THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 15, Issue of April 10, pp. 9085–9093, 1998

Printed in U.S.A.

Alteration of the Midpoint Potential and Catalytic Activity of the Rieske Iron-Sulfur Protein by Changes of Amino Acids Forming Hydrogen Bonds to the Iron-Sulfur Cluster*

(Received for publication, July 25, 1997, and in revised form, December 12, 1997)

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The crystal structure of the bovine Rieske iron-sulfur protein indicates a sulfur atom (S-1) of the iron-sulfur cluster and the sulfur atom (S_γ) of a cysteine residue that coordinates one of the iron atoms form hydrogen bonds with the hydroxyl groups of Ser-163 and Tyr-165, respectively. We have altered the equivalent Ser-183 and Tyr-185 in the *Saccharomyces cerevisiae* Rieske iron-sulfur protein by site-directed mutagenesis of the iron-sulfur protein gene to examine how these hydrogen bonds affect the midpoint potential of the iron-sulfur cluster and how changes in the midpoint potential affect the activity of the enzyme.

Eliminating the hydrogen bond from the hydroxyl group of Ser-183 to S-1 of the cluster lowers the midpoint potential of the cluster by 130 mV, and eliminating the hydrogen bond from the hydroxyl group of Tyr-185 to S_γ of Cys-159 lowers the midpoint potential by 65 mV. Eliminating both hydrogen bonds has an approximately additive effect, lowering the midpoint potential by 180 mV. Thus, these hydrogen bonds contribute significantly to the positive midpoint potential of the cluster but are not essential for its assembly. The activity of the bc_1 complex decreases with the decrease in midpoint potential, confirming that oxidation of ubiquinol by the iron-sulfur protein is the rate-limiting partial reaction in the bc_1 complex, and that the rate of this reaction is extensively influenced by the midpoint potential of the iron-sulfur cluster.

The Rieske iron-sulfur protein is a ubiquitous component of cytochrome bc_1 complexes (1–4) and has been shown to be essential for electron transfer and energy transduction by purification of the protein in a reconstitutively active form and reconstitution to iron-sulfur protein depleted bc_1 complex (5, 6). The electronic environment of the [2Fe-2S] cluster in the Rieske iron-sulfur protein differs from that in plant type [2Fe-2S] ferredoxins as evidenced by a distinct EPR spectrum (1) and a redox midpoint potential of the Rieske protein (*e.g.* +280 mV) that is much more positive than the midpoint potentials typical of the ferredoxins (*e.g.* −420 mV; Ref. 2). The high midpoint potential of the iron-sulfur cluster is essential for the function of the Rieske protein in the Q cycle mechanism of the bc_1 complex (7, 8), in which the Rieske protein is the primary electron acceptor and drives the electron transfer reaction by oxidizing ubiquinol and divergently transferring one electron to cytochrome c_1, while the ubisemiquinone that is formed from ubiquinol reduces the low potential *b* heme.

Recently, the crystal structure of the water-soluble part of the Rieske iron-sulfur protein of bovine heart mitochondrial bc_1 complex has been elucidated at 1.5 Å (9, 10). Ten β strands form three layers of anti-parallel β sheets in a flat spherical molecule as shown in Fig. 1*A*. The cluster binding fold is a small domain-like structure comprising approximately 46 residues; it consists of a distorted four-stranded antiparallel β-sheet and three loops. The loops between the strands β4-β5 and β6-β7 each contribute one cysteine and one histidine ligand to the cluster. Cysteine 139 and 158 are ligands of Fe-1 and are buried within the protein. Histidines 141 and 161 are ligands to the redox-active Fe-2 and are completely exposed to the solvent. The loops are cross-linked by a disulfide bridge formed by two additional cysteine residues in each loop. This disulfide bridge forms one side of the cluster binding fold and stabilizes it. The other side of the cluster binding fold is covered by the so called "Pro loop."

From the crystal structure several factors were identified, which contribute to the positive midpoint potential of the Rieske protein, including a net charge of 0/−1 for the oxidized and reduced iron-sulfur cluster, the electronegativity of the histidine ligands, and the proximity of Fe-2 to the surface of the protein. The midpoint potential should also be affected by the complex hydrogen bond network through which the iron-sulfur cluster is linked to the surrounding protein. With the exception of Cys-158, which has only one hydrogen bond to the nitrogen of Cys-160, all sulfur atoms are involved in two hydrogen bonds each.

The hydrogen bond network to sulfur atoms includes Ser-163 and Tyr-165. Both residues are located in strand β7 and are conserved in all Rieske iron-sulfur proteins that oxidize ubiquinol and that have been sequenced to date. As shown in Fig. 1*B*, the hydroxyl group of Ser-163 is hydrogen-bonded to S-1, at 3.2 Å. The hydroxyl group of Tyr-165 is hydrogen-bonded to the S_γ of Cys-139, a ligand of Fe-1, at 3.1 Å. Both residues are likely to contribute to the positive midpoint potential of the Rieske protein by decreasing the negative charge density around the sulfur atoms through their respective hydrogen bonds.

In the present study we have examined the effects of the hydrogen bonds from the hydroxyl of Ser-163 to the internal sulfur atom and from the hydroxyl of Tyr-165 to the sulfur of one of the cysteine ligands on the midpoint potential of the Rieske iron-sulfur cluster by introducing amino acid changes at equivalent positions (Ser-183 and Tyr-185) in the yeast iron-

