The Involvement of Threonine 160 of Cytochrome b of Rhodobacter sphaeroides Cytochrome bc1 Complex in Quinone Binding and Interaction with Subunit IV*

(Received for publication, July 20, 1995, and in revised form, September 20, 1995)

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Abstract
The cytochrome b subunit (subunit I) of the ubiquinol-cytochrome c reductase (bc1 complex) is thought to participate in the formation of two quinone/quinol reaction centers, an oxidizing center (Qo) and a reducing center, in accordance with the quinone cycle mechanism. Threonine 160 is a highly conserved residue in a segment of subunit I that was shown to bind quinone and is placed near the putative Qo site in current models of the bc1 complex. Rhodobacter sphaeroides cells expressing bc1 complexes with Ser or Tyr substituted for Thr160 grow photosynthetically at a reduced rate, and cells expressing the mutated complexes produce an "elevated" level of the bc1 complex. The Ser substitution also affects the interaction of subunit IV with subunit I. Replacement of Thr160 by Ser results in about a 70% loss of the activity in the purified complex, whereas substitution by Tyr lowers the activity by more than 80%. Both replacements lower the apparent K_m for ubiquinol. Electron paramagnetic resonance (EPR) spectroscopy shows that in the Ser substituted complex, the environments of the Rieske iron-sulfur cluster in subunit III and the high potential cytochrome b (b562) in subunit I have been modified. The spectra of the Ser and Tyr160 iron-sulfur clusters have become redox-insensitive, with a line shape resembling that of the native complex in the fully reduced state. The EPR signal of b562 in the Ser160 complex is shifted from g = 3.50 to g = 3.52, but otherwise the line shape is very similar to the spectrum of the native complex. Most of these results are consistent with current ideas regarding the structure and function of Qo in the bc1 complex, except for the alteration of the b562 EPR feature, because this heme is not thought to be located in proximity to Qo.

Immunoblotting analysis showed that the Ser or Tyr substituted complex contained significantly less than a stoichiometric amount of subunit IV. The enzymatic activity of mutated bc1 complex was found to be activable by the addition of purified subunit IV. These results indicate that Thr160 plays an important role in the structure and/or function of the bc1 complex.

Key words: cytochrome b, Rhodobacter sphaeroides, subunit I, subunit IV, ubiquinol-cytochrome c reductase, quinone binding, EPR, immunoblotting

* This work was supported in part by the Oklahoma Center for the Advancement of Science and Technology Grant HN3-008 (to M. W. M.) and National Institutes of Health Grant GM 30721 (to C.-A. Y.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Q, ubiquinone; EPR, electron paramagnetic resonance; Qo, ubiquinol-oxidizing center; PAGE, polyacrylamide gel electrophoresis.
sistent with current models, placing this region of cytochrome b at or near the quinol-oxidizing center and the intersubunit interface with the iron-sulfur subunit. However, the substitution of serine for threonine 160 also has an effect on cytochrome b160, which is not generally thought to interact directly with the quinol-oxidizing center. Thr160 may also play a role in the proper interaction of subunit IV and cytochrome b.

EXPERIMENTAL PROCEDURES

Materials—Dodecyl maltoside was purchased from Anachem. All other chemicals were of reagent grade or of the highest quality commercially available. Plasmids pUC4K (16) and pSL1180 (17) were obtained from Pharmacia Biotech Inc. Restriction endonuclease-treated DNA fragments were purchased from Promega, Life Technologies, New England Biolabs, U. S. Biochemical Corp., Perkin-Elmer, and Pharmacia. Escherichia coli strains were purchased from BRL Life Technologies (DH5α), Invitrogen (INVαr), and Promega (BMH-71-18 mutS). E. coli S17-1 (20), R. sphaeroides BC17 (21), and the plasmid pRK415 (18) were generously provided by Dr. R. R. Gennis (University of Illinois). pSupSTP (19) was a gift from Dr. T. Donahue (University of Wisconsin). Wild-type R. sphaeroides NCIB2853 was generously provided by Dr. R. Niedermaier (Rutgers University).

Growth of Bacteria—E. coli were grown at 37 °C on LB medium. Extra rich media, e.g. TYP (22), were used in procedures for the rescue of single-stranded DNA or the purification of low copy number plasmids (23). R. sphaeroides cells were grown aerobically in an enriched Sistrom's medium (24) containing 5 mM glutamate and 0.2% casamino acids. The pH of the medium was adjusted to 7.1 with a solution of 6 N NaOH and 2 N KOH to increase the sodium ion content of the medium to a more optimal level (24). Photosynthetic cultures of R. sphaeroides were grown essentially as described (25). Cells harvesting mutated fbc genes on the pRK415-derived plasmids described below were grown photosynthetically for one or two serial passages only to minimize any pressure for reversion. The inoculation volume used for photosynthetic cultures was always at least 5% of the total volume. Antibiotics were added at the following concentrations: ampicillin, 100–250 μg/ml; tetracycline, 10–15 μg/ml for E. coli and 0.75–1.0 mg/ml for R. sphaeroides; kanamycin sulfate, 25–30 μg/ml for E. coli and 20–25 μg/ml for R. sphaeroides; trimethoprim, 85–100 μg/ml for E. coli and 25–30 μg/ml for R. sphaeroides.

