Specific Mutagenesis of the Rieske Iron–Sulfur Protein in *Rhodobacter sphaeroides*

Shows That both the Thermodynamic Gradient and the pK of the Oxidized Form

Determine the Rate of Quinol Oxidation by the *bc*$_1$ Complex$^\dagger$

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ABSTRACT: In the Rieske iron–sulfur protein (ISP) of the ubiquinol:cytochrome *c$_2$* oxidoreductase (*bc$_1$* complex) of *Rhodobacter sphaeroides*, residue Tyr 156 is located close to the iron–sulfur cluster. Previous studies of the equivalent residue in both *Saccharomyces cerevisiae* [Denke, E., Merbitz-Zahradnik, T., Hatzfeld, O. M., Snyder, C. H., Link, T. A., and Trumpower, B. L. (1998) *J. Biol. Chem.* 273, 9085–9093] and *Paracoccus denitrificans* [Schorero, T., Hatzfeld, O. M., Gemeinhardt, S., Korn, M., Friedrich, T., Ludwig, B., and Link, T. A. (1998) *Eur. J. Biochem.* 255, 100–106] have indicated that mutations at this site can lead to modifications in the redox potential of the ISP. To study the effect of similar modifications on the thermodynamic behavior and kinetics of partial reactions of the *bc$_1$* complex upon flash activation, we have constructed four mutant strains of *Rb. sphaeroides* where Tyr 156 was mutated to His, Leu, Phe, or Trp. The *bc$_1$* complex was assembled and able to support photosynthetic growth in all mutants. Three substitutions (Leu, Phe, Trp) led to alteration of the midpoint potential ($E_m$) of the ISP and a slowing in rate of quinol oxidation, suggesting that electron transfer from quinol to the oxidized ISP controls the overall rate and that this step includes the high activation barrier. The Trp mutation led to an increase of ~1 pH unit in the pK value of the oxidized ISP. The pH dependence of the rate of quinol oxidation in this mutant was also shifted up by ~1 pH unit, showing the importance of the protonation state of the ISP for quinol oxidation. This provides support for a model in which the dissociated form of the oxidized ISP is required for formation of the enzyme–substrate complex [Ugulava, N., and Crofts, A. R. (1998) *FEBS Lett.* 440, 409–413].

The ubiquinol:cytochrome *c* oxidoreductase (E.C. 1.10.2.2) (*bc$_1$* complex)$^\dagger$ and the related *bf*$_1$ complex of oxygenic photosynthesis provide the central elements of the major energy conversion pathways of the biosphere, catalyzing the transfer of electrons from a quinol to a small soluble protein such as cyt *c* by a modified Q-cycle (1–3). Electron transfer is linked to generation of a proton gradient across the membrane. The rate-limiting step in the mechanism is the oxidation of quinol at the Q$_b$ site, where the first electron is transferred to a high-potential chain of single electron carriers (ISP, cyt *c$_1$* and cyt *c*) and the second electron is transferred to a low-potential chain formed by cyt *b$_h$* and cyt *b$_l$*. Mechanistic aspects of this bifurcated reaction remain controversial (4–10). Brandt and Okun (11) have suggested that the activity of the *bc$_1$* complex is controlled by two amino acid groups, with pK values of 6.5 and 9.2, which have to be dissociated and protonated, respectively, for maximal turnover. On the other hand, Ugulava and Crofts (12) have proposed that formation of the enzyme–substrate complex (ES complex) between quinol and the oxidized ISP, which precedes the quinol oxidation reaction, requires prior deprotonation of the ISP. In this scenario, the pH dependence of the rate of quinol oxidation reflects the availability of one of the substrates—the deprotonated ISP$^{\text{ox}}$. A similar model has been proposed by Snyder and Trumpower (13), in which the quinol at the Q$_b$ site displaces a proton from one of the histidine ligands to the iron–sulfur cluster of the ISP.

The information obtained from the crystal structures of the *bc$_1$* complexes (10, 14, 15) and their subunits (16, 17) provides a context for understanding the mechanism at a molecular level and resolution of this controversy. Structures of isolated ISP from mitochondria and chloroplast complexes show that, although the sequence alignment of these two proteins shows a homology of only 28%, the overall structure is quite similar, and more strikingly the small domains containing the redox cofactor are almost superimposable. All residues that are completely conserved are located in this
domain, including Tyr 165 (bovine numbering). The hydroxyl side chain of this residue is located at hydrogen-bonding distance from the sulfhydryl group of Cys 139, which is a ligand to the Fe2 of the [2Fe-2S] cluster. Previous data showed that a Cys substitution for this Tyr in yeast leads to a complete loss of activity of the bc1 complex, due to the nonassembly of the [2Fe-2S] cluster (18). On the basis of these data Iwata et al. (16) suggested an important structural role for this residue with possible implications for the assembly of the [2Fe-2S] cluster. A role in the electron transfer was also proposed by Carrell et al. (17), when they identified a nearly identical configuration in the chloroplast ISP. More recently, Denke et al. (19) and Schröter et al. (20) showed that mutations of the equivalent tyrosine in yeast and Paracoccus denitrificans could lead to modification of the ISP redox potential. However, the kinetic implications of these modifications were not investigated in detail, mainly because of the difficulties involved in studying such parameters in respiratory systems.