* This work was supported by National Institutes of Health Grant GM 20379 and by Deutsche Forschungsgemeinschaft (DFG) Priority Programme "Transition Metals in Biology and their Coordination Chemistry" (Li 474). 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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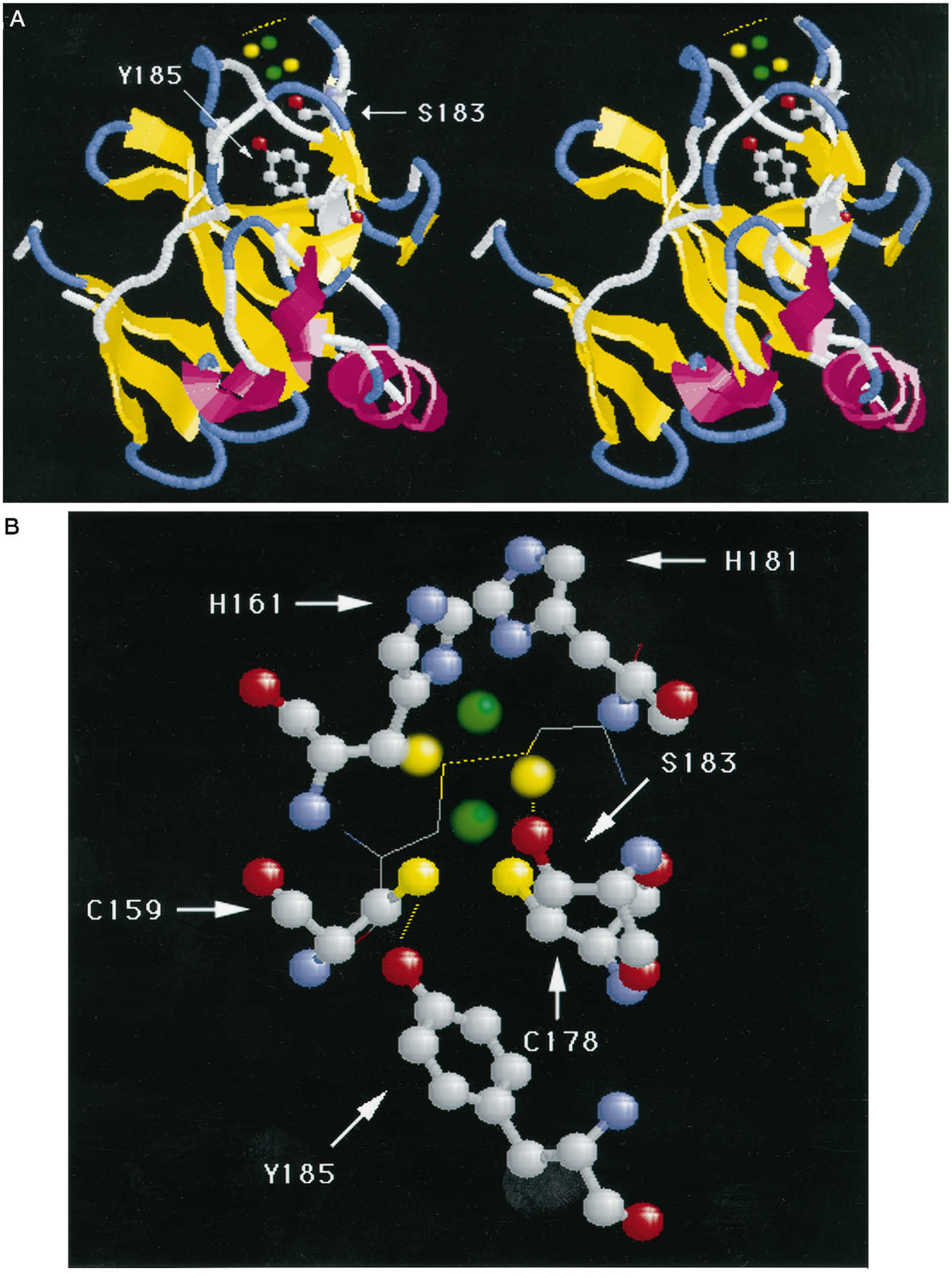

FIG. 1.

FIG. 1. **Views of the Rieske iron-sulfur protein showing the location of Tyr-185 and Ser-183.** The view in *A* shows the location of Tyr-185 and Ser-183, equivalent to Tyr-165 and Ser-163 in the bovine protein, and the [2Fe-2S] cluster. The disulfide bridge that stabilizes the cluster binding fold is shown as *yellow dots* across the top of the fold, with the "Pro loop" curving inward around the cluster on the *right*. Iron and sulfur atoms of the cluster are in *green* and *yellow*, respectively. Helices are *red*, and β-strands are *yellow*. The redox-active Fe-2 is proximal to the disulfide bridge. The C terminus of the protein and the N terminus where the protein is anchored to the membrane are to the *left* of the protein. The view in *B* shows an expanded view of the protein in proximity to the [2Fe-2S] cluster, including ball-and-stick representations of His-161 and His-181, which are ligands to the redox-active Fe-2; Cys-159 and Cys-178, which are ligands to Fe-1; and Tyr-185 and Ser-183. Also shown are the hydrogen bond between the OH group of Tyr-185 and the S_γ of Cys-159 and the hydrogen bond between the OH group of Ser-183 and S-1 of the [2Fe-2S] cluster. The disulfide bridge between Cys-164 and Cys-180 is shown in chain representation in back of the cluster.

sulfur protein through site-directed mutagenesis of the cloned gene. We show that conservative substitutions that eliminate these two hydrogen bonds lower the midpoint potential, without affecting the stability of the protein, and that these changes are accompanied by decreases in turnover numbers of the bc_1 complexes containing the altered forms of the Rieske protein.

EXPERIMENTAL PROCEDURES

Materials—SDS, acrylamide, and bisacrylamide were from Bio-Rad. Urea and agarose (Ultra Pure) were from Life Technologies, Inc. DNA was isolated from agarose gels using the QIAquick gel extraction kit from QIAGEN. Nytran™ nylon membranes were from Schleicher & Schuell. Glass beads (0.5 mm) were obtained from Biospec Products. Horse heart cytochrome *c* and the ubiquinone analog 2,3-dimethoxy-5-methyl-6-*n*-decyl-1–4-benzoquinone were from Sigma. Yeast extract, peptone, bacto-agar, and tryptone were from Difco.

Site-directed Mutagenesis—The plasmid pGEM3-RIP1, carrying the wild type *Saccharomyces cerevisiae* Rieske iron-sulfur protein gene, was used for site-directed mutagenesis with the CLONTECH Transformer mutagenesis kit (11). The mutational changes were confirmed by automated sequencing using the Dye Deoxy terminator cycle sequencing kit from Applied Biosystems Inc. A *Pst*I/*Sac*I fragment from the mutated pGEM3-RIP1 was then subcloned into the single-copy yeast vector pFL39-RIP1, from which this fragment was removed. Cells of the *RIP1* deletion strain JPJ1 were then transformed with the pFL39-RIP1 plasmid carrying the site-directed mutations (12).

Western Analysis of Mitochondrial Membranes—Yeast cells were grown 2–3 days on synthetic dextrose medium lacking tryptophan. Mitochondrial membranes were isolated as described previously (13). Protein concentrations were determined by a modified Lowry method (14, 15). Membranes were resolved by SDS-polyacrylamide gel electrophoresis (16), and iron-sulfur protein and cytochrome c_1 were detected by Western blotting (17) using monoclonal antibodies to yeast iron-sulfur protein and cytochrome c_1 (13).

Purification of Cytochrome bc_1 Complexes—For all of the yeast mutants in which the iron-sulfur protein was detectable by Western blotting of the mitochondrial membranes, the cytochrome bc_1 complexes were purified in two different laboratories and by two different methods. In one laboratory the membranes were extracted with dodecyl maltoside and the complexes purified by ion-exchange chromatography (18), and in the other laboratory the membranes were extracted with Triton X-100 and purified by hydroxyapatite chromatography (19).

Optical Spectroscopy and Enzyme Assay—Ubiquinol-cytochrome *c* oxidoreductase activities of mitochondrial membranes and purified cytochrome bc_1 complexes were assayed at pH 7.0 and 23 °C using 50 μM 2,3-dimethoxy-5-methyl-6-*n*-decyl-1–4-benzoquinol as substrate and 50 μM horse heart cytochrome *c* (5). Reduction of cytochrome *c* was monitored in an Aminco DW-2A spectrophotometer at 550 *versus* 539 nm in dual wavelength mode. Turnover numbers of the bc_1 complexes *in situ* and of the purified enzymes were calculated on the basis of the concentration of cytochrome *b*, which was determined from optical spectra of the dithionite reduced minus ferricyanide oxidized samples (20). Assays were performed in triplicate, and turnover numbers are expressed as percent of the turnover number of the bc_1 complex from the wild-type strain. Turnover numbers of the enzyme from the wild-type strain were 200–220 s^{-1} as reported previously (18).

