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\textsubscript{1}) of the iron-sulfur cluster and the sulfur atom (S\textsubscript{2}) 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\textsubscript{1} of the cluster lowers the midpoint potential by 65 mV. Eliminating the hydrogen bond from the hydroxyl group of Tyr-185 to S\textsubscript{2} of Cys-159 lowers the midpoint potential by 65 mV. Eliminating both hydrogen bonds has an approximately additive effect, lowering the midpoint potential by 130 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\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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).

The high midpoint potential of the bc\textsubscript{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. 1A. 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. 1B, 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\textsubscript{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-
FIG. 1.

Rieske Iron-Sulfur Protein Mutations
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 bc1 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 (0.5 mm) were obtained from Bio-Rad. 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 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 JFP1 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 c1 were detected by Western blotting (17) using monoclonal antibodies to yeast iron-sulfur protein and cytochrome c1 (13).

**Purification of Cytochrome b6 Complexes**—For all of the yeast mutants in which the iron-sulfur protein was detectable by Western blotting of the mitochondrial membranes, the cytochrome bc1 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 bc1 complexes were assayed at pH 7.0 and 25 °C using 50 μM 2,3-dimethoxy-5-methyl-6-n-decyl-1-4-benzoquinol as substrate and 50 μM horse heart cytochrome c5. Reduction of cytochrome c was monitored in an Aminco DW-2A spectrophotometer at 550 and 539 nm in dual wavelength mode. Turnover numbers of the bc1 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 bc1 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 bc1 Complex**—CD-monitored redox titrations of isolated cytochrome bc1 complexes were performed in an optically transparent thin layer cell with a path length of 0.1 cm 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° = −249 mV), anthraquinone-2-sulfonate (E° = −225 mV), 2-hydroxy-1,4-naphthoquinone (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-naphthoquinone (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 μM cytochrome bc1 complex in 100 mM NaCl, 35 mM MOPS, 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.

**RESULTS**

**Choice of Mutations**—The amino acid sequences of the bovine and yeast Rieske iron-sulfur proteins are almost identical throughout the regions 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.
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 bovine 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 S 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 Ser-183 and a mutation at Tyr-185 were isolated after random mutations placed both alternative hydroxyl groups 8.85 Å from the cause both of these introduce an aromatic side chain with a change in the side chain. Trp and His were chosen to replace Tyr because they would place positive and negative charges at the bottom of the cluster binding fold, and thus we have controlled for protein instability because these residues form hydrogen bonds that are likely to form a hydrogen bond 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 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 Pet/SacI fragment encompassing the mutated site was subcloned into the single-copy yeast expression vector pFL39-RIP1, from which the Pet/SacI fragment of the wild type RIP1 gene had been removed.

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 bc1 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 90%, 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
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 pet2 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 $bc_1$ complexes from yeast expressing selected mutated forms of the iron-sulfur protein and monitored the reduction status 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,Y185F.

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Eliminating the hydrogen bond from Tyr-185 to the S$_{\gamma}$ 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 $b_{c1}$ 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 $b_{c1}$ 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 $b_{c1}$ complexes containing these altered forms of the Rieske protein, which showed small variations of all three principal g values ($g_x$, $g_y$, $g_z$) but retained the characteristic features of “Rieske” clusters as well as their intensity (results not shown). These spectra indicate small perturbations of the electron 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 $b_{c1}$ 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 $b_{c1}$ 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 $b_{c1}$ 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 bc1 Complex**—Ubiquinol-cytochrome $c$ reductase assays were run under conditions such that the reaction is zero order with respect to ubiquinol 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 $b_{c1}$ complex (26) and demonstrates the extent to which the iron-sulfur cluster monitors coupling of the iron-sulfur cluster to the chiral protein environment. The CD spectra in Fig. 5 show that, with the exception of the $b_{c1}$ complex containing the S183C form of the iron-sulfur protein, in which the iron-sulfur cluster is not stably inserted due to deletion of a supernumerary subunit of the $b_{c1}$ 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 $b_{c1}$ complexes containing mutated iron-sulfur proteins.

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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 $S_Y$ 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 $bc_1$ 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 $Sy$ 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 $Sy$ 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.

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. 1B) 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).
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 bc1 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 bc1 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 bc1 complex. Thus, electron transfer from ubiquinol to iron-sulfur protein or from iron-sulfur protein to cytochrome c1 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 c1 (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 c1 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 bc1 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

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**FIG. 8. Protonmotive Q cycle mechanism of electron transfer through the cytochrome bc1 complex showing individual electron transfer reactions.** The scheme shows the branched, cyclic pathway of electron transfer from ubiquinol (QH2) to cytochrome c (C) through the four redox centers of the cytochrome bc1 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 (Q1). 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 c1, and then to cytochrome c (step 6). In step 3, the electron transferred to the bL heme group is transferred to the bH heme. In step 4, the bH heme reduces ubiquinol to the relatively stable ubisemiquinone anion (Q1). When Q1 is reoxidized by repetition of the preceding reactions, bL 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/QH2 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 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 c₁, 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 bc₁ 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 bc₁ 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 bc₁ 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. 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 similar 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.

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