membrane in vivo. On the other hand, if the Rieske subunit is eliminated, the cyt *b* and c_1 subunits form a stable two-component bc_1 complex. These findings suggested that some structural feature of cyt c_1 must be necessary to confer stability on the complex. This was further pursued by the analysis of two additional cyt c_1 mutants. First, a stop codon was fortuitously obtained at position M205, which would truncate cyt c_1 prior to its putative transmembrane anchor, and possibly allow the expression of a soluble form as has been observed by Konishi et al. (1991) for cyt c_1 from *R. sphaeroides*. However, in *R. capsulatus*, no evidence for a soluble cyt c_1 was found in *c*M205* either by spectroscopy, by immunochemistry, or by heme peroxidase activity. Redox difference spectroscopy (Figure 4) and Western blot analysis using a cyt *b*-specific monoclonal antibody (data not shown) indicated that cyt *b* is present at very low amounts in the membrane of this strain as compared to wild type and multiple lower molecular mass bands were apparent, suggesting degradation as already seen by Davidson et al. (1992) in pMT4-404/MT-RBC1. Second, to test if the putative membrane-spanning region of cyt c_1 stabilizes cyt *b*, a plasmid was constructed which deleted a major portion of the soluble region of cyt c_1. No immunological evidence for the insertion of a smaller cyt c_1-related polypeptide in the membrane was found in this mutant. Western blots using antibodies against cyt *b* of membranes isolated from this strain were also similar to both *c*M205* and pMT4-404, as expected for the absence of cyt c_1. Thus, it is possible that both the signal sequence and the bound heme are necessary for the effective translocation of cyt c_1 across the membrane and its insertion into the lipid bilayer. Such a situation occurs in the nitrogen-fixing bacterium *Bradyrhizobium japonicum* in which an intact heme binding site in cyt c_1 is necessary for the immunological detection of both cyt c_1 and cyt *b* apoproteins in the membrane (Thöny-Meyer et al., 1991). In summary, although an intact cyt c_1 is required for the proper assembly of the bc_1 complex, clearly, further work is required to locate the structural determinants of cyt *b* and c_1 stabilizing the *R. capsulatus* bc_1 complex in vivo.

The major theme of this work is an attempt to identify, through mutagenesis and biophysical characterization, the sixth axial ligand to the heme Fe of cyt c_1. The replacement of M183 with leucine is deleterious to the cell; this mutant can no longer sustain phototrophic growth; however, the replacement of M205 with valine has no effect upon cell growth. The flash-activated cyt *c* oxidation–rereduction and the DBH cyt *c* reductase assays clearly show that the Ps^- phenotype of the former mutant is due to the absence of turnover of the bc_1 complex, despite the presence of all apoproteins and cofactors as illustrated by SDS–PAGE, Western, and spectroscopic analyses of chromatophores.

The cyt c_1 redox titration data indicate that electron transfer from the Rieske cluster to *c*M183L cyt c_1 is no longer thermodynamically favorable. The ΔE_m [E_m(cyt c_1) *minus* E_m([2Fe–2S])] in this mutant is about –370 mV, resulting in a large positive ΔG (35.2 kJ mol^{-1}) for the reaction in contrast to about +20 mV and a negative ΔG (–1.9 kJ mol^{-1}) in the wild type. This effect is analogous to the observed effect following binding of stigmatellin to the complex in which an increase in the E_m value of the [2Fe–2S] cluster by approximately 150 mV occurs [to ≈+450 mV (von Jagow & Ohnishi, 1985)], inhibiting electron transfer from the cluster to cyt c_1 and yielding a Ps^- phenotype. Electron transfer from cyt c_1 to cyt c_2 in *c*M183L is thermodynamically favorable, yet whether this reaction occurs has not been measured. Additionally, the effect of the *c*M183L mutation on the formation of the cyt c_1/cyt c_2 complex is unknown.

The removal of one ligand to the heme Fe should allow binding of exogenous ligands due to a vacant position in the ligation sphere. Although CO binding to the mutant complex was observed, its direct interpretation was complicated by the unexpected CO binding to the purified wild-type complex possibly due to the damaged cyt *b* caused by protein isolation. It was therefore necessary to either isolate cyt c_1 or deplete the complex of cyt *b*. The cyt *b*-depleted *c*M183L complex did indeed bind CO while those derived from wild type and *c*M205V did not, indicating that the removal of M183 of cyt c_1 somehow rendered a position in the ligation sphere accessible to exogenous ligands. The split negative feature near 420 nm in the CO difference spectrum indicates that there may be heterogeneity in the sample due to a mixture of different forms (perhaps heme spin state) of the same protein. On the basis of the data presented here, it cannot be unequivocally discounted that cyt c_1 from *c*M183L unfolded during purification, thus allowing CO to bind. However, the basically unperturbed absorption spectrum of cyt c_1 (aside from the increase in the ratio of the Soret to α-peak, which is also observed in intact complexes and the membrane) from *c*M183L renders this possibility unlikely.

The absorption spectrum (with a sharp, intense Soret transition at 418 nm and sharp, distinctive α and β transitions at 551 and 522 nm, respectively) of the reduced *c*M183L cyt c_1 indicates that the heme is low-spin (Wood, 1984). A high-spin *c*-type ferrous heme typically has a Soret transition closer to 430 nm with a fairly broad $\alpha\beta$-region without a distinct β-band (Bartsch, 1978). If a water molecule is occupying the sixth position in cyt c_1 of *c*M183L, one would expect the heme complex to be purely high-spin since water is a weak-field ligand. It is therefore surprising that it appears low-spin. A possibility is that upon removal of M183 a different amino acid residue (acting as a strong-field ligand) replaces it in the coordination sphere, resulting in a low-spin heme. If so, then this ligand would have to be quite labile since it is displaced by CO at neutral pH.

