Ion Pair Formation between Basic Residues at 144 of the Cyt *b* Polypeptide and the Ubiquinones at the Q_o Site of the Cyt bc_1 Complex†

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ABSTRACT: Loci of spontaneous Q_o site inhibitor resistant mutants in the cyt bc_1 complex of the photosynthetic bacterium *Rhodobacter capsulatus* are M140, F144, G152, G158, and T163 of the cyt *b* polypeptide. In this report, we have studied the effects of arginine (R) substitution at these positions with a view to test for specific interactions with the [2Fe-2S] cluster, cyt b_L with Q_o site ubiquinone (Q), or hydroquinone (QH_2). All the arginine mutants displayed severely or completely impeded photosynthetic growth resulting from dysfunctional cyt bc_1 complexes. The source of dysfunction in G158R and T163R was identified by a >1000-fold decrease in the Q_o site affinity for QH_2 and Q, sufficient to empty the site in the presence of the 30 mM ubiquinone pool of the chromatophore membrane; they appear similar to the class of mutants described in the preceding paper [Ding, H., Moser, C. C., Robertson, D. E., Tokito, M., Daldal, F., & Dutton, P. L (1995) *Biochemistry 34*, 15979−15996]. The source(s) of dysfunction of M140R and G152R is not so apparent since they possess Q_o sites with normal QH_2/Q affinity; they appear to be members to the class of mutants identified and characterized in the following paper [Saribaş, S., Ding, H., Dutton, P. L., & Daldal, F. (1995) *Biochemistry 34*, 16004−16012]. The present paper focuses on the unique affects of F144R. Redox potential and EPR spectral properties of the Q_o site of F144R showed that arginine forms an ion pair with the head group of an anionic ubiquinone, tentatively suggested to be a ubihydroquinone anion (QH^-) in the Q_{os} domain. The redox potential of the putative QH^-/Q couple appears to be raised by at least 220 mV above that observed in the wild-type Q_o site. The apparent p*K* value of $R(H^+)$ in the $R(H^+)-QH^-$ ion pair is 10.1. Lysine substituted at F144 displayed parallel properties to that of F144R; the apparent p*K* value of $K(H^+)$ in the $K(H^+)-QH^-$ ion pair is 9.2. Results indicate that the strong electrostatic interaction between $R(H^+)$ or $K(H^+)$ at position 144 and the QH^- in the Q_o site is able to compensate for the opposing tendency of the neutral forms of R and K at position 144 to weaken the binding affinity of the Q_o site for Q or QH_2 by >1000-fold. In contrast, F144 substituted with the normally unprotonated histidine (F144H) neither forms an ion pair with the Q_o site occupant, at least down to neutral pH values nor measurably decreases the affinity.

The ubihydroquinon−cytochrome *c* oxidoreductase (cyt bc_1 complex)[1] accommodates two distinct ubiquinone/ubihydroquinone (Q/QH_2) binding sites, designated the Q_o and Q_i sites respectively (Mitchell, 1976). The Q_o site is flanked by the [2Fe-2S] cluster and the cyt b_L heme, which cooperate to oxidize QH_2 and initiate the energy conversion reactions of the cyt bc_1 complex. Progress toward understanding the structure and reactions of the Q_o site are detailed in Ding et al. (1992) and the accompanying paper (Ding et al., 1995). This recent work has provided evidence that the Q_o site contains two ubiquinone molecules in two contiguous domains; one is strongly bound (Q_{os} domain) and the other weakly bound (Q_{ow} domain).

Genetic and biochemical approaches have suggested that a major portion of the Q_o site structure of the cyt bc_1 complex is provided by the cyt *b* subunit [Howell & Gilbert, 1988; Daldal et al., 1989; Robertson et al., 1990; He et al., 1994; also see reviews by Brandt and Trumpower (1994) and Gray and Daldal (1995)]. Amino acid residues in the cyt *b* polypeptide identified by their inhibitor resistance as important for the Q_o site QH_2 oxidation are M140, F144, G152, G158 and T163 (Daldal et al., 1989; di Rago et al., 1989; Robertson et al., 1990; Tron et al., 1991). Of these residues, F144 and G158 have been examined extensively by site-directed mutagenesis (Atta-Asafo-Adjei & Daldal, 1991; Tokito & Daldal, 1993; Ding et al., 1995). Substitutions at F144 and G158 with neutral aromatic and aliphatic residues yield a family of Q_o site mutants that differ widely in their binding affinities for QH_2 or Q but retain similar redox

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[1] Abbreviations: cyt, cytochrome; cyt bc_1, ubihydroquinone-cyt c_2 oxidoreductase; E_h, redox potential versus the standard hydrogen electrode; E_{mx}, electrochemical redox midpoint potential at pH X; EPR, electron paramagnetic resonance; cyt b_L, low potential cyt *b* of cyt bc_1; cyt b_H, high potential cyt *b* of cyt bc_1; [2Fe-2S], 2Fe-2S cluster in cyt bc_1 complex; Q, ubiquinone; QH_2, ubihydroquinone; Q_o site, ubihydroquinone oxidizing site; Q_{ow}, ubiquinone weakly binding domain in the Q_o site; Q_{os}, ubiquinone strongly binding domain in the Q_o site; Q_i, ubiquinone reducing site.

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midpoint potentials for the Q/QH_2 couples and share the same k_{cat} for QH_2 oxidation (Ding et al., 1995).

