Interactions between the Cytochrome $b$, Cytochrome $c_1$, and Fe–S Protein Subunits at the Ubihydroquinone Oxidation Site of the $bc_1$ Complex of *Rhodobacter capsulatus*†

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ABSTRACT: Ubihydroquinone:cytochrome (cyt) $c$ oxidoreductase ($bc_1$ complex and its plant counterpart $b_{cf}$ complex) is a vital component of energy-transducing systems in most organisms from bacteria to eukaryotes. In the facultative phototrophic (Ps) bacterium *Rhodobacter capsulatus*, it is constituted by the cyt $b$, cyt $c_1$, and Rieske Fe–S protein subunits and is essential for Ps growth. Of these subunits, cyt $b$ has two nontransmembrane helices, $cd1$ and $cd2$, which are critical for its structure and function. In particular, substitution of threonine (T) at position 163 on $cd1$ with phenylalanine (F) or proline (P) leads to the absence of the $bc_1$ complex. Here, Ps* revertants of B:T163F were obtained, and their detailed characterizations indicated that position 163 is important for the assembly of the $bc_1$ complex by mediating subunit interactions at the $Q_o$ site. The loss of the hydroxyl group at position 182 of cyt $b$ was compensated for by the gain of either a hydroxyl group at position 182 of cyt $b$ or 46 of the Fe–S protein or a sulfhydryl group at position 46 of cyt $c_1$. Examination of the mitochondrial $bc_1$ complex crystal structure [Zhang, Z., Huang, L., Shulmeister, V. M., Chi, Y.-I., Kim, K. K., Hung, L.-W., Crofts, A. R., Berry, E. A., and Kim, S.-H. (1998) *Nature* 392, 677–684] revealed that the counterparts of B:G182 (i.e., G167) and F:A46 (i.e., A70) are located close to B:T163 (i.e., T148), whereas the C:R46 (i.e., R28) is remarkably far from it. The revertants contained substoichiometric amounts of the Fe–S protein subunit and exhibited steady-state and single-turnover, electron transfer activities lower than that of a wild-type $bc_1$ complex. Interestingly, their membrane supernatants contained a smaller form of this subunit with physicochemical properties identical to those of its membrane-bound form. Determination of the amino-terminal amino acid sequence of this soluble Fe–S protein revealed that it was derived from the wild-type protein by proteolytic cleavage at V44. This work revealed for the first time that position 163 of cyt $b$ is important both for proper subunit interactions at the $Q_o$ site and for inactivation of the $bc_1$ complex by proteolytic cleavage of its Fe–S protein subunit at a region apparently responsible for its mobility during $Q_o$ site catalysis.

Ubihydroquinone:cytochrome (cyt)$c$ oxidoreductase ($bc_1$ complex and its plant counterpart $b_{cf}$ complex) is an important energy transduction complex in most organisms from bacteria to eukaryotes. In purple non-sulfur photosynthetic bacteria like *Rhodobacter capsulatus*, it conveys electrons from ubihydroquinone to both cyt $c_2$ and $c_3$, and contributes to the formation of a proton gradient subsequently used for ATP production (for recent reviews, see refs 1–3).

In most bacteria, the $bc_1$ complex is composed of the Rieske Fe–S protein, cyt $b$, and cyt $c_1$ subunits encoded by $fbcF/petA$, $fbcB/petB$, and $fbcC/petC$ genes organized as an operon (see for a review ref 3). Cyt $b$ is a highly conserved integral membrane protein (4) which bears two heme groups, $b_1$ and $b_4$, with low and high redox midpoint potential ($E_{m_1}$) values, respectively. Both the Fe–S protein which contains a [2Fe–2S] cluster which can be detected by EPR spectroscopy in its reduced form and cyt $c_1$ which has a covalently attached heme group are membrane-anchored and face the periplasm. Oxidation of ubihydroquinone and reduction of ubiquinone take place in two distinct domains of the $bc_1$