Construction of Mutation(s)—Mutations were constructed by site-directed mutagenesis using the Altered-Sites system from Promega, and oligonucleotides were synthesized at the OSU Recombinant DNA/Protein Core Facility. The successfully employed oligonucleotides were CTGGGGGGGACCGCTGCTAC (for the Thr160 to Ser mutation) and CCTCTGCGGGCGCTACGTGCTAC (for the Thr160 to Tyr mutation). The template DNA fragment for mutagenesis was obtained from a previously cloned 6.7-kb pair BamHI fragment, which was isolated from R. sphaeroides NCIB 8253 and contains the fbcB operon encoding the three largest subunits of the cytochrome bc1 complex. The unique pair of NcoI–BamHI fragments containing the fbcB operon was subcloned from the BamHI done into pALTER-1 (Promega; previously designated pSELECT-1). In order to facilitate sequence verification and transfer into the expression vector following mutagenesis, the template was modified to reduce the size of the DNA fragment containing the target sites for mutagenesis. For this purpose we introduced a silent mutation creating a unique Ncol site (at position 579) in the fbcB gene and eliminated a BstEII site present outside of the coding regions in the cloned R. sphaeroides DNA fragment, so that the target region for mutagenesis is contained in a 200-base pair fragment flanked by unique BstEII and PinAI sites (Fig. 1). We also introduced a unique XbaI site between the fbcB and fbcC genes. The nucleotide sequence from just upstream of the BstEII site through the PinAI site was determined and found to be identical to the published sequence of the fbcB gene from R. sphaeroides Ga (21), except for the single base change in creating the PinAI site. This engineered fbc operon was subcloned into the expression vector (see below), conjugated into R. sphaeroides BC17 (a strain from which most of the fbcB operon has been deleted (21)), and found to support the same rate of photosynthetic growth as the original 3.5-kb pair fbc clone.

Construction of Vectors for the Expression of Mutated Complexes in R. sphaeroides—The gene encoding a trimethoprim-resistant dihydrofolate reductase from R388 (26) (excised from pSupSTP) and the engineered fbc-containing insert from pSL1180 were cloned into the multiple cloning site polylinker of pSL1180 by a series of in vitro manipulations. The resulting 4400-base pair fragment containing the dihydrofolate reductase gene and the R. sphaeroides fbc operon were subcloned together into the HindIII and EcoRI sites of the broad host range vector pRK415, producing pRKDNB3503 (Fig. 1B). For the purpose of subcloning the 200-base pair BstEII–PinAI fragments from pRKDNB3503 following mutagenesis, pRKDNB35KMB was constructed by inserting the kanamycin resistance cassette from pUC4K between the BstEII and PinAI sites of pRKDNB3503. Using pRKDNB35KMB to receive the mutated BstEII–PinAI fragments eliminates the possibility of retaining or re-cloning the wild-type fragment when attempting to subclone the mutated fragments into the fbc operon vector (27). Loss of kanamycin resistance was then used to screen for recombinant plasmids, pRKDNB3503 derivatives were conjugated into R. sphaeroides BC17 (21) from E. coli S17-1 (20) using a plate mating procedure essentially as described (28, 29).

Other Recombinant DNA Techniques—General molecular genetic methods were performed essentially as described in Sambrook et al. (30). DNA plasmids and restriction fragments were recovered from preparative agarose gels according to Qian and Wilkinson (31) after staining with methylene blue (this avoids exposure to ultraviolet light). Nucleotide sequencing was performed with an Applied Biosystems model 373 automatic DNA sequencer. Sequencing of mutagenized DNA templates was conducted by amplification of a DNA segment including the entire BstEII to PinAI sequence using polymerase chain reaction (polymerase chain reaction) primers and an undivided form of the T7 gene 6 endonuclease as described (32). The presence of engineered mutations and the absence of other changes in the template region was reconfirmed once for each mutant clone following transfer to and expression in R. sphaeroides BC17 by purifying the expression plasmid from an aliquot of a photosynthetic culture and determining the nucleotide sequence as described above.