CD-monitored Redox Titration of Isolated bc_1 Complex—CD-monitored redox titrations of isolated cytochrome bc_1 complexes were performed in an optically transparent thin layer cell with a path length of 0.1 mm built in-house and using a Jasco J-720 spectropolarimeter as described previously (21). The transparent electrode was a pyridine-3-carboxaldehyde thiosemicarbazone modified gold grid with a mesh size of 500 wires/inch and a transmission of 60% (Buckbee-Mears, St. Paul, MN). The potential was controlled through a Wenking MP 95 potentiostat (Bank Elektronik, Clausthal, Germany). To facilitate redox equilibration, methylviologen ($E°$ = −449 mV), anthraquinone-2-sulfonate ($E°$ = −225 mV), 2-hydroxy-1,4-napthoquinone ($E°$ = −145 mV), menadione ($E°$ = −13 mV), duroquinone ($E°$ = +5 mV), phenazine ethosulfate ($E°$ = +55 mV), phenazine methosulfate ($E°$ = +80 mV), trimethyl-1,4-benzoquinone ($E°$ = +99 mV), 1,2-napthoquinone ($E°$ = +144 mV), 2,6-dichlorophenol indophenol ($E°$ = +217 mV), *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine ($E°$ = +276 mV), and ferricyanide ($E°$ = +408 mV) were added at final concentrations of 25 μM each to approximately 0.5 mM cytochrome bc_1 complex in 100 mM NaCl, 35 mM MOPS,[1] 0.025% Triton X-100, pH 7.0. Neither the oxidized nor the reduced form of the mediators showed any CD signal, even at 10-fold higher concentrations. All potentials have been recalculated with reference to the standard hydrogen electrode.

Crystal Structure Calculations and Graphics—Views of the iron-sulfur protein were generated from the crystal structure coordinates of the bovine protein using the molecular graphics program RasMac, version 2.6 (Roger Sayle). Inter-atomic distances were measured, and rotamer positions of the various amino acid substitutions were examined with Swiss PDB-viewer, version 2.2 (Glaxo Institute of Molecular Biology, Geneva, Switzerland). Although this program does not calculate true energy minimization's for substituted amino acids, it does calculate a "score" for each rotamer position of a substituted amino acid according to the empirical formula $S = 4X + 3Y + 2Z - nH$, where X is the number of clashes with backbone nitrogen or carbon atoms, including the α carbon, Y is the number of clashes with backbone oxygen atoms, Z is the number of clashes with side-chain atoms, and H is the number of potential hydrogen bonds.

RESULTS

Choice of Mutations—The amino acid sequences of the bovine and yeast Rieske iron-sulfur proteins are almost identical throughout the region containing the cluster ligands and the "Pro loop" as shown in Fig. 2. The numbering of the two sequences differ by 20, so that Ser-163 in the bovine iron-sulfur protein equals Ser-183 in yeast, and Tyr-165 in the bovine protein equals Tyr-185 in yeast.[2] Only 6 of the 44 amino acids in the region surrounding the iron-sulfur cluster are not identical in the bovine and yeast proteins, and 5 of these are conservative changes. The only non-conservative change is Glu-169 in the yeast protein *versus* Asn-149 in the bovine

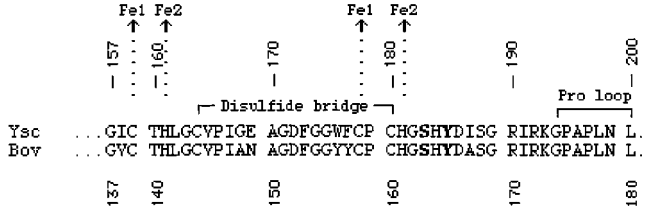

FIG. 2. **Partial sequence alignments of the bovine and yeast Rieske iron-sulfur proteins in the regions of the proteins including the cluster binding fold.** The yeast and bovine sequences differ by 20 in numbering of the residues and are numbered above and below the sequences, respectively. The residues that are ligands to the iron-sulfur cluster are indicated with *arrows*, and the Ser and Tyr residues that were changed by site-directed mutagenesis are indicated in *boldface type*. The amino acids comprising the disulfide bridge and Pro loop are also indicated.

[1] The abbreviations used are: MOPS, 3-(*N*-morpholino)propanesulfonic acid; i-ISP, intermediate iron-sulfur protein.

[2] Hereafter, unless explicitly stated otherwise, we will use the numbering system for yeast.

protein. In the bovine crystal structure, this residue is on the surface of the protein, in the loop that extends from the right of the protein in the orientation shown in Fig. 1, and distal from the cluster. It is thus unlikely that this single non-conservative change results in any substantive difference between the boine and yeast proteins.

On the basis of the high degree of sequence identity and the similar midpoint potentials and EPR spectra of the yeast and bovine proteins, we expect that the structures of the two proteins are identical in the cluster binding fold. This allows a systematic approach to explore the effects of amino acid changes in the cluster binding fold on the midpoint potential of the iron-sulfur cluster by using the bovine structure as a guide for constructing site-directed mutations in the yeast protein. Ser-183 and Tyr-185 were chosen for site-directed mutagenesis because these residues form hydrogen bonds that are likely to affect the midpoint potential of the iron-sulfur cluster. The hydroxyl group of Ser-183 is hydrogen-bonded to S-1 of the [2Fe-2S] cluster, while the hydroxyl group of Tyr-185 is hydrogen-bonded to the Sγ of Cys-159, a ligand of Fe-1.

In previous studies a mutation in the yeast Rieske protein at Ser-183 and a mutation at Tyr-185 were isolated after random mutagenesis. Replacement of Ser-183 by Leu resulted in loss of the iron-sulfur cluster and instability of the protein (22). When Tyr-185 in the yeast protein was replaced with Cys, activity of the bc_1 complex was also lost, although it was not established whether the iron-sulfur cluster was present (23).

We chose Ala, Cys, or Thr as conservative amino acid substitutions for Ser-183. Replacing Ser with Ala removes the hydrogen bond to S-1 by eliminating the hydroxyl group but minimizes steric and hydrophobicity differences, since the C_β of Ala is 3.71 Å from S-1, as compared with 3.18 Å for the oxygen of the Ser hydroxyl group. Substitution of Ser with Cys replaces the hydroxyl group with a sulfhydryl group, which is equidistant from S-1, and which could also form a hydrogen bond. We anticipated the possibility that introduction of the sulfhydryl group could result in misfolding of the protein due to formation of a disulfide bond with one of the Cys residues in the cluster binding fold, and thus we have controlled for protein instability by measuring iron-sulfur protein in mitochondrial membranes from the various mutants by Western analysis as shown below.

Tyr-185 was replaced by Phe, Trp, His, Leu, Ser, Thr, Arg, or Asp. The Phe substitution eliminates the hydroxyl group and therefore the capability to form a hydrogen bond without further changes in the side chain. Trp and His were chosen because both of these introduce an aromatic side chain with a polar group that conceivably could form a hydrogen bond to Cys-159. Replacement of Tyr with Ser and Thr introduces alternative hydroxylated amino acids, and we estimated whether these could form hydrogen bonds into the cluster. Calculations of the lowest energy rotamers of these substitutions placed both alternative hydroxyl groups 8.85 Å from the S_γ of Cys-159, *versus* 3.05 Å for the Tyr hydroxyl group. This suggests that formation of a hydrogen bond from either Ser or Thr to the sulfur would require some internal compression of the protein, and the ensuing bond length and strength would likely be different than that formed from Tyr. Arg and Asp were chosen to replace Tyr because they would place positive and negative charges at the bottom of the cluster binding fold, respectively, and these might alter the redox potential of the cluster.