In this report, we have examined F144, G158, M140, G152, and T163 substituted with basic residues, arginine (R), lysine (K), and histidine (H). The idea was to test whether potentially positively charged residues could be shown to modify the redox potentials or spectral properties of the [2Fe-2S] cluster, cyt b_L or ubiquinone associated with the Q_o site.

MATERIALS AND METHODS

Cell and Chromatophore Preparation. Site-directed mutagenesis of the cyt *b* polypeptide of *Rhodobacter capsulatus* was carried out as described in Atta-Asafo-Adjei and Daldal (1991). Wild type and mutants strains were grown under microaerobic conditions (Daldal et al., 1989). Protocols for chromatophore preparation and ubiquinone extraction/reconstitution are described in Ding et al. (1992). Redox poising and titrations were performed following the procedure described in Dutton (1978) employing redox mediators as detailed in Robertson et al. (1990) and Ding et al. (1992).

Q_o Site Occupancy. Q_o site occupancy was determined using the [2Fe-2S] cluster EPR spectrum as described previously (Ding et al., 1992, 1995). The [2Fe-2S] cluster EPR spectra were recorded with a Bruker EPR spectrometer equipped with a temperature-controlled helium cryostat from Oxford Instruments. The measurement conditions were as follows: temperature, 20 K; microwave power, 2.0 mW; microwave frequency, 9.44 GHz; modulation frequency, 100 kHz; modulation amplitude, 12.5 G.

Rate of QH_2 Oxidation in the Q_o Site. The rate of QH_2 oxidation by the cyt bc_1 complex in chromatophores was monitored by the time course of cyt b_H reduction in the presence of the Q_i site inhibitor antimycin after single flash activation. Details of the measurements are described in the accompanying paper (Ding et al., 1995). The flash activated QH_2 oxidation process in the Q_o site was treated as a pseudo-first-order reaction and the kinetic trace was fitted to one exponential plus a constant.

RESULTS

Effects on Growth of the Substitutions M140R, F144R, G152R, G158R, and T163R. Photosynthetic growth, which requires the turnover of the cyt bc_1 complex, was severely impeded in M140R, F144R, and G158R and barely detectable in G152R and T163R. In all cases, the rate of Q_o site catalyzed QH_2 oxidation measured after single flash activation was found to be low compared to that of the wild type: M140R and G152R were 10−20% active, and F144R, G158R, and T163R were less than 2% active.

Ubiquinone Occupancy of the Q_o Site. The Q_o site occupancy of the mutant chromatophores was examined when the Q_{pool} comprised only Q. The [2Fe-2S] cluster EPR spectra of Figure 1 shows three classes of mutants. The first class comprises G158R and T163R characterized by spectra a and b, which are identical to the wild type with the Q_o site depleted of ubiquinone (spectrum c). Clearly, these substitutions severely interfere with the binding of the Q/QH_2 in the Q_o site and lead to Q_{os} and Q_{ow} domains devoid of ubiquinone even in the presence of the prevailing 30 mM Q_{pool} of the chromatophore membrane (Ding et al., 1992, 1995). It has been previously shown that the dissociation constants (K_D values) for Q in the Q_{os} and Q_{ow} domains in

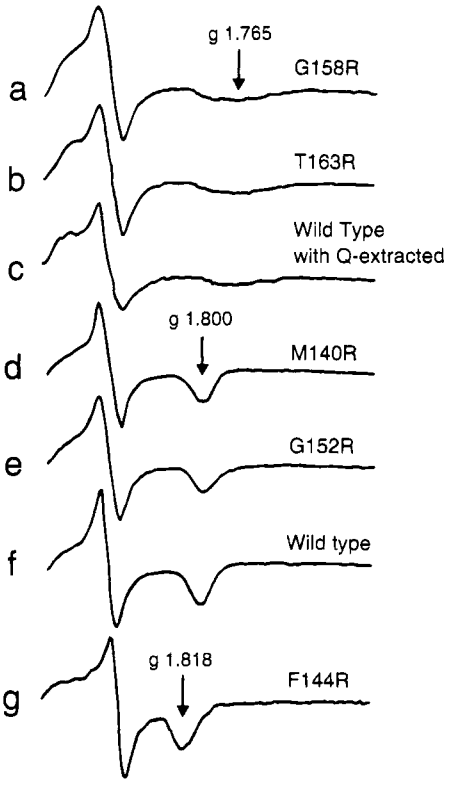


FIGURE 1: Effect of the substitution M140R, F144R, G152R, G158R, and T163R in the cyt *b* polypeptide on the [2Fe-2S] EPR spectrum of cyt bc_1 complex. Chromatophores were suspended in 100 mM KCl and 50 mM MOPS (pH 7.0) to provide a cyt bc_1 complex concentration of 25 μM. The redox potential was poised at 200 ± 10 mV so that the Q_{pool} was comprised entirely of Q.

the wild-type strain are 0.05 and 0.9 mM, respectively (Ding et al., 1992). Thus, it can be concluded that the substitutions G158R and T163R change the K_D values for Q binding in the Q_o site by well over 1000-fold. This class of mutant is well described in Ding et al. (1995).

The second class of mutant are characterized by the spectra d and e of M140R and G152R which are the same as the wild-type strain (spectrum f), indicating that the severe dysfunction caused by these substitutions is not due to weakened ubiquinone binding affinity of the Q_o site. This new class of mutant is identified and described in the following paper (Saribaş et al., 1995).