We have constructed four mutant strains of *Rhodobacter sphaeroides* in which the equivalent residue, Tyr 156, has been replaced by Phe, Trp, His, or Leu by site-directed mutagenesis. We have used these to investigate the effects of the protein environment on thermodynamic and spectral properties of the iron–sulfur cluster, assayed by EPR and CD spectroscopy. Kinetic spectrophotometry and flash excitation were used to investigate the effects on turnover of the Qb site of the bc1 complex in situ, by studying the partial reactions as a function of ambient redox potential and pH. Our results confirm that the rate of quinol oxidation is strongly dependent on the thermodynamic gradient of the reaction, as determined by the difference in redox midpoint (13, 19). More importantly, we show that the pK value of the oxidized ISP controls the pH dependence of the rate of quinol oxidation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions.** Escherichia coli strains were grown at 37 °C in LB medium supplemented with appropriate antibiotics, either ampicillin (100 μg/mL) or tetracycline (10 μg/mL). *Rb. sphaeroides* strains were grown in Sistrom medium at 30 °C either aerobically or photosynthetically. Strains containing the pRK415 derivative were maintained in the presence of 2 μg/mL tetracycline and 20 μg/mL kanamycin.

**Construction of the Mutants.** Syntheses of primers for PCR mutagenesis and sequencing of the mutant plasmids in order to confirm the mutations were performed at the Biotechnology Center of the University of Illinois. Site-directed mutagenesis was performed using plasmid pGBM as template with PCR methods as described in Kuras et al. (21). pGBM contains the whole fbc operon, cloned in pUC19, and is able to restore the WT properties of the BC17, a strain of *Rb. sphaeroides* bearing a deletion of the endogenous fbc operon (22). The fbc operon encodes the three major subunits of the bc1 complex, the Rieske ISP (fbc F), the cyt b (fbc B), and the cyt c1 (fbc C). Plasmid pGBM was first modified to introduce a six-histidine insert at the end of cyt b to facilitate rapid purification of the bc1 complex (23), leading to plasmid pGBH6B.

Four mutations of codon 156 in the fbc F gene were generated using plasmid pGBH6B, to replace the WT Tyr with either Phe, His, Trp, or Leu. For each of the mutants an insert (i156X) containing the mutagenic codon was made using the following primers: (1) (pX) 5’-CGGGATCC-CCATGGATCGCAAC-3’ -3’-GACAGTGCGGCC 3’, where the mutagenic codon [- - -] corresponds to TTC for the Phe (X = F), CAC for the His (X = H), TGC for the Trp (X = W), and CTC for the Leu (X = L) substitution, and (2) (p2) 5’-GGAATTCTATGCATCGCAAGCACGATGCGCGTG 3’.

The PCR products were digested by EcoRI and BamHI and cloned in pUC19. To introduce the mutation in the operon, an additional insert (i2) was generated using the following primers: (3) (p3) 5’-CGGGATCCTTTCTTCCG- CAAACCGATCTTCATC 3’ and (4) (p4) 5’-GGAATTCCATGGATCGCAAGCACGATGCGCGTG 3’.

The product was also digested by EcoRI and BamHI and cloned in pUC19. Standard molecular biology techniques were used to fuse each of the inserts i156X with insert i2 using the NcoI site (shown in italics, underlined). The RsrII and BstBI restriction sites were then used to introduce the fusion products containing the mutation into pGBH6B. The fbc operons were subsequently subcloneed into pRK415-1 and introduced into *Rb. sphaeroides* by conjugation with *E. coli* strain S17-1 (22).

**Biochemical Methods.** Purification of the bc1 complex and polypeptide analysis were performed as previously described (23) from chromatophore preparations (24). For each lane in Figure 1, 25 μg of the purified bc1 complex, resuspended in 66 mM DTT, 66 mM Na2CO3, 2% (w/v) SDS, 10% (w/v) sucrose, and 3 mg/mL bromphenol blue, was loaded. The protein concentration was determined from the optical spectra of the reduced bc1 complex (25). Polypeptides were visualized by Coomassie blue staining.