Isolation of Chromatophores and Purification of bc1 Complexes—Chromatophores were prepared from frozen cell paste of photosynthetically grown R. sphaeroides BC17 complement and mutant strains by proportionally scaling down the previously described procedure (33) with minor modifications. To prepare chromatophores for preliminary purification in situ, the initial clarification step by low speed centrifugation was modified by increasing the speed to 38,000 × g and the duration to 45 min (34) in order to reduce the light scattering of the chromatophore suspensions.

The cytochrome bc1 complexes were purified from chromatophores by a modification of the procedure of McCurley et al. (11). Dodecyl maltoside was used to solubilize the bc1 complexes and 20% glycerol was included during the solubilization and loading of the first column (after Andrews et al. (12)) to help stabilize mutated bc1 complexes in case they prove to be less stable to the extraction conditions. Chromatophore suspensions were adjusted to about 18 μM cytochrome b by addition of 50 mM Tris-HCl (pH 8.0 at 0 °C) containing 20% glycerol, 1 mM MgSO4, and 1 mM phenylmethylsulfonyl fluoride. Dodecyl maltoside (10% solution) was added to 50 mM Tris-HCl (pH 8.0 at 0 °C) containing 1 mM MgSO4 was added to the chromatophore suspension to 0.525 mg/ml to the cytochrome bc, and the mixture was stirred for 30 min at 0 °C and then centrifuged at 27,000 × g for 30 min. The hard precipitates at the bottom of the centrifuge tubes were discarded, and the loose pellets and supernatants were collected. 4 mM NaCl was added to a final concentration of 0.1%, and the suspension was stirred for 1 h at 0 °C. The mixture was centrifuged at 200,000 × g for 90 min. The supernatants were collected and applied to a DEAE-Biogel A column equilibrated with 50 mM Tris-HCl (pH 8.0 at 0 °C) containing 20% glycerol, 100 mM NaCl, 1 mM MgSO4, 5 mM Na3citrate, and 0.01% dodecyl maltoside (TMG buffer containing 100 mM NaCl). The column was washed with, in sequence, 3 volumes of TMG buffer containing 100 mM NaCl, 2 volumes of TMG buffer (TMG buffer without glycerol) containing 150 mM NaCl, and 2 volumes of TMG buffer containing 200 mM NaCl. The crude cytochrome bc1 complex was eluted from the column with TMG buffer containing 300 mM NaCl. The collected bc1 complex was diluted with one-half volume of TMG buffer containing 40% glycerol and applied to a DPC-membrane chromatophore CL-6B column eluted with TMG buffer containing 100 mM NaCl and then centrifuged at 27,000 × g for 30 min. The nearly pure cytochrome bc1 complex was eluted with TMG buffer containing 375 mM NaCl and concentrated using a Centriprep-10 concentrator to a final concentration of 100 μM cytochrome b or greater. Glycerol was added to about 20%, and the purified complex was stored at –80 °C. The purity of the bc1 preparations estimated by SDS-PAGE was 90–95%.

Biochemical and Spectroscopic Methods—Ubiquinol-cytochrome c re-
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Cytochrome c ductase activity was measured at 23°C by following the reduction of R. sphaeroides fbc28670 bc operon, and pRKDNB35KmBP were constructed to facilitate subcloning of the segment of the fbc operon (see "Experimental Procedures"). The millimolar extinction coefficient of 18.5 was used in calculating the reduced cytochrome c concentration. The nonenzymatic reduction of cytochrome c by ubiquinol was determined under the same conditions in the absence of enzyme. Chromatophore preparations were diluted to a concentration of 1.25–7.5 μM cytochrome b, depending on the expected specific activity of the sample, with a solution of 50 mM Tris-Cl (pH 8.0 at 0°C), 1 mM MgCl₂; bc preparations were diluted with 50 mM Tris-HCl (pH 8.0 at 0°C), 250 mM NaCl, 1 mM MgCl₂, 10% glycerol, and 0.005% dodecyl maltoside. Chromatophores and bc preparations were assayed by addition of 2–5 μl of suitably diluted samples to a 1.0-ml assay mixture containing 100 mM sodium/potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 50 μM cytochrome c, and 10 μM DAD; 5-methyl-6-(10-bromodecyl)-1,4-benzoquinol; 30 μM potassium cyanide was added to assays of chromatophores to inhibit oxidase activity. Protein was determined by the Lowry method (35) with the inclusion of 1% sodium dodecyl sulfate in the samples and standards. For accurate measurement of the protein content of chromatophores, interfering pigments were removed by acetone/methanol extraction as described (36). Cytochrome b (37), cytochrome c₃ (38), ubiquinone (39, 40), and bacteriochlorophyll (41) were determined according to published methods.