The third class of Q_o site mutant is established by F144R. Spectrum g shows that F144R displays a significantly narrowed and distorted [2Fe-2S] EPR spectrum, characterized by a g_x at 1.818 and g_y at 1.884, which is clearly the result of a novel interaction with the [2Fe-2S] cluster of the cyt bc_1 complex. Substitution of F144 with basic residues is the subject of the remainder of this report.

Role of Ubiquinone at the Q_o Site in the Interaction between F144R and the [2Fe-2S] Cluster. Figure 2 shows that extraction of ubiquinone from chromatophores of F144R leads to the replacement of the distinct g_x at 1.818 with a broadened signal with a g_x at 1.765. This signal is the same as that found in the wild type with the a ubiquinone depleted Q_o site (see Figure 1). Reconstitution of the extracted chromatophore membranes of F144R with ubiquinone-10 returns the [2Fe-2S] cluster EPR spectrum to the characteristic g_x at 1.818 and g_y at 1.884. These results show that the F144R substitution does not interact with the [2Fe-2S] cluster independently of the ubiquinone occupant in the Q_o

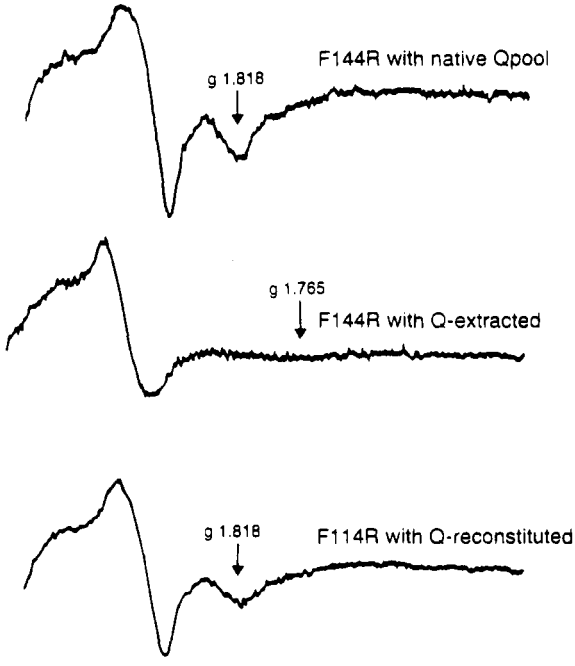


FIGURE 2: Interaction between the [2Fe-2S] cluster, arginine 144, and the ubiquinone occupants in the Q_o site of the mutant F144R. Chromatophores from F144R were examined under the conditions described in Figure 1 except that the cyt bc_1 complex concentration was 20 μM. Lyophilized chromatophores extracted of ubiquinone and reconstituted (ubiquinone 10) using the dry isooctane method, as described in Ding et al. (1992).

site which is clearly an integral part of the interaction.

Comparison with Other Neutral and Basic Residue Substitutions at F144. Figure 3 lists the [2Fe-2S] cluster EPR spectra of various F144 mutants when the Q_{pool} is all Q. The upper part describes a range of Q_o site occupancies caused by substitution with a variety of neutral aromatic and aliphatic residues. Remarkably, all these F144 mutants, even those with a nearly empty Q_o site such as F144L are able to grow photosynthetically (Ding et al., 1995). Below these neutral substitutions are the clear exceptions F144R, F144K, and F144H, which have marginal photosynthetic growth rates. The EPR spectra show that substitution of F144 with another basic residue, F144K, also yields an unusual [2Fe-2S] cluster EPR spectrum with the g_x at 1.791 and g_y at 1.894. On the other hand, substitution with the weak base, F144H, yields a [2Fe-2S] cluster EPR spectrum typical of the wild type strain; it is noted, however, that the spectrum of F144H examined when the Q_{pool} comprises QH_2 deviates from the wild type spectrum in displaying a g_x at 1.785 instead of 1.777 (see Figure 7D). Thus, the F144R, K, and H are marked by unusual [2Fe-2S] cluster spectral line shapes.

Ionic Interaction between Arginine at Position 144 and the Ubiquinone Occupants in the Q_o Site. Figure 4 shows the effect of the addition of 50 mM $MgCl_2$ or $CaCl_2$ on the [2Fe-2S] cluster EPR spectrum of F144R and the wild type. Increasing salt concentrations causes the g_x at 1.818 of F144R to broaden significantly and shift to 1.795. Similar effects were obtained with $Mg(acetate)_2$ (data not shown). In contrast, addition of 50 mM $MgCl_2$ or $CaCl_2$ induced no observable changes in EPR spectrum of the wild type strain. Likewise, the presence of KCl up to 100 mM caused no changes of the spectra of either F144R or the wild type (not shown). These results indicate that the divalent cation is the dominating factor in the salt effects on the [2Fe-2S]

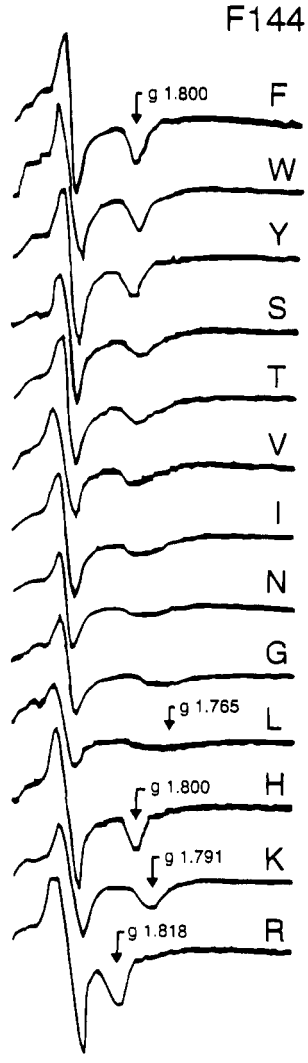


FIGURE 3: Comparison of the [2Fe-2S] EPR spectrum F144R with a range of neutral and other basic F144 substitutions of the cyt bc_1 complex. Chromatophores were prepared as described in Figure 1. The single letter on each spectrum represents the substituted residue at the position F144 of the cyt *b* polypeptide.