**Biophysical Methods.** All mutant strains were characterized through differential spectrophotometry to identify the three cytochrome components of the bc1 complex; in addition, for all chromatophore preparations, the complement of redox centers was assayed by excitation using multiple flashes in the presence of antimycin and measurement at the wavelength pairs appropriate for individual components (4, 26). The spectra and Emax values for the cytochrome components were the same within experimental error in all strains, and components were present in the same relative stoichiometry.

EPR spectra from the purified bc1 complexes were obtained using a Varian E-112 X-band spectrometer equipped with an Air Products (Allentown, PA) variable temperature cryostat and a Varian TE102 mode cavity. The conditions used...
were microwave frequency 9.05 GHz, microwave power 1 mW, modulation amplitude 12.5 G, and temperature 11 K.

CD spectroscopy was performed using a Jasco J-720 spectropolarimeter as described previously (12). The mutant bc₁ complexes were resuspended at 2 µM in the appropriate buffer in the presence of 0.01% dodecyl maltoside. Redox titrations were performed in a modified cylindrical cuvette as described in Ugulava and Crofts (12). Titrations curves were obtained in the CD spectra by abstracting values at appropriate wavelengths (500 and 470 nm). The curves were fitted with a one-electron Nernst component using the built-in nonlinear least-squares-fitting algorithm of the data analysis program Origin 4.0 (Microcal Software, Inc., Northampton, MA).

Flash-induced kinetics were measured using a home-built kinetic spectrophotometer at a constant temperature (25 °C) as described previously (4). Chromatophores were suspended at about 0.4 µM RC in 100 mM KCl containing 50 mM appropriate buffer (see below) and with additions as indicated in the figures. The ambient redox potential in the cuvette was adjusted by adding small amounts of K₃Fe(CN)₆ or Na₂S₂O₄, and the suspension was kept anaerobic by a constant flow of argon gas. The following redox mediators were used in all kinetic experiments at a concentration of 7 and 20 µM each: DAD (Eₐ 245 mV), 1,2-naphthoquinone-4-sulfonic acid (Eₐ 217 mV), 1,2-naphthoquinone (Eₐ 145 mV), 1,4-naphthoquinone (Eₐ 60 mV), and N-methylphenazonium methosulfate (Eₐ 85 mV). Pyocyanine (Eₐ −60 mV) and Fe(III) NaEDTA (Eₐ 117 mV) were used at concentrations of 7 and 20 µM, respectively. Kinetics of cyt b₃ reduction were followed at 561–569 nm (26). The rates were estimated either from the initial slope of cyt b₃ reduced after the first flash, or by deconvoluting the kinetics of cyt b₁ reduction to a single-exponential component, assuming pseudo-first-order kinetics. The two methods gave similar results, although at pH ranges where the kinetics became fast, slight deviation from a single-exponential behavior was observed. The electrochromic band shift was measured from the absorbance change at 503 nm as described in Glaser and Crofts (27). Initial rates of the slow phase were determined from the difference kinetics without and with myxothiazole.

Buffers used both for the kinetic measurements and for the redox titrations of the ISP were MES for pH 5.0–6.5, MOPS to pH 7.5, EPPS to pH 8.5, and CHES to pH 10.

Molecular modeling was performed on a Silicon Graphics Indigo2 workstation using the program package Quanta (Molecular Simulations Inc., San Diego, CA). For the homology modeling of the ISP from Rb. sphaeroides, the Modeller module from the same package was used, with the coordinates for the soluble fragment of the bovine mitochondrial ISP (PDB file 1rie) as a structural template. An alignment of 20 bacterial and mitochondrial ISPs was first generated using ClustalW, and the database was further searched to obtain the best fits for the extra sequences characteristic for the purple bacteria. The sequence of Rb. sphaeroides corresponding to the soluble part was then used to generate the molecular model of the ISP. Since the program does not take into account the special configuration of cofactors, the coordinates for the [2Fe-2S] cluster were built from the mitochondrial structure. The conformations of the side chains of the two histidine ligands found after generation of the preliminary model were modified in order to adopt the geometry necessary for liganding the cluster. The conformity of the model to appropriate physicochemical parameters was verified by comparison with a database of structures with resolution of at least 2.0 Å (Procheck 3.4), and 98.2% of the residues were found in favored regions. The modeling of the Tyr 156 substitutes was based both on the structural model of the Rb. sphaeroides ISP and on the structure of the beef heart mitochondrial ISP. In each case, the structure of the backbone was fixed, and the best rotamers were calculated using modules in the Quanta package.