SDS-PAGE was performed according to Laemmli (42) and to Schägger and von Jagow (43) using a Bio-Rad Mini-Protein dual slab gel cell. Disaggregation of the sample and resolution was best obtained using a freshly prepared solubilization buffer containing 5% SDS, 10 mM EDTA (pH 8.0), 5% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol, followed by separation on a 16.5% polyacrylamide gel as described (43). Samples were incubated for 1–2 h at 30–37°C immediately prior to loading onto the gel. Chromatophores were extracted with acetone/methanol (36) before solubilization. Western blotting was carried out as described (44) using rabbit antibody raised against purified subunit IV (33) or against purified subunit II (45). The polypeptides separated in the SDS-PAGE gel were transferred to a 0.2-μm polyvinylidenefluoride membrane for immobiloblotting. Goat anti-rabbit IgG conjugated to alkaline phosphatase or protein A conjugated to horseradish peroxidase was used as the second antibody.

Low temperature EPR spectra were obtained with a Bruker ER 200D spectrometer equipped with an Air Products flow cryostat. Some spectra were recorded at 77 K using a liquid N₂ Dewar. Instrument setting details are provided in the legends of the relevant figures.

**RESULTS**

Expression and Purification of Cytochrome bc₃ Complexes Containing Altered Cytochrome b — Expression of cytochrome b with the conservative substitution Thr₁⁶₀ → Ser (T160S) as part of an fbc operon contained on a low copy number plasmid in R. sphaeroides BC17 yields cells capable of photosynthetic growth at a retarded rate (maximal doubling rate about 50–60% of that obtained with the complement strain). The properties of chromatophore membranes isolated from these cells are summarized in Table I together with the properties of membranes from complement cells expressing the wild-type cytochrome b also encoded by a plasmid-borne gene. The specific ubiquinol-cytochrome c reductase activity of membranes from the mutant cells is significantly reduced (25–40% of the activity found in complement membranes). On the other hand, these membranes also contain an apparently elevated level of the complex, as indicated by the elevated level of cytochrome b, relative to that found in membranes prepared from complement cells (membranes from complement cells already possess...
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Comparison of the composition and enzymatic activity of purified cytochrome bc1 complexes obtained from the membranes of R. sphaeroides BC17 expressing wild-type (complement strain), Thr160→Ser, or Thr160→Tyr cytochrome b

All bc1 complexes were prepared simultaneously using the same reagents, buffers, and chromatographic media.

| Components | Complement bc1 | T160S bc1 | T160Y bc1 |
|------------|----------------|----------|----------|
| Enzymatic activity | 2.9 | 0.51 | 0.40 |
| K_m (μM cytochrome c reduced/min nmol cytochrome b at 23°C) | 1.3 | 0.60 | 0.57 |

TABLE II

Comparison of the composition and enzymatic activity of purified cytochrome bc1 complexes obtained from the membranes of R. sphaeroides BC17 expressing wild-type (complement strain), Thr160→Ser, or Thr160→Tyr cytochrome b

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Two to three times the amount of cytochrome b was found in wild-type R. sphaeroides, presumably due to a gene dosage effect; data not shown, but see Ref. 21. This enhanced level of cytochrome b was found in the membranes of each of three independently isolated mutants containing the T160S alteration.

The mutation Thr160→Tyr (T160Y) was also subsequently prepared. R. sphaeroides cells expressing this variant of cytochrome b are also able to grow photosynthetically at a reduced rate. The cytochrome b content of membranes from these cells is also elevated relative to membranes from complement cells (Table I) but to a slightly lesser extent than membranes containing the T160S complex. The specific ubiquinol-cytochrome reductase activity of these membranes was even lower than that of membranes containing the T160S complex, only 10–20% of that found in complement membranes.

The bc1 complexes were extracted with dodecyl maltoside and purified by a modification of described methods (see Experimental Procedures) from chromatophore membranes of Rs BC17 cells expressing the Thr160→Ser or Tyr mutations and from membranes of BC17 expressing the cloned wild-type complex (complement cells). The T160S complex is spectrally identical to the complement complex (Fig. 2) but retains the lower activity seen in the chromatophores. The absorbance spectrum of T160Y bc1 is also similar to that of the complement bc1, but the ratio of b to c1 is lower for T160Y. The biochemical properties of the purified enzymes are summarized in Table II. SDS-PAGE (Fig. 3) shows that the T160S and T160Y complexes contain the same four subunits as the wild-type and complement enzymes, but the T160S complex has a reduced level of subunit IV (estimated to be about one half the level present in the complement bc1 complex). Indeed, addition of purified, recombinant subunit IV to the enzyme caused a 70% increase in the activity of the T160S bc1 complex (Fig. 4). Addition of subunit IV to the complement bc1 complex also produced some stimulation of the activity. By comparison, addition of exogenous subunit IV to purified three-subunit complex (completely lacking subunit IV (46)) resulted in a 5-fold stimulation of the basal specific activity under the conditions used (Fig. 4). Apparently not all of the recombinant subunit IV preparation used was in an active conformation for interaction with depleted bc1 complexes, because full stimulation of the three-subunit bc1 required more than three molecules of subunit IV per bc1 complex. Examination of additional preparations of wild-type, complement, and mutant bc1 complexes by SDS-PAGE (not shown) indicated that the relative amount of subunit IV present varies somewhat for each preparation, but purified T160S bc1 had a consistently lower ratio of IV relative to subunits I-III than complement or wild-type complexes.

