

CD-monitored redox titration of the Rieske Fe-S protein of *Rhodobacter sphaeroides*: pH dependence of the midpoint potential in isolated bc_1 complex and in membranes

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Abstract The redox potential of the Rieske Fe-S protein has been investigated using circular dichroism (CD)-spectroscopy. The CD features characteristic of the purified bc_1 complex and membranes of *Rhodobacter sphaeroides* were found in the region between 450 and 550 nm. The difference between reduced and oxidized CD-spectra shows a negative band at about 500 nm with a half of width 30 nm that corresponds to the specific dichroic absorption of the reduced Rieske protein (Fee, J.A. et al. (1984) J. Biol. Chem. 259, 124–133; Degli Esposti, M. et al. (1987) Biochem. J. 241, 285–290; Rich, P.R. and Wiggins, T.E. (1992) Biochem. Soc. Trans. 20, 241S). It was found that the redox potential at pH 7.0 for the Rieske center in the isolated bc_1 complex and in chromatophore membranes from the R-26 strain of *Rb. sphaeroides* is 300 ± 5 mV. In chromatophores from the BC17C strain of *Rb. sphaeroides*, the E_m value measured for the Rieske iron-sulfur protein (ISP) was higher (315 ± 5 mV), but the presence of carotenoids made measurement less accurate. The E_m varied with pH in the range above pH 7, and the pH dependence was well fit either by one pK at ~ 7.5 in the range of titration, or by two pK values, $pK_1 = 7.6$ and $pK_2 = 9.8$. Similar titrations and pK values were found for the Rieske Fe-S protein in the isolated bc_1 complex and membranes from the R-26 strain of *Rb. sphaeroides*. The results are discussed in the context of the mechanism of quinol oxidation by the bc_1 complex, and the role of the iron sulfur protein in formation of a reaction complex at the Q_o -site.

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Key words: *Rhodobacter sphaeroides*; Iron-sulfur protein; Rieske; Midpoint potential; Circular dichroism spectroscopy

1. Introduction

The Rieske iron-sulfur protein (ISP) is an essential subunit of the cytochrome (cyt) bc_1 and b_6f complexes. Each Rieske protein contains a high potential [2Fe-2S] cluster, which has two cysteine and two histidine residues as ligands [4–6]. The Rieske center, characterized by an electron paramagnetic resonance spectroscopic (EPR) signal at $g = 1.90$ when in the reduced form, is a widely distributed component of electron transfer systems [7]. Most information on the Rieske center has been derived from low-temperature EPR measurements [8–10]. In contrast to the cytochromes, the Rieske protein possesses a weak and poorly resolved optical spectrum, which is completely obscured by the intense absorption bands of the cytochromes throughout the spectrum of the bc_1 complex

[10,11]. However, the reduction of the center is accompanied by the formation of the broad negative band at about 500 nm in the circular dichroism (CD) spectrum [2,3]. Among methods available for studying Rieske protein, CD-spectroscopy is one of the most direct. Several groups have previously described the CD features of the ISP either in the isolated water-soluble fragment, or in isolated bacterial and mitochondrial bc_1 complexes [1,12–16], but little work has previously been reported for the iron-sulfur protein in situ in the native membrane-bound complex.

In the present work we demonstrated that the redox properties of the ISP in membranes of *Rhodobacter sphaeroides* (R-26 and BC17C strains) can be measured by CD-spectroscopy, and show that they are the same as those of the ISP in an isolated bc_1 complex. We also show that the properties of the *Rb. sphaeroides* complex are similar to those of the ISP of mitochondrial complexes, both with respect to midpoint potential, and pH dependence, with two pK values on the oxidized form. We discuss the results in the context of the structure of the complex, and a hypothesis for the role of the ISP in which the pH dependence of electron transfer in the acidic range reflects a requirement for the dissociated form of the ISP for formation of the reaction complex from which quinol oxidation proceeds. The group in the ISP with the lower pK is tentatively identified as His-153 (*His-161* in the beef subunit).

This technique is appropriate for titrating redox potentials of the ISP from mutant strains under native conditions without the need to purify the bc_1 complex or use low-temperature EPR studies.

2. Materials and methods

The carotenoid-deficient R-26 strain of *Rb. sphaeroides* was grown photosynthetically. The BC17C strain, which is a derivative of the carotenoid-containing *Rb. sphaeroides* Ga strain with the bc_1 complex expressed in trans, was used as a control for experiments with mutant strains generated in this background. Cells were grown in the dark under vigorous aeration to minimize carotenoid biosynthesis [17].