### RESULTS

#### Choice of Mutations and Properties of the Mutant Strains

We have constructed four mutant strains of Rb. sphaeroides where Tyr 156 from the ISP was substituted by Phe, Trp, His, or Leu. The mutations were introduced in a context where cyt b contains a polyhistidine C-terminal extension (His₆ tag) to facilitate rapid purification of the bc₁ complex (23). All four strains were capable of growing photosynthetically, which indicates that none of these mutations led to an inactive bc₁ complex. The Y156H mutant showed the same growth rate as the WT (doubling time of 8 h), whereas growth of Y156F, Y156L, and Y156W was slightly slowed, with generation times of 10, 10, and 12 h, respectively. The purified bc₁ complex of the mutant strains showed all four subunits with a relative stoichiometry undistinguishable from that of the WT (Figure 1).

#### Properties of the [2Fe-2S] Cluster

The EPR spectra of the [2Fe-2S] center of ascorbate-reduced bc₁ complexes from Y156H, Y156F, and Y156L are similar to those of the WT with g₁ and g₃ bands at 1.895 and 1.800, respectively (Figure 2A and Table 1). By contrast, the signal from the Y156W mutant bc₁ complex showed a clear difference, particularly in the position of the g₃ band that was shifted downfield to

<table>
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<tr>
<th>Table 1</th>
<th>Turnover of bc₁ Complex Modified in ISP Mutants</th>
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<td><strong>EPR parameters</strong></td>
<td><strong>redox parameters</strong></td>
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<tr>
<td><strong>ascorbate reduced</strong></td>
<td><strong>in the presence of stigmatellin</strong></td>
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<td><strong>strain</strong></td>
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<tr>
<td>Y156F</td>
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<td>Y156L</td>
<td>1.895</td>
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<tr>
<td>Y156W</td>
<td>1.890</td>
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<sup>a</sup> Eₐ<sub>lim</sub> is the limiting value for Eₐ at pH < pK₁. <sup>b</sup> The rate for wild type at pH 7 was 1330 mol·(mol of bc₁)⁻¹·s⁻¹. <sup>c</sup> These values were forced and lie outside the titration range. The curve was well fit by a single pK with the same value as pK₁.
The redox properties of the Rieske iron–sulfur center in the mutant strains were investigated by CD spectroscopy. It has been shown that the Rieske ISP has a typical CD spectrum with a broad negative band around 500 nm in its reduced state (30, 31). This property has been used to probe both the redox potential of this protein and its pH dependence (12, 32). Redox titrations of the purified bc1 complexes from the mutant strains were performed at different pH values in the range from 5.0 to 10.5. All of the titration curves could be fitted to a Nernst equation with \( n = 1 \). The midpoint potentials determined at each pH for the different mutants were plotted in Figure 3. It was previously observed that, in the pH range used for our study, the \( E_m \) value of the ISP from \textit{Rh. sphaeroides} is constant below \( \text{pH} 7 \) and decreases above this point in both the purified bc1 complex and in membranes (12, 33). The data were fitted using either one or two pK values with \( pK_1 \sim 7.6 \) and \( pK_2 \sim 9.8 \). These values are similar to those found for beef heart mitochondria (32). The results of the deconvolution using two pK values are summarized in Table 1, and the fits with these parameters are shown in Figure 3. Several observations on the effect of the mutations on the redox potential of the ISP are noteworthy:

1. Neither \( pK_1 \) nor \( pK_2 \) was markedly altered by replacement with either Phe or His. In contrast, the Y156W mutation introduced an increase in the value of \( pK_1 \), which was shifted by \( \sim 1 \) pH unit to 8.5 ± 0.1. The data suggest that, if a second pK is assumed, its value might have been altered in both Y156W and Y156L mutants. However, we should note that the pH dependencies for all strains could be fit quite well with a single pK and, for these two mutants, could be fit better with only one pK value of 8.5 ± 0.1 or 7.7 ± 0.1, respectively. When fitted with two pK values, the second pK had to be fixed at higher than 11 in order to obtain the same quality of the fit and thus was beyond the range of the titration data.