In order to examine the relative amount of subunit IV initially present in the membranes of the mutant and complement cells, mutant and complement cells were grown side by side, and chromatophore membranes were prepared at the same time. Examination of these chromatophores by SDS-PAGE and subsequent Western blots developed with antibodies raised against subunit IV indicates that the membranes from the cells expressing T160S cytochrome b contain a significantly lower ratio of subunit IV to cytochrome b when compared with the complement membranes. It was estimated to be about 40% of the ratio found in the complement cells by densitometry corrected for the apparent relative transfer efficiency (see Fig. 5). T160Y membranes contain a modestly lower ratio of subunit IV to cytochrome b (estimated to be ~80% of the complement level). Complement membranes, in turn, appear to contain a lower ratio of subunit IV than membranes from wild-type R. sphaeroides (not shown). It thus appears that R. sphaeroides cells overproducing the fbc genes due to the presence of multiple copies of the operon on a plasmid and/or other factors either do not induce a concomitant increase in the levels of subunit IV or have a more rapid turnover of that subunit. This effect is
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FIG. 4. Activation of T160S and three-subunit bc1 complexes by addition of exogenous, recombinant subunit IV. Cytochrome bc1 complexes of T160S (b) and the three-subunit core complex (c) diluted to ~2.5 μM cytochrome b were mixed with varying amounts subunit IV in a total volume of 30–50 μl of 50 mM Tris-HCl (pH 8.0 at 0 °C), 250 mM NaCl, 1 mM MgCl2, 0.01% dodecyl maltoside, and 10% glycerol and incubated on ice for 1–2 h. 3-μl aliquots were withdrawn for assay of ubiquinol-cytochrome c reductase activity. The amount of subunit IV added was based on determination of the protein content by the method of Lowry (35). The concentration of bc1 complex present was reetermined after dilution by optical difference spectroscopy and was based on the cytochrome c1 content.

FIG. 5. Western analysis of chromatophore membranes from mutated and complement bc1 complexes. Membrane samples containing 75 pmol (lanes 1–4) or 150 pmol (lanes 5–9) cytochrome b were extracted with acetone/methanol to remove pigments, redissolved in loading buffer containing SDS and 2-mercaptoethanol, and subjected to tricine SDS-PAGE on duplicate gels. One gel was transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was cut into two pieces so that the upper part contained proteins with a molecular mass greater than about 23 kDa, and the bottom section contained the low molecular mass proteins. The top section of the membrane was probed with antibodies to R. sphaeroides subunit I (cytochrome c1) and developed with a horseradish peroxidase system, whereas the bottom section was probed with antibodies to subunit IV and developed with an alkaline phosphatase system. Only the Western analysis of the membrane is shown. Lane 1, chromatophores (75 pmol b) from R. sphaeroides BC17 expressing the T160S mutation; lane 2, chromatophores (75 pmol b) from BC17 expressing the T160Y mutation; lane 3, chromatophores (75 pmol b) from BC17 complement; lane 4, chromatophores (75 pmol b) from T160S strain (46) lacking subunit IV; lane 5, prestained molecular mass standards; lane 6, chromatophores (150 pmol b) from BC17 expressing the T160S mutation; lane 7, chromatophores (150 pmol b) from BC17 expressing the T160Y mutation; lane 8, chromatophores (150 pmol b) from BC17 complement; lane 9, chromatophores (150 pmol b) from T160S strain (46) lacking subunit IV.

Subunit IV ratio 0.40 0.85 1.0 0.0 0.37 0.76 1.0 0.0

FIG. 6. EPR spectra of the antimycin-sensitive ubisemiquinone radical in the T160S cytochrome bc1 complex. Oxidized complement and T160S cytochrome bc1 complexes containing about 300 μM cytochrome b in 50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 1 mM MgCl2, 0.01% dodecyl maltoside, and 20% glycerol were mixed with a fumarate/succinate mixture (40:1) such that the final concentration of succinate was ~3 mM and a catalytic amount of bovine succinate-ubiquinone reductase. After recording the spectra over a range of microwave powers, the samples were thawed and a 2-fold excess of antimycin A was added. The EPR spectra of the T160S complex at 0.2 milliwatts in the absence (1) and presence (2) of antimycin are shown together with their difference (1–2; the antimycin-sensitive radical) spectrum. The inset shows the uncorrected power saturation curves of the samples. EPR instrument parameters were a microwave frequency of 9.32 GHz, a modulation amplitude of 4 G, a time constant of 0.05 s, a scan rate of 2.5 G/s, and a temperature of 140 K.