2.1. Chromatophore preparation

Chromatophores were prepared after mechanical disruption in a French press, using differential centrifugation, as previously described [18]. Chromatophores were suspended in 100 mM KCl, 50 mM Mops buffer, pH 7.0. Membranes from the BC17C strain were prepared by the same procedure.

2.2. Isolation of the bc_1 complex

The histidine tagged bc_1 complex was purified as described in Guerova-Kuras et al., 'Expression and 1-step purification of a fully active poly-histidine tagged bc_1 complex from *Rb. sphaeroides*' (submitted to FEBS Letters).

2.3. Experimental conditions

The buffer used for measurements was 50 mM potassium phos-

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Abbreviations: ISP, Rieske iron-sulfur protein; EPR, electron paramagnetic resonance; CD, circular dichroism; cyt, cytochrome

phate, at pH 7.4 unless otherwise indicated, containing 1 mM EDTA, 0.8 M sucrose (and 0.01% dodecyl maltoside for the isolated bc_1 complex). The redox potential of the suspension was controlled by addition of small aliquots of potassium ferricyanide ($K_3Fe(CN)_6$) (for oxidation), or sodium dithionite (for reduction). Redox mediators were used at the following concentrations: 2 μ M *p*-diphenylamine-sulfonic acid (E_m 400 mV), 2 μ M *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (E_m 380 mV), 2 μ M ferrocene acetic acid (E_m 365 mV), 2 μ M 1,1'-dimethylferrocene (E_m 341 mV), 10 μ M *p*-benzoquinone (E_m 280 mV), 2 μ M TMPD (E_m 275 mV), 2 μ M DAD (E_m 245 mV), 6 μ M 2,5-dihydroxy-*p*-benzoquinone (E_m 180 mV), 6 μ M 1,2-naphthoquinone (E_m 180 mV), 2 μ M pyocyanine (E_m 80 mV). Gramicidin (1 μ M), valinomycin (1 μ M), and nigericin (1 μ M) were also added to ensure equilibration of pH across the membrane for work with the complex in situ. Redox titrations were performed in cylindrical glass cuvettes modified for redox titration under anaerobic conditions (see Fig. 1). The spectrometer was equipped with a compact induction-driven magnetic stirrer designed in-house. All titrations were performed at room temperature ($21 \pm 1^\circ\text{C}$).

2.4. CD-spectroscopy

CD-spectra were recorded using a JASCO J-720 spectropolarimeter. Experimental conditions used were as follows: bandwidth, 2 nm; scan speed, 50 nm/min; resolution, 1 nm; accumulation, 1–10 traces for averaging; path length, 1 cm; room temperature (20 – 22°C). The response time of the apparatus was varied to optimize sensitivity and spectral resolution, in the range between 0.5 and 8 s. Most experiments were performed with a response between 4 and 8 s, in order to minimize experimental time in redox titrations. Control experiments showed that no significant loss of spectral resolution resulted from the longer response time (Fig. 2), but a much larger number of traces for averaging was required to give a similar sensitivity at faster response times.

3. Results

The CD-spectra of oxidized and reduced bc_1 complex isolated from *Rb. sphaeroides* in the region between 450 and 550 nm are shown in Fig. 2A. The differential CD-spectrum shows the change on reduction of the ISP [2,3].

As can be seen from Fig. 2B, the difference between reduced and oxidized CD-spectra of R-26 chromatophores has the same features as for isolated bc_1 complex (λ_{max} at about 500 nm; width at half height, 30 nm). Fig. 2C shows similar spectra from the BC17C strain. The difference between reduced and oxidized CD-spectra for the chromatophores has similar features (λ_{max} is 494 nm, width at half height, 30 nm) as for the purified bc_1 complex and the chromatophores from the R-26 strain, but with some overlapping contributions from re-

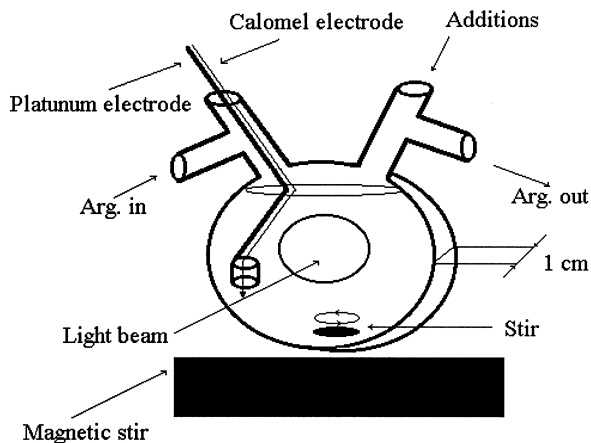


Fig. 1. Schematic representation of the modified cuvette for CD-monitored electrochemical redox titration.