Electron Transfer Properties of the bc1 Complex. Modifications in the midpoint potential of the ISP have been shown to alter the activity of the bc1 complex (18–20, 34). This is consistent with the hypothesis that the quinol oxidation by the ISP contains the rate-limiting partial reaction (13, 35). The flash-induced kinetics of quinol oxidation in the four mutants and the WT, measured at pH 7.5 through cyt \( b_{h/t} \) reduction in the presence of the Qo site inhibitor antimycin, are shown in Figure 4A. The decrease in the \( E_m \) of the ISP in mutant strains was associated with a decrease in the rate of quinol oxidation, but the correlation was clearly not linear. Indeed, a decrease of 60 mV (Y156F and Y156L) led to an \( \sim 1.7 \)-fold decrease in the rate constant whereas an additional 40 mV drop in mutant Y156W induced a 14-fold loss of activity (96 s\(^{-1}\) as compared to 1330 s\(^{-1}\) for the WT). Also noteworthy is the longer lag before onset of the maximal rate seen in the Y156W strain; a similar lag is seen in wild type at lower pH (7). Since the pK value of the ISP in this

![Figure 2: EPR spectra of the [2Fe-2S] cluster of the ISP from WT and mutant strains. (A) Purified bc1 complexes (40 μM), resuspended in 50 mM MOPS, pH 7.0, 100 mM KCl, 0.01% (w/v) dodecyl maltoside, and 15 μg/mL phosphatidylcholine, were reduced with 1 mM sodium ascorbate for 5 min before freezing. (B) Same conditions as in (A) but in the presence of 60 μM stigmatellin. The dashed line shows the position of the \( g_x \) value in the WT.](image)
mutant is also modified, and this could contribute to the rate constant by decreasing the concentration of deprotonated ISPox, we have extended our study to include the protonation state of the ISP by following the kinetics of quinol oxidation at different pHs. The pH dependencies of the quinol oxidation in the mutant strains and the WT are shown in Figure 4B. The kinetics of cyt bH reduction in the presence of antimycin were measured at different pH values at 25 °C. In all experiments, the initial relative concentration of ubiquinol was set to ~30% of the whole quinone pool by adjusting the redox potential of the reaction mixture. It has previously been shown that, with this relative amount of quinol, the rate of cyt bH reduction approaches a maximum and reflects the rate-limiting step, which corresponds to the oxidation of a quinol at the Qo site (4, 26). When the concentration of quinol was adjusted in this way, cyt bL and cyt bH were both oxidized prior to the flash over the pH range 5.5–8.0, and a saturating flash introduced the same concentration of oxidized cyt c2 to the complex, so that the initial concentrations of the reactants of the overall reaction were the same immediately after excitation at each pH. In this pH range we observed that the rate of quinol oxidation in the mutants Y156H, Y156F, and Y156L was strongly pH dependent, increasing up to a maximum at pH ~7.5, as previously observed in the WT (Figure 4B and ref 7). It should be noted that the amplitude of the absorbance change due to heme bH was similar in all strains when measured over an appropriate time scale. Interestingly, mutant Y156W showed a different pH dependence of the rate. There was practically no dependence on pH over the range 5.5–7.5, but a substantial increase between pH 7.5 and pH 8.0, suggesting that the maximum rate could be out of the pH range of these experiments (Figure 4B, open diamonds). The kinetics of quinol oxidation in this mutant in the alkaline region were measured using the electrogenic reactions of the bc1 complex. Since the midpoint potential of cyt bH is pH independent above the pK at ~7.8, while the quinone pool continues to show a pH dependence up to much higher pH, for experiments above this pH, redox conditions that provide the same initial concentration of quinol will also poise cyt bH partly in its reduced form prior to the flash. Measurement of the initial rate of reduction of cyt bH could not therefore be used to estimate the rate of quinol oxidation at pH >8.5. The electrogenic events accompanying the uninhibited turnover of the complex can be assayed through the slow phase of the carotenoid electrochromic band shift at 503 nm (4, 7, 27). The initial rate of the slow phase is determined by the rate-limiting step of the overall reaction, which is that of the Qo site, and is therefore an indirect measure of the rate of quinol oxidation (2, 4, 7, 26, 27). Using these two alternative approaches, Hong et al. (7) were recently able to measure the activation barrier for this reaction as a function of pH, thus confirming the complementarity of the two methods. The pH dependence of the rate of the slow phase in the Y156W mutant confirms the increase in the pH range 7.5–8.0 observed by the cyt b reduction (Figure 4B). The maximal rate is attained only at pH ~8.9, which is significantly higher than the optimal pH for the WT and could reflect the shift of the pK value of ISPox in this mutant (see Discussion).