Effect of Mutation on the Antimycin-sensitive Radical Associated with the Quinone-reducing Site—The purified bc1 complexes from complement and T160S-expressing cells were poised in a partially reduced state and examined by EPR spectroscopy to compare the features of the antimycin-sensitive ubisemiquinone radical. Both the mutated and the complement complexes were found to contain a ubisemiquinone radical 7.5 Gauss in width at g = 2.004 (Fig. 6), as previously reported for the wild-type complex (11). The power saturation behavior of the mutated and complement complexes were examined and found to be virtually identical (Fig. 6, inset). These results indicate that both the complement and mutated bc1 complexes have an intact quinone reducing center that stabilizes an anionic ubisemiquinone radical.

Effect of Mutation on the Rieske Iron-Sulfur Cluster—The EPR parameters of the Rieske [2Fe-2S] cluster are thought to be sensitive to the redox state of the Q pool and the quinone(s) bound at the quinol-oxidizing center (Qo) (11, 12, 47–52). EPR spectra of the T160S and complement bc1 complexes were recorded under several conditions, some of which are shown in Fig. 7. When cytochrome c1 and the [2Fe-2S] cluster are reduced by a small excess of ascorbate, the complement bc1 complex has a spectrum that is essentially the same as that previously reported for the complex from wild-type R. sphaeroides, with resonances at g1 = 2.02, g2 = 1.89, and g8 = 1.81 (11, 12). In contrast, the spectrum of the mutated complex shows a broadened signature with g1 shifted to 1.76; this change is apparently not due to the partial depletion of subunit IV found in the T160S complex, because three-subunit bc1 purified from a strain having no subunit IV (46) has an EPR spectrum very similar to that of the wild-type complex with g1 = 1.81.3 Upon partial oxidation of the samples by titration with ferricyanide to an apparent potential of about 210 mV (with cytochrome c1 (Em1/2 = 240 mV) one-fourth oxidized), no changes were found in the spectrum of either the mutant or the complement bc1 complex (not shown). Upon complete reduction by addition of dichloro-2,5-thionine, the spectrum of the bc1 complement complex is broadened, with g1 shifting to 1.76, as previously reported for the

3 Y. R. Chen, D. Tolkatchev, and C.-A. Yu, unpublished results.
Importance of Threonine 160 of Cytochrome b

Threonine 160 is a highly conserved residue in the primary sequence of cytochrome b, present in all known mitochondrial and eubacterial cytochromes b, except those from nematodes (2). Its position in the primary sequence is near residues that are altered in mutations conferring resistance to Q<sub>10</sub> center inhibitors (Gly<sup>158</sup>, Ile<sup>162</sup>, and Thr<sup>163</sup>), and it is contained within a segment of the cytochrome b polypeptide that corresponds to a peptide specifically labeled by azido-quinone in the bc<sub>1</sub> complex from beef heart (15). Current interpretations of experimental data and modeling efforts place this region in an amphipathic helix, designated as helix-cl, located on the positive side of the membrane and forming part of the Q<sub>1</sub> center of the complex. This region should also be close to cytochrome b<sub>1</sub>, which is reduced by semiquinone in the Q<sub>0</sub> center. Indeed, a mutation at the position corresponding to Ile<sup>162</sup> in Rhodobacter exhibits an altered circular dichroism spectrum for cytochrome b<sub>565</sub> of yeast (56). Furthermore, the Rieske [2Fe-2S] cluster should also be located nearby, although it is bound to a different subunit of the complex, because it is reduced by quinol in the Q<sub>0</sub> center and binds certain Q<sub>1</sub> center inhibitors. As a conserved residue in a potentially pivotal region of the complex, Thr<sup>160</sup> proves to be a good candidate for investigation using the method of site-directed mutagenesis combined with biochemical and biophysical characterization.