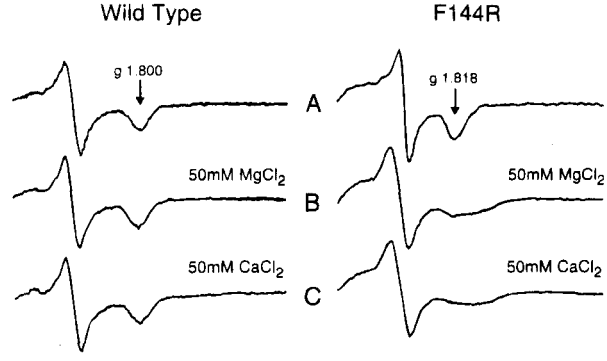


FIGURE 4: Effect of alkaline metal cations on the [2Fe-2S] EPR spectrum of F144R and the wild type. The chromatophores from F144R and the wild type were as described in Figure 1 except that the 100 mM KCl was replaced with other salts as indicated.

cluster EPR spectrum of the mutant F144R. Such ionic effects taken together with effects of the ubiquinone extraction/reconstitution are consistent with the suggestion that the unusual line shape of the F144R [2Fe-2S] cluster is due to the formation of an ion pair with ubiquinone in the Q_o site. The most likely partners in the ion pair are a protonated cationic form of arginine and an anionic and hence reduced

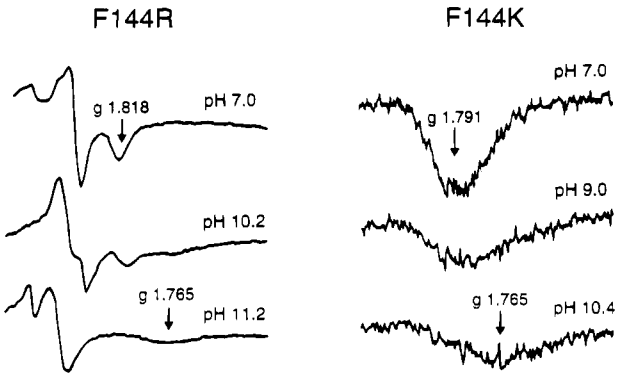


FIGURE 5: pH titration of the [2Fe-2S] EPR spectrum of F144R and F144K. Chromatophores of F144R and F144K were each suspended at 25 μM cyt bc_1 complex in 100 mM KCl with buffers 10 mM MOPS (pH 7−8), 10 mM glycine (pH 9.5−10.5), and 10 mM Tricine (pH 7.5−9.5) to cover the pH range from 7 to 11. Throughout, the pH and redox potential were poised to maintain [2Fe-2S] cluster reduced and the Q_{pool} oxidized based on the known E_m/pH relationships of these components (Prince & Dutton, 1976; Takamiya & Dutton, 1979; Ding et al., 1992). Note that only the g_x band is shown for F144K.

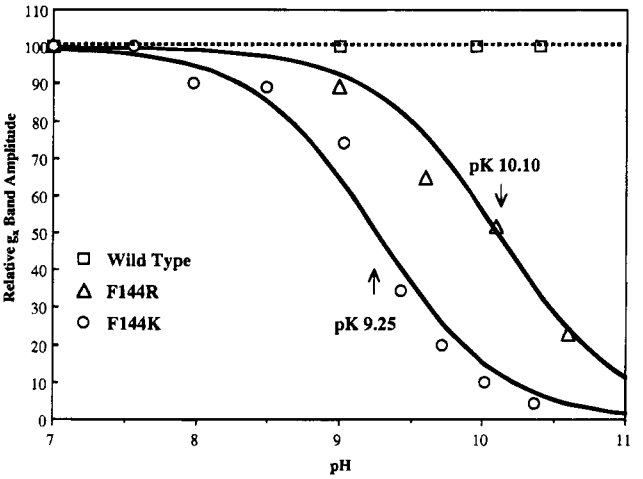


FIGURE 6: Apparent pK values of the residues R and K at position 144. Amplitudes of the characteristic g_x band of the mutants F144R (g_x at 1.818), F144K (g_x at 1.791) and the wild type (g_x at 1.800) are plotted as a function of the ambient pH values. Sample spectra are shown in Figure 5. The solid line drawn through the data points are the Henderson−Hasselbalch curves for pK values 10.10 for F144R (△) and 9.25 for F144K (○). The wild type (□) displays no spectral changes during the pH titration.

form of ubiquinone. Candidates are the ubisemiquinone anion ($Q^{\bullet -}$) and the ubihydroquinone anion (QH^-).