**DISCUSSION**

All mutations so far reported to change the redox potential of the ISP are within 7 Å of the center of the cluster. From the four substitutions of Tyr 156 reported in the present work, we found that three of them, Tyr to Leu, Phe, or Trp, decreased the midpoint potential of the cluster as summarized in Table 1. On the basis of sequence alignment, residue Tyr 156 from the *Rb. sphaeroides* ISP corresponds to residue Tyr 165 in the sequence of the beef mitochondrial ISP and to Tyr 132 in the ISP of spinach chloroplast. In the crystal structures of the proteins from both organisms, the equivalent Tyr is one of those lying close to the metal cluster (16, 17). Moreover, the hydroxyl group of the side chain was found
FIGURE 4: Kinetics of electron transfer in the mutant strains. (A) Flash-induced kinetics of cyt b reduction at pH 7.5. Chromatophores were resuspended in 50 mM MOPS (pH 7.5), 100 mM KCl, and redox mediators as indicated in Experimental Procedures; 5 μM valinomycin, 2 μg/mL gramicidin, and 5 μM antimycin were also present. The ambient potential was poised at 70 mV at 25 °C and redox mediators as indicated in Experimental Procedures; 5 μM valinomycin, 2 μg/mL gramicidin, and 5 μM antimycin were also present. The ambient potential was poised at 70 mV at 25 °C.

(B) pH dependence of the rate of quinol oxidation in the WT (○), Y156H (△), Y156F (△), Y156L (γ), and Y156W (●) mutant strains. Open symbols: rate determined from cyt b reduction in the presence of 5 μM antimycin. Closed symbols: rate determined from the slow myxothiazole-sensitive phase of the kinetics of the carotenoid band shift. (C) Dependence of the rate of quinol oxidation on E₅₀ of ISP. The rates determined from cyt b reduction at pH 7 for all mutant strains are shown as open symbols; the rate for Y156W at pH 8 is added as a closed symbol. The E₅₀ values at a specific pH were taken from the simulation curves of Figure 3. The standard deviation at each rate is shown with bars. Slopes are shown for the best fit using either data point for the Y156W strain. The dashed line shows the slope of 0.016 mV⁻¹, expected from a Marcus treatment of the model discussed in ref 7. The curvature expected from a Marcus curve is not appreciable over this range of ΔE.

The EPR spectra of the ISP in the mutants indicate that the Phe, Leu, and His substitutions introduce rather small structural perturbations. Indeed, the g₁ band, which reflects the interactions of the ISP with either the quinone or stigmatellin occupant of the Q₁ site (Figure 2), and the CD spectra (not shown) measured in these mutants were identical to those of the WT. The change in midpoint potential of the ISP in Y156L and Y156F could well reflect the inability of Leu and Phe side chains to participate in hydrogen bonding. In both mutant strains, we observed a decrease of the ISP midpoint potential to ~250 mV. Similar results were obtained in yeast and P. denitrificans where the substitution of Tyr to Phe showed a decrease in the midpoint potential of the ISP by 65 and 44 mV, respectively (19, 20). Interestingly, the midpoint potential of the ISP was not altered by the substitution to His. This result might seem surprising since in the model structure the mobile hydrogen from the imidazole ring appears to be too far away from Cys 129, the sulphydryl ligand, to allow the formation of an H-bond.

However, it should be noted that the hydrogen bond network around the metal center is not as rigid as originally indicated (19); in the structures of the ISP reported by Iwata et al. (10) for the bovine bc₁ complex in P₆₆ and P₆₆:22 crystal forms, the distance between the hydroxyl group of Tyr 165 and the sulphydryl liganding group of Cys 139 (bovine numbering) is different. The distance increased to 4 Å when the ISP was close to cyt b (Int position, found in one monomer in P₆₆ crystals) as compared to 2.9 Å, when the ISP was close to cyt c₁ (in P₆₆:22 crystals) or in the soluble fragment (16). Thus, the Tyr is too far away from the Cys in the Int position to form a hydrogen bond. Interestingly, Iwata et al. (10) observed an alternative H-bond network between the cluster binding and the base region in the Int conformation as compared to the cyt c₁ conformation or the structure of the soluble fragment. We could interpret these differences in several ways. It seems possible that even if the His substituent does not allow the same H-bonding pattern as the native structure, its imidazole ring might still allow participation in a H-bond network, either directly or through a bridging H₂O molecule. Alternatively, it may be that formation of a H-bond is not the critical factor in determining the redox potential but that polarity, contributed either by the imidazole ring or by the tyrosine –OH, is important.

For both the leucine and histidine mutants, our results contrast strongly with those obtained with the equivalent substitutions in yeast, where it was reported that both...
mutations prevented the accumulation of the ISP and thus impaired the activity of the bc$_1$ complex. Additionally, although the Y156W mutant protein was accumulated in both the yeast and bacterial systems, the yeast protein was reported to be less stable. Since none of our mutants showed any indication of an unstable ISP subunit, we have been able to better characterize these strains, taking advantage of the stability in Rb. sphaeroides. When estimated from the ratio RC/bc$_1$ complex, determined from flash-induced kinetics, the amount of functional bc$_1$ complex in our mutant strains appeared similar to that of the WT (not shown), and the amplitudes of the kinetic traces for all cytochromes, and EPR spectra, were similar. Moreover, the subunit composition and purification parameters for the mutant bc$_1$ complexes were similar to those of the WT, leading in every case to a purified bc$_1$ complex in which the four subunits were present with a similar stoichiometric ratio (Figure 1). Taken together, these results argue against the presence of partially assembled bc$_1$ subcomplexes that lack the ISP protein in any of our mutant strains.