Expression of the T160S mutation in cytochrome b results in a bc<sub>1</sub> complex having a significant loss of activity partially compensated by a small decrease in the apparent K<sub>m</sub>. Interestingly, cells expressing this mutated cytochrome b during photosynthetic growth appear to induce an increased level of the three largest subunits of the bc<sub>1</sub> complex. This induction could be a regulatory response of cells expressing the mutated complexes to lowered electron transfer activity or an elevated "redox poise." Alternatively, the effect could be caused by a spontaneous second-site mutation that leads to enhanced expression and is positively selected by photosynthetic growth. The latter possibility is unlikely, however, because three T160S clones independently isolated following mutagenesis all displayed elevated levels of the bc<sub>1</sub> complex during the first round of photosynthetic growth following initial amplification by aerobic growth in the dark. The purified T160S complex contains a significantly reduced amount of subunit IV, relative to the bc<sub>1</sub> complex purified from complemented cells. Addition of recombinant subunit IV to the partially depleted preparations results in an increase in activity; however, the final activity of reconstituted T160S bc<sub>1</sub> complex still remains lower than that of the complement bc<sub>1</sub> complex. Given the apparent low content of subunit IV in the T160S bc<sub>1</sub> preparations, the less than 2-fold activity increase seen upon incubation with added exogenous subunit IV is surprising. One possible explanation is that a large fraction of the purified T160S bc<sub>1</sub> complex is in a confor-

**DISCUSSION**

Threonine 160 is a highly conserved residue in the primary sequence of cytochrome b, present in all known mitochondrial and eubacterial cytochromes b, except those from nematodes (2). Its position in the primary sequence is near residues that are altered in mutations conferring resistance to Q<sub>10</sub> center inhibitors (Gly<sup>158</sup>, Ile<sup>162</sup>, and Thr<sup>163</sup>), and it is contained within a segment of the cytochrome b polypeptide that corresponds to a peptide specifically labeled by azido-quinone in the bc<sub>1</sub> complex from beef heart (15). Current interpretations of experimental data and modeling efforts place this region in an amphipathic helix, designated as helix-cl, located on the positive side of the membrane and forming part of the Q<sub>1</sub> center of the complex. This region should also be close to cytochrome b<sub>1</sub>, which is reduced by semiquinone in the Q<sub>0</sub> center. Indeed, a mutation at the position corresponding to Ile<sup>162</sup> in Rhodobacter exhibits an altered circular dichroism spectrum for cytochrome b<sub>565</sub> of yeast (56). Furthermore, the Rieske [2Fe-2S] cluster should also be located nearby, although it is bound to a different subunit of the complex, because it is reduced by quinol in the Q<sub>0</sub> center and binds certain Q<sub>1</sub> center inhibitors. As a conserved residue in a perhaps pivotal region of the complex, Thr<sup>160</sup> proves to be a good candidate for investigation using the method of site-directed mutagenesis combined with biochemical and biophysical characterization.

Expression of the T160S mutation in cytochrome b results in a bc<sub>1</sub> complex having a significant loss of activity partially compensated by a small decrease in the apparent K<sub>m</sub>. Interestingly, cells expressing this mutated cytochrome b during photosynthetic growth appear to induce an increased level of the three largest subunits of the bc<sub>1</sub> complex. This induction could be a regulatory response of cells expressing the mutated complexes to lowered electron transfer activity or an elevated "redox poise." Alternatively, the effect could be caused by a spontaneous second-site mutation that leads to enhanced expression and is positively selected by photosynthetic growth. The latter possibility is unlikely, however, because three T160S clones independently isolated following mutagenesis all displayed elevated levels of the bc<sub>1</sub> complex during the first round of photosynthetic growth following initial amplification by aerobic growth in the dark. The purified T160S complex contains a significantly reduced amount of subunit IV, relative to the bc<sub>1</sub> complex purified from complemented cells. Addition of recombinant subunit IV to the partially depleted preparations results in an increase in activity; however, the final activity of reconstituted T160S bc<sub>1</sub> complex still remains lower than that of the complement bc<sub>1</sub> complex. Given the apparent low content of subunit IV in the T160S bc<sub>1</sub> preparations, the less than 2-fold activity increase seen upon incubation with added exogenous subunit IV is surprising. One possible explanation is that a large fraction of the purified T160S bc<sub>1</sub> complex is in a confor-
mation that no longer interacts with subunit IV, perhaps due to aggregation or partial denaturation. Another possibility is that the low level of subunit IV found in the T160S membranes in the first place, as well as any conformational changes, in some measure results from a decreased affinity of subunit IV for the modified cytochrome b. The possibility that Thr160 in cytochrome b is directly or indirectly involved in the binding of subunit IV to the bc1 complex is currently under investigation. In contrast to the expression levels observed with T160S, membranes isolated from T160Y contain levels of subunit IV more comparable with the complement chromatophores. Possibly this more hydrophobic substitution has an effect on the interaction of subunit IV with cytochrome b different from that of the T160S change.

The EPR spectra of the T160S complex displayed altered signatures for both the Rieske iron-sulfur center and cytochrome b$_{562}$. Interestingly, the broad g = 3.50 signal attributed to b$_{562}$ is shifted to lower field, whereas the g = 3.75 signal due to b$_{565}$ appears to be essentially unchanged in the spectra of the mutated complex. This is somewhat surprising, because it is b$_{565}$ that is thought to be located near the quinol-oxidizing center and the positive side of the membrane, whereas b$_{562}$ resides in the central portion of the membrane or closer to the negative side and the quinone-reducing center of the complex (3, 5, 6).