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∥† Abbreviations: cyt, cytochrome; $bc_1$ complex, ubiquinol:ubiquinone cytochrome $c$ oxidoreductase; $Q_h$, ubiquinol ubiquinone; $Q$, ubiquinone; $Q_o$, ubiquinone oxidation site; $Q_i$, ubiquinone reduction site; [2Fe–2S], two iron–two sulfur cluster of the Rieske Fe–S protein; EPR, electron paramagnetic resonance; $b_l$, low-potential $b$-type heme; $b_h$, high-potential $b$-type heme; $E_{m_1}$, ambient potential; $E_{m_2}$, redox midpoint potential at pH 7.0; Ps, photosynthesis; $E_{a,7}$, ambient potential; Ps, photosynthesis; Res, respiration; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ONPG, 4-methylumbelliferyl-β-D-galactoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside; DAD, 2,3,5,6-tetramethyl-p-phenylenediamine; PMS, N-methylphenazonemethosulfate; PHES, N-ethylendibenzoprazine ethosulfate; MOPS, (N-morpholinopropanesulfonic acid; TMPD, [1,2-naphtho-4-sulfonic acid; EDTA, N,N′,N′-tetramethyl-p-phenylenediamine; 1,2-NQ-4S, 1,2-naphthoquinone–4-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DBH, 2,3-dimethoxy-5-decyl-6-methyl-1,4-benzoquinone; SDS, standard hydrogen electrode.

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complex, called Q\(_o\) and Q sites. These sites are located near the hemes b\(_1\) and b\(_2\) on the outer and inner sides of the cytoplasmic membrane, respectively. Earlier indications about the location of these sites within the bc\(_1\) complex came from genetic analysis of spontaneous inhibitor resistance mutations in several species, mainly in yeast and bacteria (for a review, see ref 5). In the case of the Q\(_o\) site, most of these mutations were confined to a highly conserved portion of cyt b called the Q,I domain encompassing residues M140–T163 (6) (Figure 1). The Q,I domain is located between helices C and D of cyt b and bears a structural resemblance to the quinone binding (Q\(_a\) and Q\(_b\)) domains of the bacterial photosynthetic reaction center (RC) (7). Biochemical, biophysical, and genetic studies have revealed that residues F144, G152, and G158 affect the Q/QH\(_2\) occupancy of the Q\(_o\) site (7–11), that M140 and Y147 are essential for electron transfer from the Q\(_o\) site to the [2Fe-2S] cluster and to cyt b\(_1\) (12), and that G146 is critical for proper packing of heme b\(_1\) (13) (Figure 1).

The mitochondrial bc\(_1\) complexes from both chicken heart and bovine heart have been crystallized (14–16), and their complete structures are now solved (17, 45). With respect to cyt b, the structure has confirmed the previously predicted eight transmembrane helices structure (for a review, see ref 1) and further revealed the presence of two non-transmembrane helices (cd1 and cd2) around the Q\(_o\) site (17, 45). A great deal of structural information, such as the distances between the redox centers in the bc\(_1\) complex, is now available. Unexpectedly, it was found that the Fe–S protein can occupy distinct positions in different crystals of the bc\(_1\) complex, hinting at its mobility (17–19, 45).

Currently, there is a fair amount of data about the role of several residues affecting Q/QH\(_2\) binding or electron transfer reactions at the Q\(_o\) site (reviewed in ref 5). However, little is known about the residues coordinating the protein interactions between the subunits and their postulated movement during catalysis. The nature of the electronic events within the bc\(_1\) complex and the available data clearly suggest that the Q,I region of cyt b interacts with the [2Fe-2S] cluster region of the Fe–S protein around non-transmembrane helices cd1 and cd2 (Figure 1). Indeed, a few mutations located in the Q,I domain have been found to affect binding of the Fe–S protein to the cyt b subunit in yeast (20), or of the accessory fourth subunit in Rhodobacter sphaeroides (21). Furthermore, it has been shown that mutants lacking the Fe–S protein subunit still form a cyt b–cyt c\(_1\) subcomplex (22), which can be purified as a reconstitutively active entity (M. Valkova-Valchanova et al., in preparation). On the other hand, mutants lacking cyt b yield only low amounts of cyt c\(_1\), and those without cyt c\(_1\) contain no bc\(_1\) complex in their cytoplasmic membranes (23).

An important insight into the formation of the Q\(_o\) site became available by the observation that among the many Q,I region mutants only the B:T163F and -P substitutions abolished the assembly of the bc\(_1\) complex (9). This finding, in light of the work of Davidson et al. (23), strongly suggested that these mutations must also perturb the interactions between cyt b and cyt c\(_1\) subunits, since otherwise they would have contained a cyt b–cyt c\(_1\) subcomplex. Revertants of the B:T163F mutation were therefore selected, and the intragenic and intergenic second-site suppressors located in cyt b, cyt c\(_1\), and the Fe–S protein were characterized in detail. The double mutants contained considerable amounts of a soluble, truncated form of the Rieske Fe–S protein with wild-type physicochemical properties. These findings indicated for the first time that non-transmembrane helix cd1 of cyt b is critical not only for the assembly of the bc\(_1\) complex but also for its proteolytic inactivation via cleavage of the Fe–S protein subunit at a region thought to be important for its mobility during Q\(_o\) site catalysis.