In contrast to the other substitutions, the EPR spectra of the ISP in the Y156W mutant appeared substantially modified as compared to that of the WT, although the strain retained the characteristic Rieske-type signal and its sensitivity to stigmatellin or interaction with quinone (Figure 2). When the Trp substitution was modeled in the structure of the soluble ISP, the side chain, even in the optimal position, formed close contacts with neighboring residues. Interestingly, the best rotamer of the Trp showed only two close contacts, both with Ser 163 (bovine numbering), a hydrogen bond donor to the [2Fe-2S] cluster. Mutations of Ser 163 have previously been shown to decrease the midpoint potential of the ISP by 130 mV (19). It is therefore possible that the structural rearrangements needed to accommodate the Trp residue in the Y156W mutant also perturbed the hydrogen bonding of Ser 154 (Rb. sphaeroides numbering) and led to the substantially lower midpoint potential of Y156W as compared to Y156F and Y156L. Molecular modeling of the latter two substitutions showed no close contacts with the neighboring residues.

The pH dependence of the ISP redox potential, first observed in chromatophores from Rb. sphaeroides and mitochondrial membranes by Prince and Dutton (33), was fitted with a $pK_a$ value of 8.0 for the oxidized ISP. A similar pH dependence of the ISP redox potential has been reported in numerous other systems (12, 32, 33, 36, 41–47), although some authors have shown that the pH dependence of the ISP redox potential could also be fitted with two $pK_a$ values. Attribution of the $pK_a$ on the oxidized form to dissociation of a histidine comes from detailed spectroscopic studies. Changes in the Mossbauer spectrum of the ISP with pH (36) were interpreted as showing that one of the histidine ligands was responsible for the pH dependence of the $E_m$ of the Rieske ISP. This was later supported by resonance Raman studies of the Rieske-type ISP from Thermus thermophilus, in which the spectral transitions sensitive to pH were identified as Fe(III)–N(His) stretching motions, showing that between pH 7.3 and pH 10.1 the imidazole undergoes deprotonation (37). The structures of the bovine and chicken mitochondrial bc$_1$ complexes showed that His 161 (bovine numbering) forms a hydrogen bond with the propionate of the heme c in cyt c$_1$ (10, 15) or stigmatellin bound in the Q$_o$ site (15) depending on the conformational state of the complex. The identification of His 161 as the residue controlling the pH dependence of the $E_m$ of the ISP is generally well accepted (12, 16, 32, 35). The value of $pK_1$ was altered substantially in the Y156W mutant, showing an increase of almost 1 pH unit. As $pK_1$ was only slightly or not altered by the Phe, His, or Leu substitutions, the change in the $pK_1$ observed in the Y156W mutant could not be attributed simply to the replacement of Tyr 156, or loss of the H-bond, and would have to reflect more general structural perturbations in the overall cluster binding region, perhaps through the steric interaction with Ser 154, as discussed above.