Apparent pK Values of Arginine and Lysine in Position 144. Figure 5 shows [2Fe-2S] cluster EPR spectra of F144R and F144K at different pH values under conditions where the [2Fe-2S] cluster was reduced and the Q_{pool} comprised of only Q. As the pH was changed from 7 to 11.2, the characteristic spectrum of F144R with g_x at 1.818 and g_y at 1.884 was gradually replaced by a broader spectrum with g_x at 1.765 and g_y at 1.901, familiar as a ubiquinone depleted Q_o site. This change of the [2Fe-2S] cluster EPR line shape argues that, if the arginine becomes deprotonated and hence neutral, the anionic ubiquinone vacates the Q_o site. A similar experiment done with F144K shows that as the pH was increased, the g_x at 1.791 shifted to g_x at 1.765, again indicating that a deprotonated lysine 144 residue results in a Q_o site devoid of ubiquinone. In contrast, the mutant F144H and the wild type showed no change in their spectra over a similar range of pH change.

Figure 6 shows the amplitudes of the characteristic g_x band for F144R (g_x at 1.818), F144K (g_x at 1.791), and the wild type (g_x at 1.800) as a function of ambient pH. The bold lines drawn through the data points are Henderson−Hasselbalch fits with corresponding pK values as indicated. The apparent pK values are 10.10 and 9.25 for F144R and F144K, respectively. No acid/base dependent spectral transition was indicated by the wild type signal or that of F144H (not shown).

Redox Titrations of the [2Fe-2S] Cluster, Cyts b_H and b_L and Q_o Site Q/QH$_2$ Couples of the Mutants F144R, F144K, and F144H. Redox titrations (not shown) of the [2Fe-2S] EPR spectrum of F144R, F144K, and F144H all display a Nernst curve (n = 1) with an E_{m7} value of 290 ± 10 mV, which compare with 280 ± 10 mV determined in the wild type (Prince & Dutton, 1976). Similarly, redox titrations of the cytochromes b show that the redox properties of cyt b_L and cyt b_H hemes in F144R are unaltered from those of the wild type. These straightforward results indicate that under the conditions examined, none of the F144R, K, and H substitutions have any significant effect on the redox properties of the [2Fe-2S] cluster and cyt b_L that flank the Q_o site.

In contrast there are major effects on the redox potentials of at least one Q/QH$_2$ couple of the Q_o site of F144R and K. Figure 7A represents a typical [2Fe-2S] cluster spectra from a redox titration of the Q/QH$_2$ couples in the wild type Q_o site at pH 7.0. The transition from g_x at 1.800 (two Q in the Q_o site) to g_x at 1.777 (two QH$_2$ in the Q_o site) yields an E_{m7} value of 85 ± 10 mV (Ding et al., 1992). A similar measurement of the mutant F144H (Figure 7D) shows that this mutant possesses a similar E_{m7} value at approximately 100 mV. In contrast, the g_x bands of the mutants F144R (Figure 7B) and F144K (Figure 7C) do not change during a similar redox titration. The absence of any indication of a redox transition over the redox titration range (pH 7.0) from −450 to 350 mV (350 mV is the upper limit for the use of the EPR signal of the [2Fe-2S] cluster because of its own oxidation) indicates that the E_{m7} values of the Q_o site occupants of F144R and K are greatly altered and lie outside this range.

In an attempt to expand the range of examination of the E_m values of Q/QH$_2$ couples in the Q_o site of the mutant F144R, the measurements were performed above pH 8.0 to provide another 60 mV of scope to measure a high E_m of the Q/QH$_2$ couple in the Q_o site. This opportunity arises from the pH independence of the E_m value of the [2Fe-2S] cluster up to pH 8.0 (Prince & Dutton, 1976) while that of the Q/QH$_2$ couple of the Q_{pool} (Ding et al., 1992), and presumably of the Q_o site, is lowered by 60 mV.

Figure 8A shows representative [2Fe-2S] cluster EPR spectra of the mutant F144R at pH 9.0. The overall signal amplitude decreases as the redox potential increases, as expected for the oxidation of the [2Fe-2S] cluster and consistent with the expected E_{m9} at 220 mV (n = 1). However, it is apparent that the amplitude of the g_x at 1.818, which predominantly records the occupancy of the putative ubiquinone anion form in the Q_o site, decreases more steeply at lower potentials than the g_y band which records the redox state of the [2Fe-2S] cluster generally. Figure 8B compares