MATERIALS AND METHODS

Growth Conditions and Strains. R. capsulatus and Escherichia coli strains were grown in MPYE and LB media, respectively, supplemented with appropriate antibiotics as needed (24). The strains that were used are listed in Table 1.

Preparation of R. capsulatus Electrocompetent Cells and Electroporation. R. capsulatus strains (MT1131 and its derivatives) were grown in MPYE medium until an OD\(_{620}\) of 0.4–0.5 was reached for the preparation of electrocompetent cells and harvested by centrifugation at room temperature for 10 min at 5 K. They were washed twice with sterile double-distilled water and once with 10% glycerol. The final pellet was resuspended in 10% glycerol, distributed into 200 \(\mu\)L aliquots, rapidly frozen in a dry ice–ethanol bath, and stored at −80 °C. For electroporation, 40 \(\mu\)L of competent cells (in a 0.2 cm gap cuvette) and 10–100 ng of plasmid DNA isolated from R. capsulatus were incubated for 10 min at 4 °C. After electroporation with a Bio-Rad Gene Pulser set at 25 mF, 2.5 kV, and 400 W with a time constant of >8 s, cells were incubated in MPYE medium supplemented with 10 mM MgCl\(_2\) and 10 mM MgSO\(_4\) for 4 h at 35 °C, centrifuged at 4 K for 5 min,
and conjugation into MT-RBC1, five independent Ps
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and the corresponding ambient redox potentials are also indicated.

resuspended in 100 µL of MPYE medium, spread on MPYE plates, and incubated under selective growth conditions.

Isolation and Analysis of Ps
+ Revertants of the B:T163F Mutant of R. capsulatus. A total of 1790 spontaneous Ps
+ revertants of the B:T163F mutant (9) were isolated from six independent cultures on MPYE plates supplemented with tetracycline and pooled together. Their plasmid DNA was extracted and electroprociated into the R. capsulatus strain MT-RBC1, and the Ps
+ colonies thus obtained were repooled for plasmid DNA preparation. The BstXI–EcoRI, EcoRI–Styl, and StyI–Stul fragments containing almost the entire fbcF, fbcB, and fbcC genes were purified by gel electrophoresis from this pooled DNA and exchanged with their appropriate counterparts on pPET-B:T163F containing the B:T163F mutation (9). After subsequent ligation to pRK404 and conjugation into MT-RBC1, five independent Ps
+ colonies were retained in each case, and the fragments thus exchanged were resequenced to confirm the presence of the B:T163F mutation and to identify the nature of the additional mutation conferring the Ps
+ phenotype. These analyses revealed that the clones obtained using the BstXI–EcoRI, EcoRI–Styl, and StyI–Stul DNA fragments contained a single mutation at position 46 [Ala(GCC) to Thr(ACC)] of the Fe–S protein (fbcF), 182 [Gly(GGC) to Ser(GAC)] of cyt b (fbcB), and 46 [Arg(CGC) to Cys(TGC)] of cyt c1 (fbcC), respectively (Figure 2). These plasmids were designated p(B:T163F+B:G182S), p(B:T163F+C:R46C), and p(B:T163F+F:A46T); their fbcFB operons were resequenced to ensure that the second-site revertants (B:G182S, B:T163F and B:T163F+F: A46T) contained only these mutations.

Construction of the Single Mutants Carrying Only the Second-Site Suppressor Mutations. For the fbcF:A46T fragment, the 530 bp BstXI–EcoRI fragment and, for the fbcB:G182S and fbcC:R46C mutations, the 1430 bp Stul–SpilI fragment were excised from appropriate plasmids and exchanged with their counterparts on pPET1 bearing a wild-type copy of fbcF (8). The plasmids thus obtained were designated pPET-F:A46T, pPET-B:G182S, and pPET-C: R46C, respectively, ligated into pRK404 using their unique HindIII restriction site, and introduced into R. capsulatus strain MT-RBC1 via triparental crosses selecting for Tet R.