Thus, the effect of the T160S substitution on the b$_{562}$ spectrum would appear to be indicative of a long range interaction between the oxidizing center and b$_{562}$ or of a geometry placing the two in closer proximity than current models. It is noted that long-range effects of mutations at other positions in cytochrome b have been previously reported, e.g. the redox midpoint potentials of cytochrome c$_1$ were affected by substitutions in cytochrome b (27).

The substitutions at T160 in cytochrome b also affected the EPR signature of the Rieske [2Fe-2S] center, which is located in a separate subunit of the complex. The iron-sulfur subunit is thought to bind in the general vicinity of b$_{565}$ on the positive side of the membrane to form part of the quinol-oxidizing center, because the cluster is a primary electron acceptor from the quinol. The particular line shape observed for the [2Fe-2S] cluster is thought to be mediated by the oxidation state of the quinol present in the Q$_o$ center (11, 12, 50–53, 57). When oxidized quinol is present, the EPR signal is sharper than when quinol is present. The change is most apparent in the case of the g$_y$ resonance, which in the bc$_1$ from R. sphaeroides is found at g = 1.81 when oxidized ubiquinone is present but shifts to 1.76 and becomes much broader when ubiquinol is present. The substitutions at Thr160 resulted in a broadened [2Fe-2S] EPR signature with g$_x$ = 1.76, which is unaltered by accessible changes in the redox state of the samples. There was no detectable difference between the EPR spectra of the mutated complexes and the “reduced state” spectrum of the complement bc$_1$ complex. The effect of the mutations on the iron-sulfur cluster spectrum suggests that the Thr160 residue of cytochrome b interacts with the quinol-oxidizing center and/or the [2Fe-2S] cluster. This idea is consistent with current models of the structure and function of this part of the complex.

The effect of the T160 substitutions on the iron-sulfur cluster is also reminiscent of the change observed for the substitution of Leu for Phe144 (F144L) in the cytochrome b from R. capsulatus (58). The F144L bc$_1$ complex in R. capsulatus chromatophores was reported to have a very low turnover rate and a dysfunctional Q$_o$ center (having less than 10% of the wild-type activity for the ubiquinol to cytochrome b$_{5}$ reduction step). The EPR spectrum of F144L was broadened and insensitive to redox state with g$_x$ assigned a value of 1.765. It was suggested that these properties of the F144L complex resulted from a reduced affinity for quinone and quinol exhibited by the Qo center of the mutated complex. In a subsequent study of the effect of the extraction of ubiquinone from chromatophore membranes on the iron-sulfur cluster, Ding et al. (52) found that the g$_y$ signal became very broad and was located at approximately 1.765 upon depletion of ubiquinone from R. capsulatus chromatophore membranes. These workers were able to distinguish the “depleted state” spectrum having g$_x$ ~ 1.765 from the “reduced state” spectrum with g$_y$ = 1.777 and found that the line shape of the quinone-depleted state was broadened considerably beyond that seen in the presence of either ubiquinone or ubiquinol. With our EPR instrumentation we cannot assign g values with such a high degree of precision given the broadness of the g$_y$ feature in the reduced and the putative quinone-depleted states. However, there was no significant difference in the width or depth of the g$_x$ = 1.76 signals of the R. sphaeroides T160S and T160Y bc$_1$ complexes relative to the spectrum of the complement bc$_1$ in the reduced state. Thus, the changes in the iron-sulfur cluster EPR spectra resulting from the T160 substitutions in the R. sphaeroides system do not exhibit the extremely broad line shape reported for the quinone-depleted state and are probably not due to a complete absence of quinone and quinol binding to the Q$_o$ center. The nature of these interactions will be the subject of future investigations. One possibility, which would account for the reduced turnover of the mutant complexes and their “reduced state” EPR spectra, is that ubiquinol binds more tightly to the quinol-oxidizing center of the mutated complexes than ubiquinone, raising its effective redox potential beyond the optimal range for transfer to the [2Fe-2S] cluster. Destabilization of the transient ubisemiquinone state required at the Q$_o$ center would also inhibit turnover.

Acknowledgments—We express our thanks to Dr. Yeong-Renn Chen and Tian Hua for providing protein and chromatophore samples and valuable assistance in the analysis thereof. We also thank Dr. Dmitri Tolkachev for help in operation of the EPR spectrometer and interpretation of the EPR data. We are also grateful to Lisa McReynolds for excellent technical assistance and to Dr. Roger Koeppe for critical review of the manuscript.

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