The plasmid pGEM3-RIP1 was used as the template for site-directed mutagenesis, after which a *Pst*I/*Sac*I fragment encompassing the mutated site was subcloned into the single-copy yeast expression vector pFL39-RIP1, from which the *Pst*I/*Sac*I fragment of the wild type *RIP1* gene had been removed.

TABLE I

Growth phenotypes, midpoint potentials of iron-sulfur clusters, and cytochrome c reductase activities of mitochondrial membranes from yeast strains with mutations in the Rieske iron-sulfur protein

Yeast strain	Phenotype	$E_{m,7}$	Activity
			%
WT	**Pet**$^+$	+285	100
S183T	**Pet**$^+$	+259	80
S183A	*pet*$^-$	+155	10
S183C	*pet*$^-$	ND	0
Y185F	**Pet**$^+$	+217	40
Y185W	**Pet**$^+$	+195	30
Y185H	*pet*$^-$	ND	0
Y185L	*pet*$^-$	ND	0
Y185S	*pet*$^-$	ND	0
Y185T	*pet*$^-$	ND	0
Y185R	*pet*$^-$	ND	0
Y185D	*pet*$^-$	ND	0
Y185F, S183A	*pet*$^-$	+105	2

Amino acids are numbered according to the sequence of the yeast protein. WT is the deletion strain JPJ1 transformed with pFL39 carrying wild-type *RIP1*, which served as a positive control. S183 and Y185 correspond to S163 and Y165, respectively, in the bovine protein. **Pet**$^+$ and *pet*$^-$ indicate growth or lack of growth on ethanol/glycerol medium. Cytochrome *c* reductase activities were measured on isolated mitochondrial membranes. Midpoint potentials are average values determined by potentiometric titrations of purified cytochrome bc_1 complexes as shown in Fig. 5. ND, not determined.

The resulting constructs were used to transform JPJ1, a yeast strain in which the endogenous *RIP1* gene has been deleted (24). JPJ1 has no ubiquinol-cytochrome *c* oxidoreductase activity and is unable to grow on non-fermentable carbon sources. When JPJ1 is transformed with a single-copy plasmid carrying the wild-type *RIP1* gene, activity of the bc_1 complex and ability to grow on the non-fermentable carbon source are restored (24). Transformation of JPJ1 with the single-copy pFL39 plasmid carrying the site-directed *rip1* mutants allows characterization of any mutant phenotypes without interference from the iron-sulfur protein encoded by the wild-type *RIP1* gene.

Growth Characteristics and Ubiquinol-Cytochrome c Reductase Activities—To monitor effects of the *RIP1* mutations on respiratory activity, transformants were grown on solid medium containing ethanol/glycerol at 25, 30, and 37 °C (Table I). Only three mutants, carrying the S183T, Y185F, and Y185W mutations, had sufficient respiratory activity to allow growth (Pet$^+$) at all three temperatures. The other mutations resulted in a petite phenotype (*pet*$^-$), such that the yeast were unable to grow on ethanol/glycerol at any of the three temperatures. When grown on ethanol/glycerol at 30 °C in liquid culture, the doubling time of the wild-type strain was 3.6 h, and the doubling times of the mutants with the S183T, Y185F, and Y185W forms of the iron-sulfur protein increased to 4.0, 4.4, and 9.2 h, respectively.

Measurements of ubiquinol-cytochrome *c* oxidoreductase activities indicated that mitochondrial membranes from the three mutants able to grow on the non-fermentable carbon source, carrying the S183T, Y185F, and Y185W mutations, had 80%, 40%, and 30% of wild-type activity, respectively (Fig. 3). Mitochondrial membranes from the yeast carrying the S183A mutation and the S183A,Y185F double mutation had 10% and 2% of wild-type cytochrome *c* reductase activities, but these low activities were insufficient to allow growth on non-fermentable carbon sources. The S183A,Y185F double mutation in the iron-sulfur protein had a larger effect on the ubiquinol-cytochrome *c* activity compared with the single mutations. Membranes from the yeast strains carrying the other iron-sulfur protein mutations possessed no detectable cytochrome *c* reductase activity.

Western Blot Analysis of Mitochondrial Membranes from

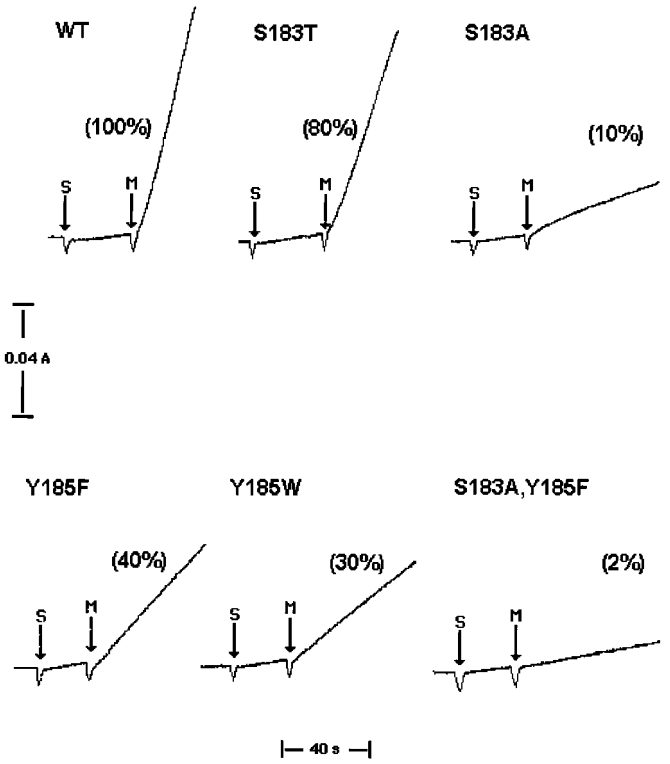

FIG. 3. **Ubiquinol-cytochrome *c* reductase activities of mitochondrial membranes from yeast expressing wild-type and mutated forms of the iron-sulfur protein.** The tracings show reduction of cytochrome *c* *versus* time. The non-enzymatic rate of reduction was measured by addition of 40 μM quinol substrate (*S*), after which the enzymatic rate was initiated by addition of membranes (*M*). *WT* indicates membranes from yeast containing wild-type Rieske iron-sulfur protein. Membranes from yeast containing mutated forms of the iron-sulfur protein are identified by the number of the amino acid residue and the amino acid change made at that position. Cytochrome *c* reductase activities, expressed as a percent of the turnover number of the enzyme in the membranes from the wild-type strain are indicated in *parentheses*.