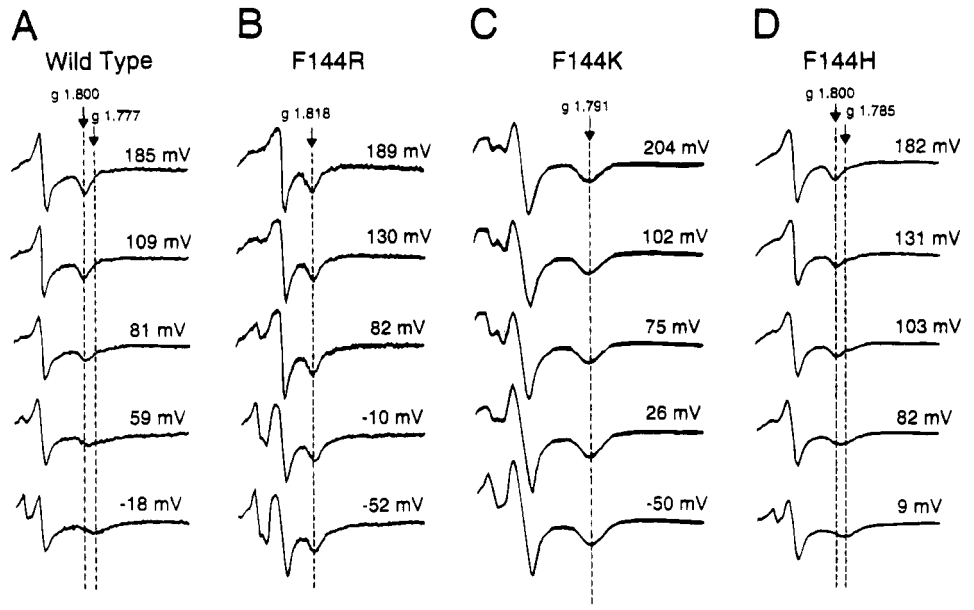


FIGURE 7: Redox titrations of the ubiquinone occupants in the Q_o site at pH 7.0. Chromatophores were suspended at a 20−25 μM cyt bc_1 complex in 100 mM KCl and 50 mM MOPS, pH 7.0. The redox range examined was 250 to −250 mV. Representative [2Fe-2S] EPR spectra of the redox titrations for the Q/QH_2 couples in the Q_o site of the wild type F144R, F144K, and F144H are shown. Note in the case of F144H that when Q_{pool} is reduced, its [2Fe-2S] EPR spectrum g_x band is at 1.789, instead of 1.777 seen in the wild type.

the amplitude of the g_y at 1.884 with the amplitudes of the g_x at 1.818 after correction for the redox state of the [2Fe-2S] cluster as a function of the redox potential. Although difficult to establish with certainty because of the accompanying oxidation of the [2Fe-2S] cluster and loss of the overall spectrum, the spectral alterations are consistent with a change of the g_x at 1.818 to g_x at 1.765 before the [2Fe-2S] cluster signal is finally lost. This implies that once the putative anionic QH^- is oxidized and neutralized to form Q in F144R, the Q vacates the Q_o site. This result is analogous to the effect of neutralizing the cationic charge in the arginine which also led to QH^- vacating the site (Figure 5).

The course of the loss of the g_x at 1.818 as a function of redox potential indicates an E_{m9} of 190 ± 20 mV for the couple in the Q_o site, and an n value of 2 is suggested, consistent with the oxidation−reduction of a QH^-/Q couple. A similar measurement has not been done on F144K, but it may be reasonable to suggest from the experiment done at pH 7.0 that the E_{m9} value of the putative QH^-/Q couple in the Q_o site of F144K has been increased similarly to that of the mutant F144R.

QH_2 Oxidation Kinetics in the Q_o Site of the Mutants F144R, F144K, and F144H. In order to further confirm the results from the redox titration of the Q_o site Q/QH_2 couple(s) measured using the [2Fe-2S] cluster EPR line shape of the mutants F144R, F144K, and F144H, we examined the redox potential dependency of the rate of the QH_2 oxidation in the Q_o site after single flash activation in chromatophore membranes. Table 1 compares the single flash induced QH_2 oxidation rate at the Q_o site in the wild type and mutants F144R, F144K, and F144H. The rates for the wild type are 1650 ± 50 or 150 ± 20 s^{-1} when the Q_{pool} is poised to be essentially all QH_2 or Q prior to activation, respectively (Ding et al., 1995). The midpoint potential of the kinetic transition matches those determined for the Q_o site Q/QH_2 couples (Figure 7A) and the Q_{pool} (Ding et al., 1992). F144H, although slower than the wild type, shows a similar midpoint potential for the kinetic transition which also confirms the results from the redox titrations of the Q_o site Q/QH_2 couples (Figure 7D). Likewise, although F144R and F144K display only a slow QH_2 oxidation rate, it is unaffected over the redox potential range examined, again confirming the results shown in Figure 7B and C that the redox state of ubiquinone occupants in the Q_o site are unchanged during the redox titration at pH 7.0.

DISCUSSION

An amino acid residue that is normally charged when free in aqueous solution can assume different guises when introduced into a protein, depending on the environment, neighboring residues, and redox cofactors and on the protein stability. Placement of arginine (R), lysine (K), or histidine (H), the residues examined in the present study, simply on the exterior of a protein at neutral pH can be expected to retain pK values close to 12, 10, and 6, respectively, and to have only minor effects on charge interactions with the protein interior or on the protein stability. Introduction into the interior of the wild-type protein structure will tend to favor the uncharged, unprotonated form, and hence the pK value will appear to decrease. The magnitude of the pK decrease will be modulated in general by the polarity of the environment and the extent of protein destabilization but also possibly by specific interactions through hydrogen bond or ion pair formation with adjacent charge complementing residues or acid−base or redox cofactors. Probing proteins of unknown structure with a basic amino acid residue can be an uncertain exercise unless such specific close-order interactions can be identified and quantified. The cyt bc_1 complex studied here has no high-resolution structure at this time and hence is vulnerable to interpretive uncertainties regarding the source of the alteration induced by the basic residue substitution. However, after a survey of the arginine mutants of M140, F144, G152, G158, and T163 of the *R. capsulatus* cyt *b* polypeptide, our results suggest that F144 is sufficiently close and suitably oriented to permit the