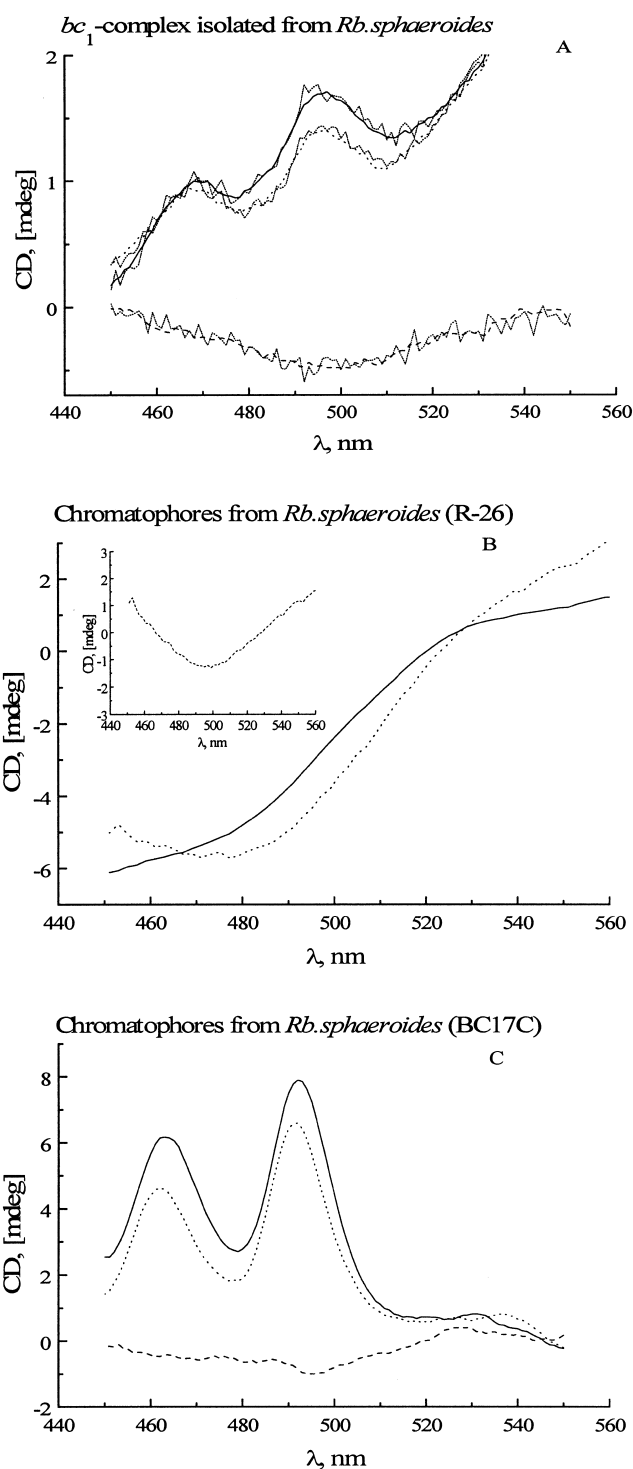


Fig. 2. CD-spectra in the region 450–550 nm of (A) the isolated bc_1 complex, (B) chromatophores from the R-26 strain, and (C) chromatophores from BC17C from *Rb. sphaeroides*. Solid lines: oxidized spectra (taken at E_h 400 mV); dotted lines: reduced spectra (taken at E_h 200 mV); dashed lines: reduced minus oxidized, which correspond to the reduction of the ISP (experimental conditions: bandwidth, 2 nm; scan speed, 50 nm/min; response, 8 s; resolution, 1 nm; accumulation, 1–3). In A, short dashed lines show CD-spectra of isolated bc_1 complex measured bandwidth, 2 nm; scan speed, 50 nm/min; response, 0.5 s; resolution, 1 nm; accumulation, 10. For all difference spectra, λ_{max} was around 500 nm, half-width was 30 nm. Final concentration of bc_1 complex was 2 μ M. Other conditions were as described in Section 2.