Construction of the fbcFB′::lac Z Fusions and β-Galactosidase Assays. For the fbcFB′::lac Z operon fusions, the
promoter-cloning vector pGD499 (25) was digested with HindIII and treated with mung bean nuclease. The blunt-ended vector DNA was then digested with BamHI, and the 29.1 kb DNA fragment containing a promoterless lacZ gene was isolated by using “elutips” according to the manufacturer’s instructions (Schleicher and Schuell, Keen, NH). It was then ligated to the purified 1.7 kb BamHI–SmaI fragments isolated from the appropriate plasmids, pMTS1 (wild-type) (12) and its derivative carrying B:T163 which contained the promoter of fbcFBC, the entire fbcF, and part of fbcB (Figure 2). The plasmids thus obtained, pSmaFB′-Z and pSmaFB(T163F)-Z, were introduced into E. coli HB101 cells using CaCl2 transformation and conjugated into MT-RBC1 selecting for Tet8. The β-galactosidase activity of these transconjugants was monitored by the appearance of a blue color on MPYE plates containing X Gal. Appropriate isolates were grown in MPYE liquid medium supplemented with tetracycline. Cells were then harvested at an OD630 of 0.45–0.48, resuspended in a phosphate buffer (60 mM Na2HPO4, 40 mM NaH2PO4·H2O, 10 mM KCl, 2 mM MgSO4, and 0.25% β-mercaptoethanol), and sonicated briefly. Cell debris was eliminated by centrifugation at 14000g using a microfuge, and 100 μl aliquots of the supernatants were assayed using ONPG (4 mg/mL). Development of the yellow color was monitored (vs 20–25 min for the background) at 35 °C by addition of 1 mL of 1 M sodium carbonate. The β-galactosidase specific activity was calculated as millimoles of ONPG hydrolyzed per minute per milligram of total proteins.

Preparation of Chromatophore Membranes and Supernatants. Chromatophore membranes were prepared in 50 mM MOPS (pH 7.0) buffer containing 100 mM KCl, 1 mM PMSF, and 1 mM EDTA using a French pressure cell as described earlier (8), except that for spectroscopic analyses they were washed three times with the same buffer. Bacteriochlorophyll and protein concentrations were determined spectrophotometrically using an ε775 of 75 mM−1 cm−1 and according to Lowry et al. (26), respectively. Membrane-free supernatants were prepared by extensive ultracentrifugation of chromatophore membrane supernatants (spin twice for 16 and 5 h, respectively, at 4 °C and 258000g) to ensure that all membrane debris was eliminated. They were then concentrated about 15-fold by using Amicon Centriprep 3 concentrators with a molecular mass cutoff of 3000 Da.

SDS–PAGE and Western Blot Analysis. Laemmli-type SDS–PAGE (with a 15 or 17% acrylamide concentration) was used to analyze the presence of the bc1 complex subunits in chromatophore membranes and their supernatants. After being stained with Coomassie blue, gels were scanned with a Molecular Dynamics Personal Scanner and analyzed using the IP-GEL documentation software (Molecular Dynamics). Western blot analyses were performed as described in Atta-Asafo-Adjei and Daldal (8) using antibodies raised against the Fe–S protein cyt b and cyt c subunits of the R. capsulatus bc1 complex.

Flash-Activated Single-Turnover Kinetics. Qo site catalysis was monitored by flash-activated, time-resolved single-turnover kinetics essentially as described earlier (27), except that a single wavelength spectrophotometer was used (Biomedical Instrumentation Group, University of Pennsylvania, Philadelphia, PA). Flash-induced (8 μs actinic light pulse) cyt b, and cyt c re-reduction kinetics were measured using chromatophore membranes which contained approximately 0.20 μM bacterial RC, in the presence of 3 μM valinomycin, 2.5 μM PMS, 2.5 μM PES, 6 μM DAD, 10 μM 2-hydroxy-1,4-naphthoquinone, and 10 μM FeCl3−EDTA. The RC concentration was determined by measuring the optical absorption difference between 605 and 540 nm after four flashes at an E6 of 370 mV and using an extinction coefficient ε605 of 29.8 mM−1 cm−1. Samples were poised at desired E6 values using sodium dithionite or potassium ferricyanide, and the bc1 complex inhibitors antimycin A (10 μM) and myxothiazol (5 μM) were added as needed. Cyt b reduction and cyt c re-reduction were monitored at 560 minus 570 nm and 550 minus 540 nm, respectively, and the single-turnover electron transfer rates were calculated as in Gray et al. (27).