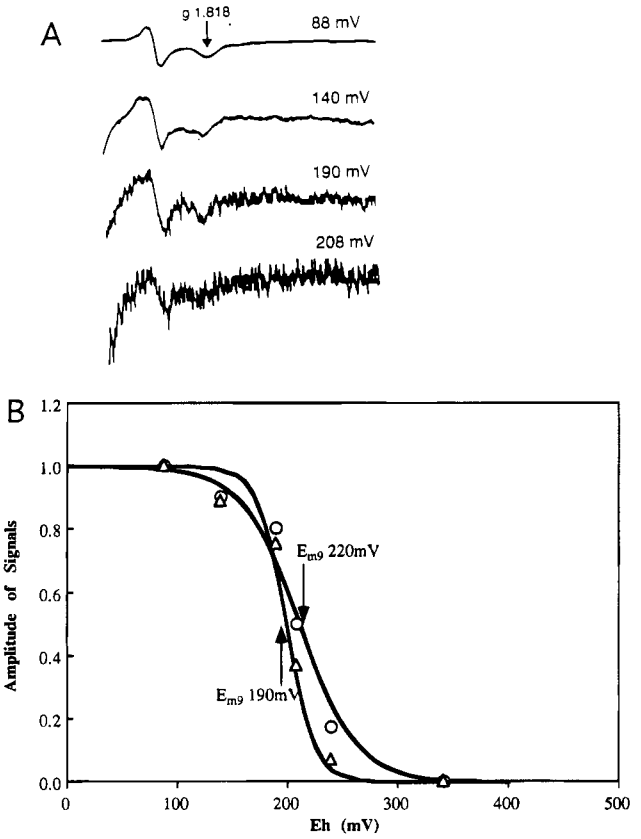


FIGURE 8: Redox titration of the Q_o site ubiquinone occupant of the mutant F144R at pH 9.0. Chromatophores from F144R were suspended at 15 μM cyt bc_1 complex in 100 mM KCl and 50 mM glycine, pH 9.0. Part A shows representative [2Fe-2S] EPR spectra of the mutant F144R at different redox potentials. The signals shown at redox potentials of 190 and 208 mV are amplified 2- and 4-fold, respectively. Part B shows the redox potential dependence of the amplitudes of the g_y band at 1.884 (○) and the g_x band at 1.818 (△) after correction to the diminished amplitude of overall spectrum due to [2Fe-2S] cluster oxidation. The amplitude of the g_y at 1.884 predominantly reflects the total amount of the reduced [2Fe-2S] cluster and was fitted to the Nernst curve with an E_{m9} value of 220 mV ($n = 1$). The amplitude of the g_x at 1.818 reflects the reduced state of the putative ubihydroquinone anion occupant in the Q_o site of the mutant F144R and was fitted to a Nernst fit with an E_{m9} value of 190 mV ($n = 2$).

Table 1: QH_2 Oxidation Rates in the Wild Type and Basic F144 Mutant Strains

redox state of Q_{pool}[a]	rate of the first QH_2 oxidized (s^{-1})			
	wild type	F144H	F144K	F144R
QH_2	1650 ± 50	250 ± 24	45 ± 10	3.0 ± 1
Q	150 ± 20	50 ± 5	40 ± 7	3.5 ± 1

[a] Chromatophores were suspended in 100 mM KCl and 50 mM MOPS, pH 7.0. The redox potential was poised to establish the Q_{pool} either >95% QH_2 (E_h 50 mV) or virtually all Q (E_h 200 mV) prior to activation.

formation of an ion pair between the positive, protonated forms of R and K at 144 and an anionic form of ubiquinone at the Q_o site. This finding identifies a rather specific and short-range interaction that is informative even in the absence of a atomic structure of the cyt bc_1 complex.

The absence of significant shifts in the E_m values of the [2Fe−2S] cluster or cyts b_H and b_L induced by the substitution of arginine or lysine at F144 is consistent with the presence of strong, local dipolar interactions between the R and K cations and the Q_o site anion. Moreover, the effects

Table 2: Interactions of the Q_o site Occupants and the [2Fe-2S] cluster in Cyt bc_1 Complex

occupants in the Q_o site	*g* bands of the [2Fe-2S] cluster			reference
	g_x	g_y	g_z	
WT (Q extracted) X	1.765	1.901	2.032	Figure 1
WT 2Q	1.800	1.894	2.028	Figure 1
WT 1Q	1.783	1.890	2.026	Ding et al. (1992)
WT 2 or 1 QH_2	1.777	1.890	2.020	Ding et al. (1992)
F144R·H^+···QH^-	1.818	1.884	2.033	Figure 1
F144K·H^+···QH^-	1.791	1.894	2.028	Figure 3
F144H···QH_2	1.785	1.889	2.025	Figure 3
F144R·H^+···QH^- (M^{2+})	1.795	1.889	2.034	Figure 4
Q extracted				Figure 2
F144R···X RH^+ deprotonated	1.765	1.901	2.032	Figure 5
QH^- oxidized				Figure 8A

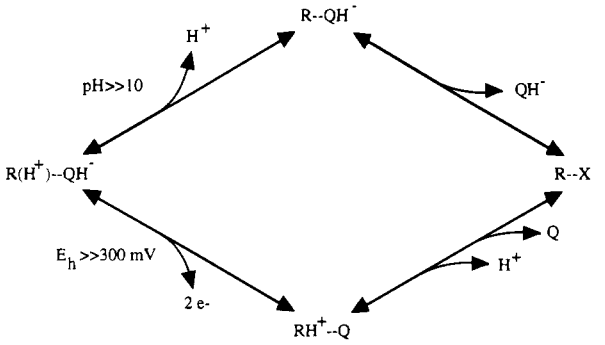
of the divalent cations on the ion pair interaction in the Q_o site of the mutant F144R suggests that at least one of the partners of the ion pair is accessible from the aqueous phase. Such an effect directed to QH^- would be consistent with the proposed nature of the native Q_o site function where upon the oxidation of QH_2, two protons are released toward bulk aqueous solution [see Ding et al. (1995)].