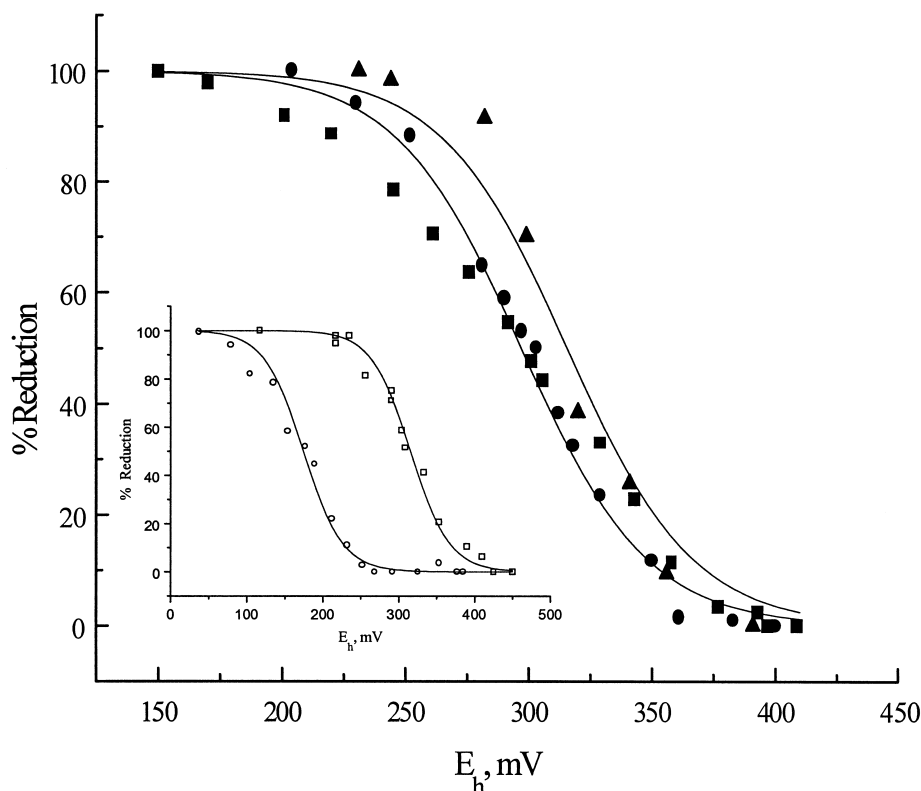


Fig. 3. CD-monitored electrochemical redox titrations of the isolated bc_1 complex of *Rb. sphaeroides* (●), chromatophores of the R-26 strain (■), and membranes from aerobically grown strain BC17C (▲) under pH=7.5. Each point was obtained by subtraction of points from the CD-spectra at 500 and 470 nm. 100% reduction corresponded to a CD change of 0.5–1.5 mdeg., depending on preparation. The data were fitted by a Nernst curve with $n=1$. ●, Isolated bc_1 complex, E_m 300 mV; ■, chromatophores from R-26, E_m 300 mV; ▲, chromatophores from BC17C, E_m 315 mV. Conditions were described in Section 2 and in Fig. 2. Inset: Similar experiments at pH 5.0 (open circles) and 9.0 (open squares), to show the pH dependence of the titrations.

sidual carotenoids. BC17C was grown under dark conditions with vigorous aeration to minimize carotenoid biosynthesis [17], but the contribution from the small amount of residual carotenoid caused an apparent shift of λ_{max} . Nevertheless, the data show that redox characteristics of the ISP in membranes of carotenoid containing *Rb. sphaeroides* strains can be monitored by CD-spectroscopy, despite the strong CD-spectrum of carotenoids in situ, as long as the contribution from these bands is minimized.

The negative band with a λ_{max} about 500 nm has been titrated by redox-potentiometry (Fig. 3). It was found that the redox mid-potential at pH 7.4 for the ISP both in the isolated bc_1 complex and in membranes of the R-26 strain was ~ 300 mV, while in membranes of the BC17C strain of *Rb. sphaeroides* the value obtained was 315 mV. We attribute the slightly higher E_m value to the imprecision resulting from the presence of residual carotenoids. Titrations were made over the pH range between 5.0 and 10.0 (see inset for example titrations).