It is interesting that a single amino acid change (M183L) affects the equilibrium redox midpoint potential of a heme to such a dramatic degree. The highly positive redox potential for the Fe(II)/Fe(III) couple in the wild-type protein indicates that the ferrous form of the Fe is stabilized. In contrast, the relatively negative redox potential for cyt c_1 from *c*M183L indicates that the oxidized state of the Fe is stabilized. There are several factors which control the relative stability of the oxidized and reduced states of hemes within proteins [reviewed in Cusanovich et al. (1987), Moore et al. (1986), and Moore and Pettigrew (1990)]. Among these determinants is the hydrophobicity of the heme binding pocket, with a hydrophobic environment resulting in a highly positive redox potential. Second, the identity of the axial ligands plays a role; a methionine sulfur is a good π-electron acceptor and stabilizes the reduced state, resulting in a more positive redox potential as compared to bis(imidazole) coordination. Therefore, the observed decrease in the E_m value of the heme group of cyt c_1 from *c*M183L is consistent with loss of a sulfur ligand. Model compounds (with the fifth position occupied by imidazole) in which the sixth position is either vacant (Harbury & Loach, 1960) or occupied by imidazole (Harbury et al., 1965) typically have redox potentials more negative (by about 160 mV) than methionyl ligation (Marchon et al., 1982). Moreover, the pentacoordinate cytochromes *c*′ have E_m values ranging from 0 to 150 mV (Meyer & Cusanovich, 1989).

However, the heme in cyt c' is high-spin, and there are other structural differences between the soluble cyt c' and cyt c_1 which could account for some of the decrease in the E_m value.

Other factors besides the ligand environment, such as protein conformation and complex electrostatic interactions, also are involved in the relative stabilization of the oxidized and reduced states. It is thus possible that the removal of the sulfur in *c*M183L accounts for only a portion of the drop in E_m observed whereas it may also be partially due to electrostatic effects caused by an increase in the exposure of the heme group to the external solvent or more hydrophilic amino acid side chains. This increased exposure may be caused by a partial unfolding of the protein around the heme when the ligand is removed, thereby "loosening" the structure. The cyt c_1 of *c*M183L is more susceptible to proteolytic degradation as evidenced by the extra heme-stained band seen in Figure 3, and increased proteolytic degradation is indicative of a loss of tertiary structure. Perhaps the methionyl ligand to the heme Fe "locks" the protein into a more rigid structure, excluding water and hydrophilic groups as well as directly modulating the thermodynamic properties of the heme.

There have been other studies related to the axial ligand replacement in *c*-type cytochromes using various approaches. Nakai et al. (1990) used site-directed mutagenesis to replace M164 (corresponding to M183 in *R. capsulatus*) of cyt c_1 from the yeast *Saccharomyces cerevisiae* with leucine and lysine and observed that these mutants were unable to grow on nonfermentable substrates. While they could detect the presence of the apoprotein in Western blots of total cell homogenates, they were unable to detect an α-band in redox difference spectra for M164L but M164K had a very small α-band. Hampsey et al. (1986) reported missense mutants of yeast iso-1-cyt *c* at M85 which is the sixth axial ligand. In contrast to the mutations in the consensus N-terminal heme binding motif, these latter mutants were partially functional as indicated by growth on nonfermentable substrates. Raphael and Gray (1989, 1991) used semisynthesis to replace the sixth axial ligand (M80) of horse heart cyt *c* with histidine, leucine, and cysteine and obtained results very similar with those reported in this work. These substitutions resulted in a reduction of the midpoint potential of over 600 mV for M80C, 300 mV for M80L, and 220 mV for M80H. The mutant proteins reacted with CO, and M80H and M80L had electronic absorption spectra similar to that of the wild-type protein. While the heme ligation in M80C was confirmed to be His–Cys by MCD measurements, it was proposed that the probable ligation in M80L was His–H_2O. However, the optical absorption spectrum of M80L is no different from the wild-type protein, suggesting that the heme is low-spin which would be inconsistent with His–H_2O ligation. Wallace and Clark-Lewis (1992) have recently presented data on several substitutions including both natural and nonnatural amino acids at M80 of horse heart cyt *c* also using semisynthesis. Replacement of M80 with the aliphatic amino acids norleucine (Nle) and alanine did not result in a high-spin heme, and it was suggested that the protein provides an alternative strong-field ligand, as may also be the case in *c*M183L of *R. capsulatus*. However, the E_m value of M80Nle is very much similar to that of the wild-type cytochrome, in contrast to both our results and those of Raphael and Gray (1991) for a methionine to leucine substitution. Wallace and Clark-Lewis (1992) further suggested that this may be due to the more structurally disruptive branched-chain nature of leucine.

In conclusion, the data presented in this work are consistent with M183 being the sixth axial ligand to the heme Fe in cyt c_1, and all of the effects observed can be accounted for by the removal of a methionyl sulfur from the ligation sphere of a heme. Clearly, more detailed structural and spectroscopic analyses are needed to further establish the nature of the sixth ligand of cyt c_1 and to answer some of the questions raised in this study.

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