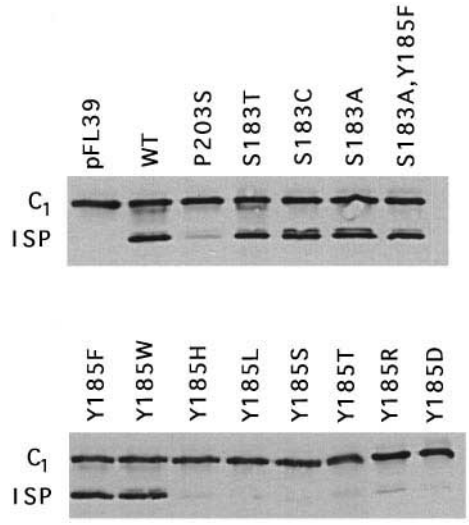

FIG. 4. **Immunoblot analysis of mitochondrial membranes from yeast expressing wild-type and mutated forms of the iron-sulfur protein.** Mitochondrial membranes from wild-type and mutant yeast were probed with antibodies against iron-sulfur protein (*ISP*) and cytochrome c_1 (c_1). pFL39 indicates membranes from JPJ1 transformed with the control vector. The abbreviations for the wild-type and mutant yeast strains are as in Fig. 3.

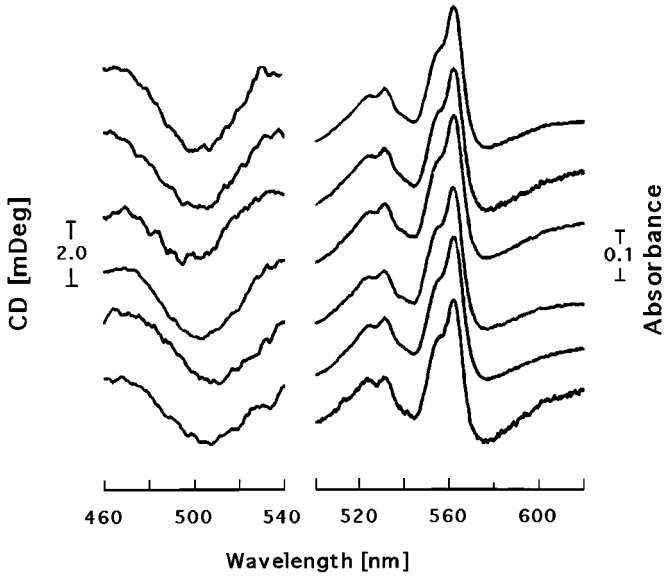

FIG. 5. **CD and optical spectra of bc_1 complexes isolated from yeast strains expressing wild-type or mutated forms of the iron-sulfur protein.** The spectra on the *left* are the reduced minus oxidized CD spectra of the iron-sulfur cluster in purified bc_1 complexes, and those on the *right* are the reduced minus oxidized optical spectra of the cytochromes in the same complexes. From *top* to *bottom*, the spectra are of bc_1 complexes containing the following forms of Rieske iron-sulfur protein: wild type, S183T, Y185F, Y185W, S183A, and S183A,185F.

Yeast Strains with Altered Rieske Proteins—Mitochondrial membranes of JPJ1 expressing the mutated genes from pFL39-RIP1 were assayed for their content of iron-sulfur protein by Western blotting with monoclonal antibodies to iron-sulfur protein, using cytochrome c_1 as an internal control to establish that equal amounts of mitochondrial protein were loaded onto the gel. As expected, the deletion strain JPJ1 transformed with the pFL39 plasmid lacking any *RIP1* gene showed no immunodetectable Rieske protein (Fig. 4).

Substitutions of Ser-183 with Ala, Thr, or Cys resulted in essentially wild-type levels of the Rieske protein. Likewise, membranes from yeast expressing the Y185F and Y185W forms of the iron-sulfur protein contained the same amount of iron-sulfur protein as those from cells expressing wild-type protein. Substitutions of Tyr-185 with Arg resulted in a marked decrease in the amount of immunologically detectable iron-sulfur protein similar to that in a previously described *pet*$^-$ mutant, P203S, in which a mutation in the C terminus resulted in a temperature-sensitive protein (Fig. 4 and Ref. 20). All other substitutions of Tyr-185, including Y185D, Y185H, Y185L, Y185S, and Y185T resulted in essentially undetectable amounts of iron-sulfur protein (Fig. 4).

Determination of Midpoint Potential of Mutated Iron-Sulfur Protein Using Circular Dichroism—In the reduced state the Rieske protein displays a strong negative CD band at 500 nm, which is free of interfering signals in isolated bc_1 complex (21). We isolated cytochrome bc_1 complexes from yeast expressing selected mutated forms of the iron-sulfur protein and monitored the reduction status of the iron-sulfur cluster by CD spectroscopy as the applied redox potential was varied (Fig. 5). From these titrations, we determined the midpoint potentials of the [2Fe-2S] clusters, as shown in Fig. 6.

The midpoint potential of the Rieske iron-sulfur protein in wild-type bc_1 complex was +285 mV, in agreement with previously reported values (2). The most pronounced effect on midpoint potential from a single exchange was observed with the S183A mutation, which lowered the midpoint potential by 130 mV to +155 mV, coincident with elimination of the hydrogen bond from the Ser-183 hydroxyl group to S-1 of the cluster.

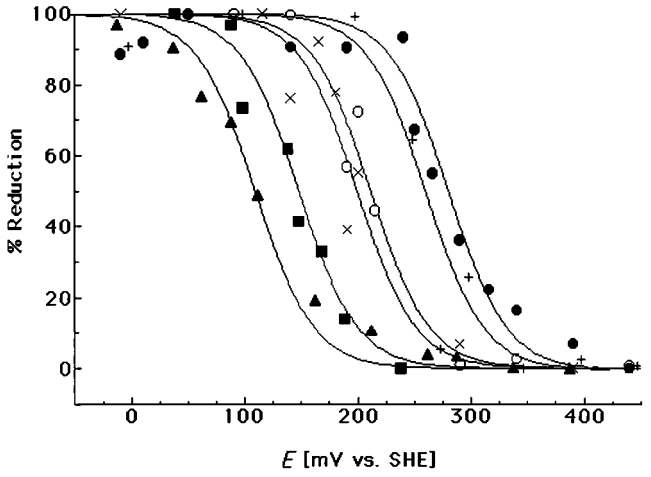

FIG. 6. **Determination of the oxidation-reduction midpoint potentials of the Rieske iron-sulfur clusters by CD-monitored electrochemical redox titrations of bc_1 complexes isolated from yeast strains expressing wild-type or mutated forms of the iron-sulfur proteins.** The degree of reduction of the Rieske cluster was obtained from the CD intensity at 500 nm and fitted using a single Nernstian curve with $n = 1$. The data points are as follows: ●, wild type enzyme, $E_m = 279$ mV; ○, Y185F, $E_m = 211$ mV; ×, Y185W, $E_m = 195$ mV; □, S183A, $E_m = 147$ mV; ▲, S183A,Y185F, $E_m = 111$ mV.