EPR Spectroscopy. The soluble and membrane-bound forms of the Fe–S protein in chromatophore membranes and their supernatants of the B:T163F+G:182S mutant were titrated in the presence of 10 μM TMPD and 50 μM 1,2-NQ-4S between the E6 values of 200 and 400 mV, and the amplitudes of the g1 signals were normalized and used to calculate the E60 values of their [2Fe-2S] clusters. In general, chromatophore membranes of the revertants and single mutants were prepared for EPR spectroscopy with the addition of 20 mM sodium ascorbate. The measurements were carried out using a Bruker spectrometer model ESP-300E, equipped with a helium cryostat under the following conditions: temperature, 20 K; microwave power, 2–5 mW; modulation amplitude, 12.5–20 G; modulation frequency, 100 kHz; and microwave frequency, 9.4 GHz.

Chemicals. PMS, PES, 3,5,6-trimethyl-p-phenylenediamine (diaminodurene), antimycin A, myxothiazol, stigmatellin, 2-hydroxy-1,4-naphthoquinone, and pyocyanine were obtained as described earlier (27), and oligonucleotides were synthesized by the Nucleic Acid Synthesis Facility of the University of Pennsylvania (Philadelphia, PA). All other chemicals were reagent grade and were purchased from commercial sources.

RESULTS

The Absence of the bc1 Complex in the B:T163F Mutant Is Not a Consequence of the Transcriptional Polarity of This Mutation. First, the transcriptional fusions pSmaFB′-Z and pSmaFB(T163F)-Z described in Materials and Methods were used to probe for any transcriptional effect of the B:T163F mutation (Figure 2). These constructs were introduced into the R. capsulatus strain MT-RBC1 [A(fbcFBC)] (Table 1), and the transconjugants obtained were assayed for their β-galactosidase activities. Both of the strains produced similar amounts of such activity (data not shown), indicating that the B:T163F mutation had no polar effect on the transcription of fbcFBC. In addition, since among the various mutations at position 163 only the F and P substitutions affected the assembly of the bc1 complex (9), it was concluded that the defect observed in the latter mutants was post-translational.

Molecular Nature of the Ps+ Revertants of the B:T163F Mutant. Ps+ revertants of B:T163F were then sought to provide further insight into the molecular nature of the events leading to the absence of the bc1 complex in this unusual mutant. The revertants were isolated and their molecular
nature was analyzed as described in Materials and Methods. Two extragenic and one intragenic second-site suppressor mutations, A46T, R46C, and G182S located in the Fe–S protein, cyt c1, and cyt b subunits, respectively, were found (Figure 2). The suppressor mutants B:T163F+B:G182S, B:T163F+C:R46C, and B:T163F+F:A46T and their derivatives carrying only the second-site mutations (B:G182S, B:F:A46T, and C:R46C constructed as described in Materials and Methods) were retained for further analysis.

Phenotypic Characterization of the B:T163F Revertants. The double mutants B:T163F+B:G182S, B:T163F+C:R46C, and B:T163F+F:A46T formed smaller colonies under Ps growth conditions and exhibited slightly longer doubling times (Ps growth rates in MPYE-enriched medium were 170, 210, and 150 min, respectively, vs 120 min for a wild-type strain). On the other hand, the single mutants carrying the second-site suppressor mutations B:G182S and F:A46T had growth rates almost identical to that of a wild-type strain while that of C:R46C was slightly slower (Table 1). Reduced minus oxidized optical difference spectra of the chromatophore membranes of all revertants were virtually identical to those of a wild-type strain (data not shown), and SDS–PAGE (Figure 3A) and Western blot analyses (data not shown) indicated that they contained wild-type amounts of the Fe–S clusters (not shown). The revertants B:T163F+B:G182S, B:T163F+C:R46C, and B:T163F+F:A46T revealed that B:T163F mutants had significantly smaller EPR signals at 1.80 when the Q pool was fully oxidized, indicating that G182S contained as little as 30% Fe–S protein subunits. On the other hand, in these strains, the amplitudes of the signal for cyt b reduction, or cyt c re-reduction, normalized to the amount of RC were also smaller (not shown), consistent with the stoichiometry of their Fe–S protein subunits. On the other hand, in the double mutant B:T163F+F:A46T and the single mutants F:A46T, C:R46C, and B:G182S, these rates were affected much less (Table 1).