The pH-dependence of ISP redox potentials is presented in Fig. 4 for the region between pH 5.0 and 10.0. The maximal E_m (315 ± 5 mV) for ISP was seen at low pH (5.0). Above pH 7, increasing the pH caused a decrease of E_m value. The data were well fitted by either one pK or by two pK values, using the relationship previously suggested for the mitochondrial complex [19]. The two pK values found to best fit the titration curves were pK_1 7.6 and pK_2 9.8, with similar values both in the isolated bc_1 complex and the complex in situ. These values

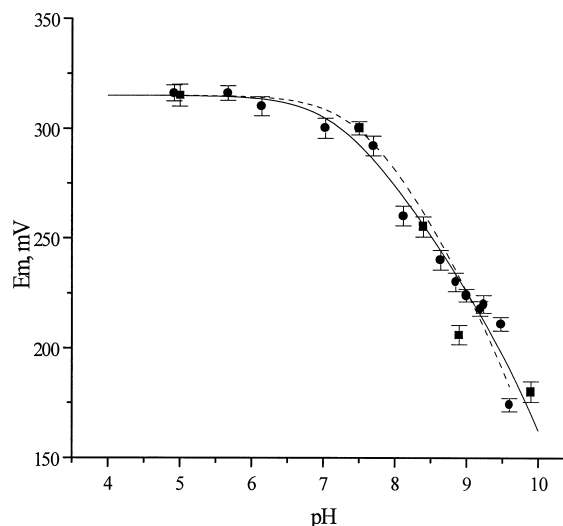


Fig. 4. Dependence on pH of redox potential for the ISP in isolated bc_1 complex (●) and in membranes of the R-26 strain (■) of *Rb. sphaeroides*. The dashed line drawn through the points is a theoretical curve derived using the equation suggested by Link [25] with values for E_m (low pH) 315 mV; $pK_1 = 7.6 \pm 0.05$; $pK_2 = 9.8 \pm 0.25$ ($\chi^2 = 74$). The solid line is for a theoretical curve using a single pK of 7.5 ± 0.045 ($\chi^2 = 56$).

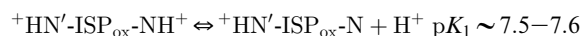
were close to those previously obtained for the water-soluble fragment of the Rieske protein from beef heart mitochondria [19]. If one pK was used, a value of 7.5 gave the best fit. The quality of the data does not allow us to distinguish between these fits; however, we can exclude a fit by two dissociable groups with pK values closer than 2 pH units in the range above 7.5.

4. Discussion

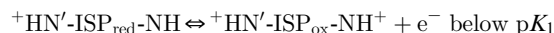
In this work we have demonstrated that the redox properties of the ISP of the bc_1 complex, both in situ in membranes from *Rb. sphaeroides* and in the isolated His-tagged complex, can be readily measured by CD-spectroscopy. The modified cuvette for CD-monitored electrochemical redox titration makes it possible to investigate the thermodynamic features of different proteins under physiological conditions in the native system or in mutant strains, without the need to purify the bc_1 complex or use low-temperature EPR studies.

The results for E_m and pK values of the ISP show titrations similar to those obtained previously for the Rieske protein from mitochondrial complexes from different organisms under different conditions [14,19,20]. Following the arguments of Prince and Dutton [7] and Link et al. [14,19], the $pK(s)$ can be attributed to dissociable group(s) on the oxidized form of the ISP (ISP_{ox}), with the following reactions (assuming two dissociable groups [19]):

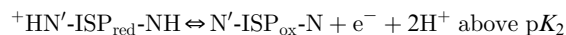
Dissociation:



Redox:



between pK_1 and pK_2



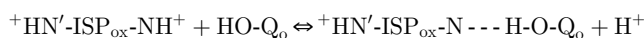
For the dissociable group in the neutral range, our titrations show a somewhat lower pK than that previously determined for the pH dependence of the E_m values of the ISP in *Rb. sphaeroides* by EPR, in which the data were fitted by a single pK of 8.0 on the oxidized form of the protein [7].

Sequence similarity and homology modeling of the *Rb. sphaeroides* ISP subunit suggest that the structure is similar to that of the beef enzyme (Guergova-Kuras, M. and Crofts, A.R., unpublished). In the beef ISP subunit [22], dissociable groups within 10.0 Å of the 2Fe2S center are the side chains of the two histidine ligands (His-132 (*His-141*) (beef numbering in italics) and His-153 (*His-161*)). Other nearby dissociable groups are His-156 (*His-164*) (11.02 Å from Fe2) and Lys-165 (*Lys-173*) (14.2 Å from Fe2), conserved between mitochondria and *Rb. sphaeroides*, are partly compensated by neighboring Asp-144 (*Asp-152*). In the beef enzyme, the dissociable group of *Arg-118* is within 11.0 Å, but is not conserved. As suggested by Link et al. [19], the two liganding histidines are most likely the groups determining the pK values that respond

to redox change. Since our titrations both in the isolated complex and in the complex in situ are similar to those found in the isolated ISP extrinsic domain fragment [14], it is unlikely that groups from the other subunits affect the pK values under the conditions of the titration. Since the value of pK_2 lies at the high pH end of the titration, and the data were well fit by a single dissociable group, we cannot assign a value to the second pK with any confidence, and it could lie outside the titration range.