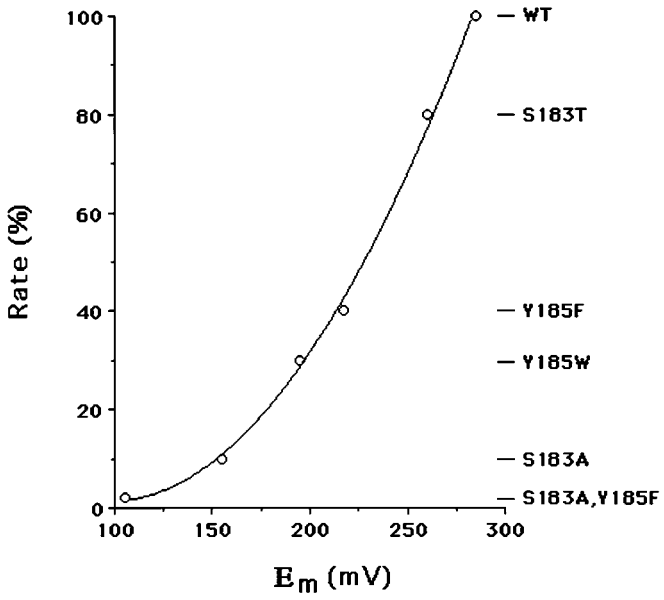

FIG. 7. **Ubiquinol-cytochrome *c* reductase activity *versus* midpoint potential of the Rieske iron-sulfur protein in mitochondrial membranes from yeast expressing mutated forms of the iron-sulfur protein.** Cytochrome *c* reductase activities were measured as described in Fig. 3, and midpoint potentials were measured as described in Fig. 6.

Eliminating the hydrogen bond from Tyr-185 to the S_γ of Cys-159 lowered the midpoint potential by approximately 65 mV, to +217 mV. Replacing Tyr-185 with Trp resulted in a slightly greater decrease in midpoint potential, to +195 mV. Notably, replacing both hydrogen bonds by the S183A,Y185F double mutation lowered the midpoint potential by 180 mV to +105 mV, indicating that these two hydrogen bonds have an approximately additive effect on the electronegativity of the cluster.

The CD spectrum of the cytochrome bc_1 complex isolated from the yeast strain carrying the S183C mutation revealed that this change resulted in the absence of a CD signal from the [2Fe-2S] cluster. The absence of the redox center accounts for the absence of cytochrome *c* reductase activity in this mutant (Table I), even though the apoprotein is stably present (Fig. 4).

Effects of Mutational Changes on the Structure of the Iron-Sulfur Protein—In addition to allowing determination of the midpoint potential, CD absorption arising from the iron-sulfur cluster monitors coupling of the iron-sulfur cluster to the chiral protein and the non-planar geometry that depends on the protein environment. The CD spectra in Fig. 5 show that, with the exception of the bc_1 complex containing the S183C form of the iron-sulfur protein, in which the iron-sulfur cluster is missing, the shape and intensity of the signature resonance from the iron-sulfur cluster in the 500 nm region is not significantly altered by the Y185F, Y185W, S183T, S183A, or Y185F,S183A mutations. The spectra are good evidence that the conservative changes in the altered forms of the iron-sulfur protein in which the cluster has been retained have not caused any structural rearrangement in the protein environment surrounding the iron-sulfur cluster.

This is further supported by the EPR spectra of the purified bc_1 complexes containing these altered forms of the Rieske protein, which showed small variations of all three principal *g* values (g_z, g_y: <0.01; g_x: <0.04) but retained the characteristic features of "Rieske" clusters as well as their intensity (results not shown). These spectra indicate small perturbations of the electronic distribution within the cluster, as would be expected if the hydrogen bonds from Ser-183 and Tyr-185 participate in electron delocalization, but exclude significant structural perturbations.

Optical spectra of the cytochromes provide a sensitive monitor of the interaction between the domain of the iron-sulfur protein that includes the iron-sulfur cluster and the domain of cytochrome *b* that includes the low potential *b* heme at center P. In mutants where the iron-sulfur cluster is absent due to mutational changes that eliminate one of the liganding histidines or cysteines (13) or where the iron-sulfur cluster is not stably inserted due to deletion of a supernumerary subunit of the bc_1 complex (*cf.* Fig. 1 in Ref. 25), there is a loss of absorption in the 560–562-nm region of the optical spectrum, presumably due to indirect effects on the cytochrome *b* environment resulting from these changes in the iron-sulfur protein. The optical spectra in Fig. 5 show that no such changes are evident in the purified bc_1 complexes containing mutated iron-sulfur proteins.

Perhaps the best evidence that the conservative mutational changes have not altered the structure of the protein is that the protein remains stably associated with the cytochrome bc_1 complex and the iron-sulfur cluster remains intact during purification, which involves detergent extraction and ion-exchange (18) or hydroxyapatite (19) chromatography, as indicated by the fact that there was no decrease in turnover of the complex as a result of the purifications. The apparent lack of structural changes in the protein environment surrounding the iron-sulfur cluster is consistent with the highly constrained nature of the iron-sulfur fold as revealed by the crystal structure (10).

Relationship between Midpoint Potential of the Rieske Iron-Sulfur Protein and Activity of the Cytochrome bc_1 Complex—Ubiquinol-cytochrome *c* reductase assays were run under conditions such that the reaction is zero order with respect to quinol and cytochrome *c*; consequently, the rate of cytochrome *c* reduction reflects the rate of electron transfer within the enzyme. As shown in Fig. 7, there is an exponential relationship between ubiquinol-cytochrome *c* reductase activity and the midpoint potential of the Rieske iron-sulfur protein as the latter varies from +100 to +280 mV. In the range between 220 and 280 mV, the rate increases 2.5-fold with an increase of 60 mV. As discussed below, this confirms that oxidation of ubiquinol by the iron-sulfur protein is the rate-limiting partial reaction in the bc_1 complex (26) and demonstrates the extent to

which the rate of electron transfer from bound quinol to cytochrome c_1 is controlled by the midpoint potential of the iron-sulfur cluster.

DISCUSSION

The Rieske iron-sulfur protein has a redox potential that is about 400–700 mV higher than the redox potentials of other [2Fe-2S] proteins. The crystal structure of the protein shows that the iron-sulfur cluster is embedded in a complex hydrogen bond network. We expected that the hydrogen bond network should contribute to the positive midpoint potential. To study the effects of the hydrogen bond network on the redox potential of the iron-sulfur protein, we introduced replacements for Ser-183 and Tyr-185, which contribute two of the seven hydrogen bonds of this network, by site-directed mutagenesis of the cloned iron-sulfur protein gene.

The most striking result from this mutational analysis is the finding that two hydrogen bonds formed by these two residues increase the midpoint potential of the Rieske [2Fe-2S] cluster by approximately 180 mV, with the hydrogen bond from the Ser-183 hydroxyl group to S-1 of the iron-sulfur cluster contributing 130 mV and the hydrogen bond from the Tyr-185 hydroxyl group to Sγ of Cys-159 contributing 65 mV. The presence of these two hydrogen bonds in the wild-type protein would decrease the electron density in the cluster and facilitate delocalization of the electron in the cluster during reduction of Fe-2. The potential of the iron-sulfur protein with the S183A mutation is comparable to that observed in menaquinol oxidizing *bc* complexes (27), where alanine instead of serine has been found in the native sequences (28).

Substitution of Ser-183 with Thr resulted in retention of 80% of the wild-type bc_1 activity, while substitution of Ser-183 with Ala resulted in only 10% of wild-type bc_1 activity (Table I). Since modeling these substitutions indicated that the rotamer scores for Ser, Thr, and Ala were identical, we deduce that the S183T substitution retained the hydrogen bond to S-1. The slight decrease of the redox potential in the S183T form of the protein is probably due to some subtle steric distortion resulting from the additional methyl group in the side chain of the Thr residue. This would alter the length and or angle of the hydrogen bond from the Thr hydroxyl to S-1, resulting in a weakening of the hydrogen bond, although this was not obvious when the Thr rotamers were viewed in the computer program. A previously described yeast mutant (22) in which Ser-183 of the Rieske protein is replaced by Leu showed significantly less immunologically detectable iron-sulfur protein, suggesting that insertion of a bulkier side chain results in an unstable protein.