Next, the EPR g1 signal of the [2Fe-2S] cluster of the Fe–S protein (Figure 4A) was used to semiquantitatively estimate its stoichiometry in mutant bc1 complexes. This analysis revealed that B:T163F+B:G182S and B:T163F+C:R46C mutants had significantly smaller EPR g1 signals per the amount of total membrane proteins (Figure 4B) than a wild-type strain. Again, the B:T163F+F:A46T double and B:G182S and C:R46C single mutants had about 2-fold less, while F:A46T had almost wild-type levels of EPR g1 signals. The EPR spectra, in combination with the SDS–PAGE data described above, illustrated the fact that the second-site suppressor mutations located in cyt b and cyt c1 restored less effectively the stoichiometry of the Fe–S protein subunit. Furthermore, the revertants B:T163F+B:G182S and B:T163F+C:R46C also exhibited weaker EPR g1 signals at g = 1.80 when the Q pool was fully oxidized, indicating that their Qo sites were also partially occupied, unlike the double mutant B:T163F+F:A46T and the single mutants B:G182S, C:R46C, and F:A46T (Figure 4A). Thus, among the three second-site suppressor mutations, the one located on the Fe–S protein was most efficient in overcoming the defect inflicted by the B:T163F mutation.
The Substoichiometry of the Fe–S Protein Subunit in B:T163F Revertants Is Not Induced by Increased Susceptibility of the [2Fe-2S] Cluster to Oxidative Damage. The low $b_{c1}$ complex activity found in the double mutants was unstable (although to a lesser extent in B:T163F+F:A46T) and often vanished within 2–3 days upon storage at 4 °C, unlike the wild-type activity which was stable over a period of 10 days under the same conditions. This observation led us to examine whether in these mutants the [2Fe-2S] cluster was more susceptible to oxidative damage upon exposure to air, as recently seen with several Fe–S protein mutants (28). For this purpose, the weakly suppressed double mutant B:T163F+B:G182S was chosen for further analysis (Figure 5). When the chromatophore membranes were prepared in the presence of 0.16 mg/mL sodium dithionite from anaerobically (Ps) grown cells of this mutant, the $b_{c1}$ complex activity was at most 2-fold higher than that observed in the membranes derived from respiratory-grown cells of the same mutant. In addition, when the ratio of the EPR $g_z$ signal to the amount of the Fe–S protein per total protein (estimated by SDS–PAGE) was determined, no more than a 15–20% difference was found between the values obtained using Ps- or Res-grown cells of a given mutant (Figure 5A,B). Thus, although a slight increase in the susceptibility of the [2Fe-2S] cluster to oxidative damage could be detected, this was not sufficient to account for the low and variable amount of the Fe–S protein found in the B:T163F revertants.

A Soluble Form of the Rieske Fe–S Protein. We reasoned that the substoichiometry of the Fe–S protein subunit in B:T163F+B:G182S and B:T163F+C:R46C (Figure 3) may be due to its proteolytic degradation. If this were the case, then its degradation products could be detected in the supernatants of chromatophore membranes. Indeed, upon analysis of concentrated membrane-free supernatants by SDS–PAGE and immunoblotting, a 20 kDa polypeptide was readily recognized by monoclonal antibodies specific for the $R. capsulatus$ Fe–S protein (29) (Figure 6). This band was also present, but to a much lesser extent, in the chromatophore membranes of both the wild-type strain pMT0-404/MT-RBC1 overproducing the $b_{c1}$ complex and the single mutants B:G182S, C:R46C, and F:A46T (data not shown). Conversely, the double mutants B:T163F+B:G182S and B:T163F+C:R46C contained approximately 3–5-fold higher amounts of this protein in the supernatants of the chromatophore membrane supernatants, while they exhibited similarly decreased amounts of the Fe–S protein in their chromatophore membranes (Figure 4B). The supernatants used in these experiments were devoid of membrane fragments, as confirmed by the absence of cyt $c_1$ using specific antibodies (data not shown).

The EPR $g_z$ signal corresponding to the [2Fe-2S] cluster of the Fe–S protein could be readily detected in the supernatants of the chromatophore membranes prepared from the revertants B:T163F+B:G182S (Figure 7, inset) and B:T163F+C:R46C (data not shown). This signal was very similar to that observed in chromatophore membranes (Figure 4A), indicating that the Fe–S protein detected in the supernatants contained a wild-type-like [2Fe-2S] cluster. Furthermore, the soluble form of the Fe–S protein could not respond to stigmatellin and had no pronounced EPR $g_z$ signal at $g = 1.80$ as shown earlier for the soluble bovine subunit (30). Potentiometric titration of the corresponding EPR $g_z$ signal indicated that the $E_m$ values of the membrane-bound Fe–S protein subunit present in intact $b_{c1}$ complexes and that found in a soluble state in chromatophore supernatants were identical (about 285 mV for B:T163F+B:G182S) (Figure 7).