The pH titrations of the [2Fe-2S] cluster line shapes of the mutants F144R and F144K in the presence of the reduced anionic form of ubiquinone in the Q_o site yielded pK values of 10.1 and 9.25 for F144R and F144K, respectively. Corollary redox titrations done at pH values to maintain the residue R (K not done) at position 144 in a protonated cationic form has provided indications of a Q_o site ubiquinone couple displaying an E_{m9} value of +190 ± 20 mV and an n value of 2. The E_{m9} value is some 220 mV higher than that predicted for the wild type (−30 mV), suggesting that, in the presence of the protonated residue arginine cation, the ubiquinone reduced form is stabilized in the Q_o site by an amount approaching 10^4 fold (5.1 kcal/mol) over that of the oxidized form. The candidates for the reduced ubiquinone anionic form include the ubisemiquinone anion ($Q^{\bullet -}$) and the ubihydroquinone anion (QH^-). Available evidence allows a tentative discrimination to be made between the two possibilities pointing to the ubihydroquinone anion (QH^-) as the more likely candidate. This is based on; (a) the failure to detect by EPR a semiquinone free radical in the Q_o site of F144R (data not shown) and (b) the indication of an n value of 2 for the ubiquinone couple in the Q_o site. However, more work on this matter is needed.

An unusually high E_{m9} value for the putative QH^-/Q couple in the Q_o site of +190 ± 20 mV, expected to be at least 250 mV at pH 7.0, impacts on the interpretation of the EPR spectra of the [2Fe-2S] cluster of the mutants F144R and K. Since these spectra were taken at redox potential of 200 mV at pH 7.0 (Figures 1−3), it is reasonable to suggest that they are the result of an interaction between the reduced [2Fe-2S] cluster and a ubihydroquinone (QH^-) in the Q_o site, despite the presence of only ubiquinone in the fully oxidized Q_{pool}. Table 2 summarizes the various interactions between the ubiquinone occupants in the Q_o site and the [2Fe-2S] cluster EPR spectrum observed in the wild type and the mutants described in this work.

It is significant that when either the pH is raised to above the pK value of the residue R(H^+) or K(H^+) to remove the cationic proton charge, or possibly the redox potential is

Scheme 1



raised above the E_m of the Q_o site QH^- to remove the anionic charge, the Q_o site appears to be empty as shown in Scheme 1.

These findings are consistent with the results of the accompanying paper (Ding et al., 1995), which show that similar sized neutral aliphatic residues introduced at position F144 substantially weaken the affinity of the Q or QH_2 in the Q_o site domains. For example, compared to the wild type, the affinity of the Q_{os} and Q_{ow} domains of the mutants F144I and F144L (somewhat smaller than K and R) is weakened by approximately 3.4 and 6.8 kcal/mol, respectively [see Ding et al. (1995)]. In the mutants F144R and F144K, the 5.1 kcal/mol stabilization of the putative QH^- by these basic residues seen in the E_m shift is evidently sufficient to compensate for any opposing bulk steric or polar effects to ubiquinone binding at the Q_o site that are evident when R or K are present in their uncharged forms.

In considering the apparent p*K* values of the residues R and K at 10.1 and 9.25 in the mutants F144R and F144K, respectively, it is of interest to note that in recognizing that the ion pair will stabilize of cationic protonated state of the residue arginine or lysine by an anionic Q_o site occupant, this means that their p*K* values are expected to be *raised* compared to that when the Q_o site is empty of ubiquinone. Since the interaction is mutual, the raised E_m value of the putative Q/QH^- couple in the Q_o site by +220 mV should cause an equivalent of 3.6 pH units *increase* in the apparent p*K* values of the residue arginine or lysine. Hence, without the QH^- in the Q_o site of the mutants F144R and F144K, it may be deduced that the apparent p*K* values of the residues R and K could be as low as 6.5 and 5.6, some 5.5 and 4.4 pH units lower than the values in the free aqueous solution, respectively. These considerations also suggest an explanation for the different character of the mutant F144H. This can now be understood with the suggestion that the residue histidine at position 144 has not been examined in its protonated cationic form in the pH ranges examined in this paper (pH $\leq$ 7.0). If the behavior of F144H parallels those of F144R and F144K, then the protonation and ion pair formation can be predicted to be seen at pH values $\leq$4.

The results shown in this work do not identify which of the ubiquinone molecules of the strongly (Q_{os}) and weakly binding (Q_{ow}) domains in the Q_o site may be interacting with the residue 144 R or K. The Q_o site model, which describes the two domains as contiguous with the Q_{os} domain closest to the [2Fe-2S] cluster (Ding et al., 1995), would explain the emptying of both domains by the deprotonation of the residue 144 R or K by suggesting that the proposed QH^- in the Q_{os} domain is the partner to the arginine at position 144 of the ion pair formation.

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