Replacement of Ser-183 with Cys resulted in a Rieske protein lacking iron-sulfur cluster, although the apoprotein was present in essentially normal amounts (Fig. 4). This is the first example of a mutation in the Rieske iron-sulfur protein where the protein appears fully stable in the absence of iron-sulfur cluster. Since the Ser-183 hydroxyl group is not essential for formation of the cluster, as evidenced by the presence of the cluster in the S183A form of the protein, and the rotamer scores for the Cys substitution were not significantly different than for Ser, we conclude that the presence of an additional Cys residue interferes with cluster formation, possibly at the point of iron insertion.

Among the eight substitutions for Tyr-185, only Y185F and Y185W resulted in functionally active iron-sulfur protein. The change in midpoint potential was slightly greater in the Y185W form of the protein (+195 mV) than in the Y185F form (+217 mV). We considered the possibility that the Y185W mutation might have allowed an alternative hydrogen bond from the indol nitrogen to the Sγ of Cys-159, resulting in an additional decrease in the midpoint potential (Fig. 4). However, modeling of the Trp substitution indicates that, of the 15 possible rotamers, those with the lowest two energies only bring the indol nitrogen within 5.02 and 5.51 Å of the Sγ of Cys-159, and none of the rotamers bring the indol nitrogen less than 4.40 Å from the sulfur. The difference between the Trp and Phe substitutions is thus probably due to distortions of the cluster environment resulting from the introduction of the bulkier indol ring in comparison to the phenyl group. Consistent with this interpretation, we observed that the iron-sulfur cluster in the Y185W form of the protein was not stable during freezing of the mitochondrial membranes or the isolated cytochrome bc_1 complex, whereas the protein with the Y185F substitution was stable.[3]

Yeast mutants in which Tyr-185 of the Rieske protein was replaced with His, Leu, Thr, Ser, Arg, or Asp were only able to grow fermentatively. Western blotting of mitochondrial membranes established that the amount of Rieske protein was drastically diminished in the yeast carrying the Y185R mutation and undetectable in membranes from the yeast with the Y185H, Y185L, Y185T, Y185S, and Y185D forms of the protein (Fig. 4). The absence of Rieske iron-sulfur protein in the yeast with the Y185H, Y185L, Y185T, Y185S, and Y185D iron-sulfur protein mutations is not simply due to failure to insert iron-sulfur cluster or a secondary consequence of the loss of an unstable iron-sulfur cluster, since the mature apoprotein was present in the yeast with the S183C form of the protein (Fig. 4), for which the CD spectrum indicated the cluster was absent. Likewise, in a previous study, immunologically detectable iron-sulfur protein was also present, although in decreased amounts, in the mitochondrial membranes of yeast mutants in which the Cys and His ligands required for formation of the [2Fe-2S] cluster were eliminated by site-directed mutagenesis (13). The conservative changes in the protein introduced by these mutations thus differ markedly from the mutation characterized by Gatti and co-workers (22), where only a small change in midpoint potential (50 mV) was accompanied by instability of the protein and significant loss of iron-sulfur cluster.

The lack of immunologically detectable Rieske protein in mitochondrial membranes from yeast with the Y185H and Y185D forms of the protein is somewhat surprising, since the profile of rotamer scores indicates that there are more stable rotamers for these substitutions than for the wild-type protein. One possible explanation for the absence of protein in these mutants is that the imidazole nitrogens of the histidine and carboxyl groups of the aspartate form hydrogen bonds during the protein folding process that result in aberrantly folded protein, which is then degraded. An additional source of instability in the Y185D form of the protein is that the carboxylate anion may be excluded from the otherwise hydrophobic environment surrounding Tyr-185 during protein folding.

The absence of stably formed Rieske protein in the yeast with the Y185L, Y185T, and Y185S forms of the protein can be explained by the large number of prohibited rotamers, in the case of Y185L, and by an unstable cavity proximal to the Cys-159 and Cys-178 residues that coordinate the cluster (see Fig. 1*B*) when the small Thr and Ser residues are substituted for the bulkier Tyr. From modeling these substitutions, the resulting protein instability appears to be because it is necessary to maintain the tight packing of the cluster binding fold, which is thought to protect the protein against attack by proteases (10).

[3] T. Merbitz-Zahradnik, O. M. Hatzfeld, and T. A. Link, unpublished results.

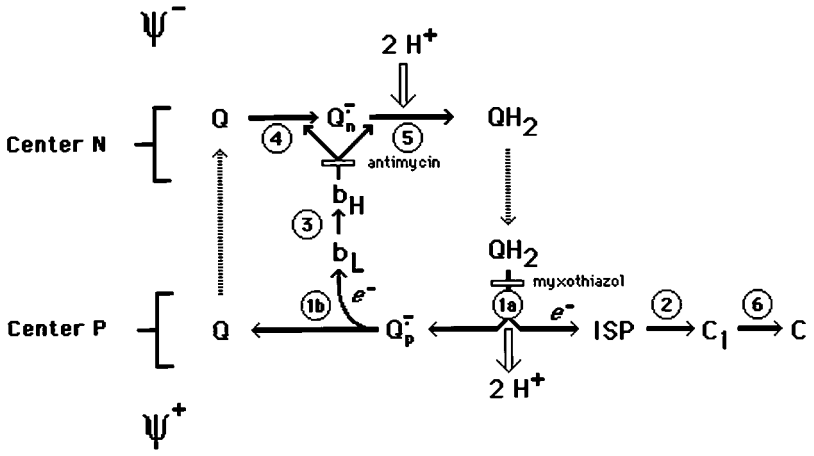

FIG. 8. **Protonmotive Q cycle mechanism of electron transfer through the cytochrome bc_1 complex showing individual electron transfer reactions.** The scheme shows the branched, cyclic pathway of electron transfer from ubiquinol (QH_2) to cytochrome *c* (*C*) through the four redox centers of the cytochrome bc_1 complex. The *circled numbers* designate electron transfer reactions. *Dashed arrows* represent movement of ubiquinol and ubiquinone (*Q*) between the site where ubiquinol is oxidized at the positive side of the membrane ("center P") and the site where ubiquinone and ubisemiquinone are reduced at the negative side of the membrane ("center N"). In step 1, ubiquinol is oxidized at center P in an essentially concerted reaction, in which one electron is transferred from ubiquinol to iron-sulfur protein (step 1a), generating a low potential ubisemiquinone anion (Q_p), which immediately reduces the b_L heme group (step 1b). Two protons are released at the P surface of the membrane coincident with oxidation of ubiquinol to ubisemiquinone anion. In step 2, the electron transferred to iron-sulfur protein is transferred to cytochrome c_1 and then to cytochrome *c* (step 6). In step 3, the electron transferred to the b_L heme group is transferred to the b_H heme. In step 4, the b_H heme reduces ubiquinone to the relatively stable ubisemiquinone anion (Q_n). When b_H is rereduced by repetition of the preceding reactions, b_H reduces ubisemiquinone anion to ubiquinol (step 5). Reduction of ubisemiquinone anion to ubiquinol at center N consumes two protons at the N surface of the membrane. The divergent oxidation of ubiquinol at center P (step 1) and electron transfer through the two *b* hemes to the Q/QH_2 couple at center N occurs twice during one complete *Q* cycle. One complete *Q* cycle thus deposits four protons on the positive side of the membrane, reduces two cytochrome *c* molecules, and consumes two protons from the negative side of the membrane. The *open boxes* show the sites at which myxothiazol and antimycin inhibit electron transfer reactions within the complex.