Next, the soluble form of the Fe–S protein was immunoprecipitated from the membrane-free supernatants of chromatophore membranes of B:T163F+B:G182S using specific
monoclonal antibodies and purified by SDS-PAGE, and its amino-terminal amino acid sequence was determined as described earlier (29). The data obtained revealed that the soluble form of the Fe–S protein started with a valine followed by a KAMASIFVDVSAV sequence identical to positions 44–57 of its membrane-bound form. We therefore concluded that this subunit was cleaved between its amino acid residues aspartate (D) 43 and valine (V) 44, hence releasing it from the membrane, and rendering it substoichiometric in the bc1 complex. It was noticed that no further degradation of the cleaved portion of the Fe–S protein was observed, indicating that it must be folded properly to resist further proteolysis.

DISCUSSION

Detailed biochemical and genetic analyses of an unusual mutation located at position 163 of cyt b allowed us to reveal specific interactions between the subunits of the bc1 complex around non-transmembrane helix cd1 of cyt b. Earlier, intragenic second-site suppressor mutations located in the subunits of the bc1 complex have been reported, and the information obtained was useful in delimiting the interactions within them (8, 31, 32). In this work, extragenic second-site suppressor mutations between the different catalytic subunits of the bc1 complex were studied in detail.

Various cyt b mutations affecting the assembly, or the steady-state stability, of the bc1 complexes have been described previously. In bacteria, the amino acid residues located near the packing domains of the heme groups have severe effects, like the G48D substitution located near heme bH (33) or the R114Q substitution located at the beginning of the bc loop between the helices B and C of cyt b (34). In yeast mitochondria, two cyt b mutations, L282F (305 in R. capsulatus) located near the highly conserved PEWY sequence and G304E predicted to be buried in the membrane, lacked the bc1 complex (35). In addition, slight perturbations of the assembly of the bc1 complex have been observed with mutations at positions 137 and 256 (152 and 279 in R. capsulatus).
Figure 7: EPR redox titrations of chromatophore membranes and chromatophore membrane supernatants of the B:T163F+BG182S mutant grown by respiration. Redox titration for the [2Fe-2S] cluster of the Fe−S protein was performed by adding aliquots of sodium ascorbate in the presence of 10 μM TMPD and 50 μM 1.2-NQ-4S. The amplitude of the signal was recorded at various values, normalized, and fitted to an Nernst equation: (●) the membrane-bound form of the Fe−S protein (chromatophore membrane supernatants, 21.7 mg of protein/mL) and (△) fit the soluble form of the Fe−S protein (chromatophore membrane supernatants, 12.2 mg of protein/mL). EPR conditions were as described in the legend of Figure 4 except that the microwave power was 5 mW. The inset shows a sodium ascorbate-reduced EPR spectrum of chromatophore membranes (10.8 mg/mL) and (●) the membrane-bound form of the Fe−S protein (chromatophore membrane supernatants, 21.7 mg of protein/mL) and (△) fit the soluble form of the Fe−S protein (chromatophore membrane supernatants, 12.2 mg of protein/mL). EPR conditions were as follows: modulation amplitude, 20 G; microwave frequency, 9.49 GHz; microwave power, 10 mW; and modulation frequency, 100 kHz.

capsulatus) located in the cd1 and ef loops of yeast cyt b, respectively (20, 36). However, the B:T163F mutation described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here appears to be unique. Its effect is clearly post-translational and allele-specific, since among the several described here. The B:T163F revertants of B:T163F must restore both the assembly of the bc1 complex and not the functional impairment of its subunits.