Zhang et al. [21] have recently shown from crystallographic evidence that the ISP occurs in several different positions in the bc_1 complex from chicken and bovine heart mitochondria, and have suggested that the different conformations indicate that the extrinsic domain moves during catalysis between docking domains on cyt c_1 and cyt b . There are potential H-bonding partners at both docking sites.

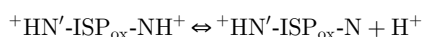
In the presence of stigmatellin [21], *His-161* of the ISP forms a contact with the inhibitor bound in the Q_o -site, which Zhang et al. modeled as a H-bond ligand between the Ne of *His-161* and carbonyl and hydroxy O-atoms of the inhibitor. Such a complex had been previously suggested by Iwata et al. [22] on the basis of evidence for formation of a complex between the ISP and such inhibitors, and the exposed position of *His-161*. A similar configuration is found in the beef complex containing stigmatellin or UHDBT [23]. The stigmatellin and UHDBT complexes likely mimic the reaction complex formed between ubiquinone (quinol) and the oxidized ISP [24,25]. Formation of such a reaction complex would involve a similar H-bond between the -OH of the quinol ring and the Ne of *His-161*. In this complex, the Ne would act as a H-bond acceptor, requiring that, below pK_1 , the associated H be displaced by the H of the quinol -OH:



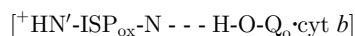
where H-O- Q_o represents the -OH of quinol bound at the Q_o -site, and $\cdots H-O-Q_o$ represents the H-bond between ISP_{ox} and the quinol -OH.

Brandt and colleagues [26,27] have shown in beef and yeast bc_1 complexes that the rate of quinol oxidation is markedly pH dependent. They have argued that the pH dependence reflects dissociation of QH_2 to QH^- (with $pK \sim 11.3$) as a prerequisite for electron transfer, as previously suggested by Rich [28], and noted that quinol oxidation showed a pH-dependent activation energy expected of a reaction involving proton release against this high pK . The rate of electron transfer through the Q_o -site on flash activation of chromatophores from *Rb. sphaeroides* also shows a strong pH dependence in the range 5.5–7.0, but with no marked pH dependence of activation energy [29]. We suggest, as an alternative to the Brandt-Rich hypothesis, that the pH dependence of the rate over the acidic range reflects the concentration of the dissociated *His-161*, determined by pK_1 , and that the electron transfer rate depends on the probability of formation of the reaction complex between bound QH_2 and ISP_{ox} in the dissociated form. At neutral pH, dissociation of the histidine ($pK \sim 7.5$) would provide a reaction pathway more probable than dissociation of QH_2 ($pK \sim 11.3$) by a factor of $\sim 10^{3.8}$.

1. Dissociation of *His-161*



2. Formation of the reaction complex with bound quinol:



where the reaction complex is indicated by square brackets. It seems likely that above pK_1 , the concentration of the dissociated form is not rate limiting, but becomes increasingly limiting as the pH is lowered below pK_1 . From the structure [21], His-153 (*His-161*) would form the bridging H-bond, and we can tentatively attribute pK_1 to this residue. On dissociation of the reaction complex to products, the ISP would carry an electron and proton to cyt c_1 , acting as a H-carrier, rather than the electron carrier assumed in most models.

At the cyt c_1 interface, *His-161* of the ISP in P6522 crystal forms of the beef bc_1 complex is close enough to one of the propionate side-chains of heme c_1 to form a H-bond or an ionic interaction, and it seems likely that this is the configuration in which electron transfer to cyt c_1 occurs [21,30]. The strength of the bond would likely depend on the redox state of both centers, and on the dissociation state of *His-161* and the propionate. Assignment of values to the bond energies would be speculative without more information, but it seems possible that binding of the ISP at this reaction interface might be modulated by the redox status of the complex, and by pH, and that this will need to be taken into account in future discussions of the movement of the ISP. At pH above pK_1 , release of a proton to the aqueous phase would accompany oxidation of ISP_{red} .

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