The use of Western blots to screen for the presence of the Rieske protein allowed mutations resulting in an unstable protein to be recognized. The Western blots also reveal an important aspect of iron-sulfur protein assembly. In *S. cerevisiae* mitochondria, the Rieske iron-sulfur protein is post-translationally processed in two steps by two peptidases during import and assembly into the cytochrome bc_1 complex (29). The intermediate iron-sulfur protein (i-ISP) that is formed during the first step of this processing is normally detectable in only small amounts in mitochondrial membranes or purified cytochrome bc_1 complex (Fig. 4; see also Fig. 5 in Ref. 23). In the current study, we have observed that significant amounts of incompletely processed i-ISP are visible in mitochondrial membranes from the mutants with both the S183A and S183C forms of the protein (Fig. 4). This species, which migrates slightly more slowly than the mature sized protein, was previously identified as i-ISP by N-terminal sequencing of the protein (23). Since i-ISP does not accumulate in mitochondrial membranes of any of the yeast mutants with changes at Tyr-185 of the Rieske protein and in which the protein is immunologically detectable, we conclude that the accumulation of i-ISP in the mitochondrial membranes of the yeast with the mutations at Ser-183 reflects a facilitative effect of the Ser-163 hydroxyl group on formation of the [2Fe-2S] cluster. The accumulation of i-ISP in the yeast with the replacements at Ser-183 is evidence that insertion of the cluster precedes conversion of i-ISP to mature iron-sulfur protein, but is not obligatory for this final step in Rieske iron-sulfur protein maturation (13).

The relationship between ubiquinol-cytochrome *c* reductase activity and Rieske midpoint potential confirms that oxidation of ubiquinol by the Rieske protein is the rate-limiting partial reaction in the bc_1 complex when the enzyme is operating catalytically (26). To illustrate this, we have depicted electron transfer from ubiquinol to cytochrome *c* through the Q cycle mechanism as consisting of seven electron transfer steps in the bc_1 complex in Fig. 8. Since the cytochrome *c* reductase assays are performed under conditions where the reaction is zero order with respect to cytochrome *c*, reaction 6 can not be limiting. If any of the iron-sulfur protein independent electron transfer steps within the Q cycle, reactions 1b, 3, 4, or 5, were significantly slower than those involving the iron-sulfur protein, reactions 1a and 2, changing the potential of the iron-sulfur cluster would not alter the activity of the bc_1 complex. Thus, electron transfer from ubiquinol to iron-sulfur protein or from iron-sulfur protein to cytochrome c_1 must be the rate-limiting partial reaction within the enzyme.

The changes in midpoint potential of the iron-sulfur protein could affect either or both of two electron transfer reactions, from bound quinol to the iron-sulfur cluster (see Fig. 8; reaction 1a) or from the iron-sulfur cluster to cytochrome c_1 (reaction 2). Since the rate decreases as the potential decreases, and there is no theoretical basis on which to explain how increasing the increment in potential between the iron-sulfur cluster and cytochrome c_1 could lead to a decrease in electron transfer rate, the decline in rate that accompanies the decrease in iron-sulfur cluster potential must reflect a decrease in the rate of electron transfer from ubiquinol to the iron-sulfur cluster.

Between 220 and 280 mV, the turnover number of the enzyme increases 2.5-fold as the midpoint potential of the iron-sulfur cluster increases 60 mV. If the rate dependence on midpoint potential is predictable from Marcus electron transfer theory, it would be expected to change 3.1-fold for a 60-mV change in potential. The less than predicted change indicates that under conditions where the activity is zero order with respect to ubiquinol and cytochrome *c*, the activity of the bc_1 complex is influenced extensively, although not solely, by the midpoint potential of the iron-sulfur cluster. If deprotonation of ubiquinol is prerequisite to electron transfer (30), the protonic chemistry must be sufficiently fast as not to impede significantly the potential dependence of the cytochrome *c* reductase rate.

Since the decline in ubiquinol-cytochrome *c* reductase rate with declining midpoint potential of the Rieske protein results from a decline in the rate of electron transfer from ubiquinol to

the iron-sulfur cluster, it is not surprising that the rate approaches zero as the midpoint potential of the Rieske protein approaches 100 mv, since the midpoint potential of the ubiquinol/ubiquinone couple is approximately 100 mV (31). The fact that the rate does not continue to decline in a linear manner below 220 mV and reach zero at approximately 160 mV suggests that a second parameter becomes operative at limiting values of Rieske protein potential. One such possibility is that rapid electron transfer from the Rieske iron-sulfur protein to cytochrome c_1, for which the rate has been estimated to be greater than $5 \times 10^5\ s^{-1}$ (32), maintains the Rieske iron-sulfur clusters in these mutants in a highly oxidized state, so that the operative redox potentials are higher than the midpoint potentials determined from equilibrium titrations. A second possibility is that this results from the concerted nature of the divergent two-electron transfer reaction at center P (reactions 1a and 1b in Fig. 8), that involves contributions from a domain on cytochrome *b*, in addition to the Rieske protein (8).

The changes of the redox potential of the iron-cluster should affect the population of the semiquinone intermediate at center P, which, according to the "proton-gated affinity change" mechanism, is stabilized by the reduced iron-sulfur cluster (33). The non-linear dependence of electron transfer rate on redox potential is consistent with this interpretation, but a more detailed characterization of the kinetics of the bc_1 complexes in these mutants will be required to unravel this aspect of the mechanism of ubiquinol oxidation.

Although 30% of the normal ubiquinol-cytochrome *c* reductase activity in the yeast mutant with a Y185W replacement in the Rieske iron-sulfur protein was sufficient for the yeast to grow on non-fermentable carbon sources, 10% of normal bc_1 activity in the mutant with a S183A replacement in the iron-sulfur protein was not sufficient to allow growth on non-fermentable carbon sources. The activities of the bc_1 complexes in these two mutants thus bracket a threshold activity between 10 and 30% of that present in wild-type yeast required to support respiratory growth. A threshold of approximately 15% of wild type activity was observed in studies of revertants of yeast mutants deficient in cytochrome oxidase activity.[4] Since the boundary activities of 10–30% are linked to midpoint potentials of +155 and +195 mV, respectively, they indicate a critical midpoint potential of the Rieske iron-sulfur protein required to support respiratory growth. Notably, there are no examples of a respiratory competent yeast in which the midpoint potential of the Rieske iron-sulfur protein is below 160 mV (22).

Note Added in Proof—Results simular to those reported here have been obtained in *Paracoccus denitrificans,* T. Schroeter, O. M. Hatzfeld, S. Gemeinhardt, M. Korn, T. Friedrich, B. Ludwig and T. A. Link, submitted for publication.

[4] C. Ortwein, B. Meunier, A. M. Colson-Corbisier, and T. A. Link, unpublished results.

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