The decrease of the $E_m$ value of the ISP in the mutant strains led to a decrease in the activity of the bc$_1$ complex (Figure 4C). Such a correlation was already observed in mutants of yeast (19, 38), Rhodobacter (34, 39), and P. denitrificans (20). In most cases it is difficult to correlate
directly the change in the $E_m$ with the decrease in activity of the complex since the mutation may introduce additional perturbations that can obscure the effect of the $E_m$ change. For example, a decrease of 20 or 104 mV in the $E_m$ value in mutants of *Rh. capsulatus* ISP where Leu 136 (L142 bovine numbering) was changed respectively to either His or Gly led to an identical decrease (96%) in the activity of the $bc_1$ complex which was attributed to a perturbed docking interface between the ISP and cyt b in the region of the Q$_o$ site (39). Even so, studies of ISP mutants showing a shifted $E_m$ value can provide useful insights about the mechanism of quinol oxidation. The $E_m$ value determines the driving force for the electron transfer reaction (through the contribution to $\Delta G^\circ$) and also the occupancy of the intermediate semiquinone state. The activation barrier for the overall two-electron reaction of quinol oxidation was shown to be $\sim 45-65$ kJ mol$^{-1}$ (7, 40), but the location of the activated step has been controversial. Crofts and Wang (4) suggested that this would be in the first electron transfer and had speculated that the resulting semiquinone might represent the transition state. Kim et al. (51) have suggested that the two electron transfer reactions occur “simultaneously”, implying a similar mechanism. In either case, assuming that the $E_m$ of the QH$_2$/SQ couple was unaffected by mutation, the value for $\Delta G^\circ$ would be modified in direct proportion to the change in $E_m$ of the ISP. If this were so, then, from a simplistic Arrhenius approach, a slope closer to $FRT$ (or 0.039 mV$^{-1}$) would be expected in the plot of Figure 4C. The weaker slope observed makes these mechanisms seem unlikely. Two of the slopes in Figure 4C are at least squares linear fits to the data, one obtained using the data point for the Y156W mutant measured at pH 7.0 (with slope 0.0179 mV$^{-1}$) and the other (0.011 mV$^{-1}$) using the rate measured at pH 8.0, to allow for the lower concentration of the dissociated form of ISP$^{\text{ox}}$ expected from the increased pK$_1$ in this strain. Both slopes are in the range expected if the overall rate was determined by the rate of the first electron transfer, using appropriate parameters from Marcus theory ($\sim 0.011-0.016$ mV$^{-1}$, depending on model and assumptions; 7, 48–50), as shown by the third slope (dashed line). A more detailed discussion in terms of Marcus theory for electron transfer is given in a separate paper (7). Additional ambiguity in interpreting the effect of the pK change is introduced by the possibility that the driving forces for electron transfer and proton transfer might contribute separately in determining the rate (50; see below). Because the driving force for proton transfer is determined by the pK values of the groups contributing to the H-bond through which transfer occurs, the change in pK of the bridging histidine would be expected to change this contribution.

Since the pH profile for rate of reaction was shifted up to reflect the higher pK, our results with the Y156W mutant provide support for the suggestion that the enzyme–substrate complex involves binding of the dissociated oxidized ISP and that the ISP acts as a hydrogen carrier in the $bc_1$ complex (7, 12, 13, 35, 40). A possible role of the ISP as a proton carrier in $b_{cf}$ complexes has been discussed by Carrell et al. (17) in the context of a buried water channel in cyt f (52) and a possible role in H$^+$ transfer from the Q$_o$ site. It is therefore of interest to consider if a mechanism similar to that proposed by Ugulava and Crofts (12) might also control the rate of electron flow in the chloroplast $b_{cf}$ complex. In chloroplasts, the ΔpH component of the proton gradient is thought to contribute a larger fraction and to play an important role in regulation. Some discrepancy exists between values for the pK on the oxidized form of the chloroplast ISP. Earlier studies of the pH dependence of the redox potential in the chloroplast ISP indicated a pK value similar to that found for mitochondria (41), but more recently, Zhang et al. (42) found a lower pK value of 6.5 for the isolated chloroplast ISP fragment. This latter value is more in line with the pH dependence of the rate of plastoquinol oxidation by the $b_{cf}$ complex. Hope et al. (53) showed a pK$_f$ of 6.1 for quinol oxidation. Similar results for the rate of cyt f recombination in intact chloroplasts, which reached a maximum at pH 6.5, were reported by Nishio and Witmarsh (54). From these results we would suggest that the rate of plastoquinol oxidation in chloroplasts might also be determined by the availability of the dissociated oxidized ISP for formation of the ES complex.

This is the first report of a pK shift induced by mutagenesis in the Rieske protein and has profound implications not only for the mechanism of the $bc_1$ complex but also for the family of related proteins in which a similar 2Fe-2S cluster is found, but with different redox properties. For the mechanism of quinol oxidation, it seems possible that the first electron transfer is proton coupled and occurs through an ETPT mechanism [as defined by Cukier and Nocera (50)]. The slow rate for coupled electron and proton transfer through the short distance of a bridging H-bond and the high value of λ observed experimentally (7) are diagnostic of such a reaction. If so, this represents the first well-characterized reaction of this type in a biochemical system. In the theoretical treatment these authors suggest (50), the contributions from proton transfer and electron transfer to the driving force for the reaction, appearing in the Franck–Condon term of the Marcus equation, are separated. The former (proton transfer) contribution can be determined from the pK values of the groups contributing to the H-bond through which transfer occurs. The determination of the pK value for ISP$^{\text{ox}}$ and its change in a mutant strain therefore represent the first step toward a deeper understanding of the mechanism, since the approach opens the possibility of studying the effect on reaction rate of a change in the driving force for the proton transfer. In the absence of additional data points, a more complete treatment in the present paper seems premature. However, we anticipate that strains with different pK values might be obtained through additional mutagenesis and look forward to a more complete treatment in that context.

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