Our earlier work on the steady-state stability of the bc1 complex (23) suggested that the B:T163F mutation must somehow affect not only the interactions between the Fe−S protein subunit and cyt b but also those between cyt b and cyt c1 subunits to abolish the assembly of all subunits. Examination of the three-dimensional structure of the chicken heart bc1 complex resolved by Berry and co-workers (45) indicated that the mitochondrial counterparts of R. capsulatus position B:T163 (i.e., T148 in the mitochondrial structure) and its suppressors, B:G182 (i.e., G167) and F:A46 (i.e., A70), described here are close to each other (Figure 8A). It is noteworthy that both of the two independent suppressor mutations reintroduce a hydroxyl group, suggesting that such a group may be necessary for the proper assembly of the bc1 complex. However, these residues are not within hydrogen bonding distances of each other (Figure 8B), and various other substitutions at position 163, like V, L, I, and A, also yield inhibitor-resistant mutants (9). Thus, perhaps it is the presence of a bulky aromatic side chain (such as that of F) that abolishes the steady-state presence of the bc1 complex in chromatophore membranes. On the other hand, the third suppressor position in the cyt c1 subunit of R. capsulatus, C:R46 (i.e., R28 in the mitochondrial structure), is located remarkably far from B:T163 (i.e., B:T148) and also from the other suppressor positions in the bc1 complex structure from the chicken heart mitochondria (Figure 8A) (18). How this mutation, which behaves as a global suppressor, compensates for the assembly defect of the bc1 complex at such a distance is at present unclear. A possibility is that perhaps the overall folding of R. capsulatus cyt c1 is distinct from that of its mitochondrial counterpart, which is unlikely in light of their pronounced homologies. Nonetheless, it would be of interest to define the exact location of C:R46C of cyt c1 in the structure of the bc1 complex from R. capsulatus when it becomes available.

Of the suppressor positions, G182 of cyt b is highly conserved in all species while R46 of cyt c1 is only present in the bc1 complexes and substituted with hydrophobic residues in the bgf complexes (4). Position A46 of the Fe−S protein is also conserved in all Fe−S proteins except in those of some cyanobacteria. However, none of these residues is essential for either the structure or function of the bc1 complex since their introduction into an otherwise wild-type background has no deleterious effect. Finally, we note that the Ps4+ revertants of B:T163F must restore both the assembly of the bc1 complex and the function of its Qo site, and further dissection of these two intertwined effects needs appropriate triple mutants. In this respect, noteworthy is the fact that the only triple mutant so far available, B:T163F+FL136G+F:A46T, compensates preferentially for the assembly defect of the bc1 complex and not the functional impairment of its Qo site (G. Brasseur and F. Daldal, unpublished data).

A soluble Fe−S protein subunit similar to that encountered in this work has been obtained earlier from purified Neurospora crassa or the beef heart mitochondrial bc1 complex by using limited chymotrypsin (37, 38) or trypsin (39) digestion in vitro. Recently, a soluble form of the Fe−S protein subunit has also been obtained from beef heart mitochondria (30) using thermolysin which cleaves it at position V68 (corresponding to V44 in R. capsulatus), and its three-dimensional structure has been solved by Iwata et al. (40). Finally, Zhang et al. (41) have also obtained a soluble Fe−S protein by proteolytic cleavage from the bgf complex and solved its three-dimensional structure (42). On the other hand, obtaining a soluble form of the bacterial Fe−S protein subunit using molecular genetics has been difficult. While Van Doren et al. (43) have cloned and expressed the R. sphaeroides fbcF operon. Furthermore, the upstream and downstream locations of the second-site suppressor mutations in fbcFBC are consistent with the lack of any effect of B:T163F on the translation of the bc1 complex subunits.
for example a specific protease, remains unknown. To what extent this proteolysis takes place during cell growth in vivo or during preparation of the chromatophore membranes in vitro is also unclear. Its production may simply be amplified in B:T163F revertants since even in pMT0-404/MT-RBC1 overproducing the wild-type bc1 complex small amounts of the cleaved product can be detected. In any event, it is important to underline the fact that the cleavage site (V44) is only two residues away from position 46 where the strongest of the suppressor mutations described here (F: A46T) is located. Moreover, intragenic suppressors of the L136G and -H mutations located next to the [2Fe-2S] cluster ligands of the Fe-S protein subunit (28) have also been found at both position 44 and 46 (32). Finally, recent
structural data suggest that this very same region might also mediate movement of the Fe–S protein subunit in response to stigmatellin (17–19, 45). Therefore, the flexible aminoterminal portion of the Fe–S protein encompassing residues M62 SASADVLA70 (corresponding to M38 NASADVKA46 in R. capsulatus) is important not only for the assembly of the bc1 complex but also for the structure and function of its Qo site.

In conclusion, T163 of cyt b is the very first example of critical residues affecting cleavage of the Fe–S protein subunit at a region thought to be responsible for its mobility during Qo site catalysis. Whether this cleavage will turn out to be the basis of a molecular mechanism regulating cellular energy transduction by proteolytic inactivation of the bc1 complex remains to be seen.

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