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Affinity and activity of non-native quinones at the Q_B site of bacterial photosynthetic reaction centers

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Abstract

Purple, photosynthetic reaction centers (RCs) from *Rb. sphaeroides* bacteria use UQ₁₀ as primary (Q_A) and secondary (Q_B) electron acceptors. Many quinones reconstitute Q_A function, while few will act as Q_B. Nine quinones were tested for their ability to bind and reconstitute Q_A and Q_B function. Only ubiquinone (UQ) reconstitutes both Q_A and Q_B function in the same protein. The affinities of the non-native quinones for the Q_B site were determined by a competitive inhibition assay. The affinities of benzoquinones (BQ), naphthoquinone (NQ) and 2-methyl-NQ for the Q_B site are 7±3 times weaker than for the Q_A site. However, di-ortho substituted NQs and anthraquinone bind tightly to the Q_A site (K_d 200 nM) and 1000 times more weakly to the Q_B site, perhaps setting a limit on the size of the site. With a low potential electron donor (2-methyl, 3-dimethylamino-1,4-Naphthoquinone (Me-diMeAm-NQ)) at Q_A, Q_B reduction is 260 meV more favorable than with UQ as Q_A. Electron transfer from Me-diMeAm-NQ at the Q_A site to NQ at the Q_B site can be detected. In the Q_B site the NQ semiquinone is estimated to be ≈ 60–100 meV higher in energy than the UQ semiquinone, while in the Q_A site the semiquinone energy level is similar or lower with NQ than with UQ. Thus, the NQ semiquinone is more stable in the Q_A than Q_B site. In contrast, the native UQ semiquinone is ≈ 60 meV lower in energy in the Q_B than the Q_A site, stabilizing forward electron transfer from Q_A to Q_B.

Keywords

Type II Reaction Centers; Q_B replacement; low potential quinone; redox potential; electron transfer

Introduction

Quinones are membrane soluble, redox active cofactors that carry electrons within and between the proteins that produce transmembrane electrochemical gradients. The quinones are reduced by lower potential substrates such as NADH in complex I, or by light initiated electron transfer in the photosynthetic proteins such as Photosystem I (Fromme et al. 2001) and Photosystem II (Diner et al. 1999; Diner and Rappaport 2002). The reduced quinones

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A quinone with a subscript A or B indicates the quinone is bound at the Q_A or Q_B site respectively. For example Me-diMeAm-NQ_A:NQ_B has Me-diMeAm-NQ in the Q_A site and NQ in the Q_B site.

are then oxidized by higher potential cofactors in the same protein or, following release into the cell membrane quinone pool, when bound to proteins such as the cytochrome bc_1 (Hunte et al. 2003) or b_6f complexes (Stroebel et al. 2003; Kurisu et al. 2003; Cramer et al. 2004). The quinone dependent electron transfer reactions add to the proton gradient by having proton coupled quinone reduction to the dihydroquinone occurring in binding sites on the high pH, N-side of the membrane. Dihydroquinone oxidation, which releases two protons, is carried in binding sites on the P-side of the membrane (Mitchell 1975).

A small number of different quinones are used in biological systems. Different quinones can be used in proteins that carry out similar functions. For example, the b_6f complex uses two plastoquinones, while the bc_1 complex uses two ubiquinones (UQ). The type II reaction centers, which are the focus of the study here, bind two quinones and carry out sequential electron transfer from the primary quinone, Q_A , to the secondary quinone, Q_B . In PSII both sites bind plastoquinone. In the bacterial reaction centers (RCs) studied here, UQ is almost always used as Q_B , while different bacteria can use UQ, menaquinone or rhodoquinone in the Q_A site. Two phylloquinones are found in PSI. Plants and bacteria may have several types of quinones in their membranes, so each quinone binding site needs to select the appropriate compound from the mixture. In addition, some proteins, such as the type II reaction centers or the bc_1 complex bind two identical quinones that carry out different functions. Here each quinone binding site must modify the quinone behavior so it can play its distinct roles in the reaction sequence.

The quinone-dependent electron and proton transfers in RCs from *Rhodobacter sphaeroides* bacteria have been studied intensively (Okamura et al. 2000; Paddock et al. 2003; Wraight 2004). The purple non-sulfur bacterial reaction centers (RCs) have three polypeptides, L, M and H. The L and M polypeptides bind the four bacteriochlorophylls (BChl), two bacteriopheophytins (BPh), two quinones, Q_A and Q_B , and a non-heme iron atom Fe that are responsible for light absorption and the subsequent electron transfer reactions (Heathcote 2002; Sadekar et al. 2006). In the *Rhodobacter sphaeroides* RCs the L and M polypeptides are 33.9% identical and both Q_A and Q_B sites bind ubiquinone-10 (UQ_{10}) (Williams et al. 1986). However the two binding sites are sufficiently dissimilar that the two quinones play different roles in the reaction sequence (Li et al. 2000a). Q_A , the primary quinone electron acceptor is tightly bound so it does not leave the protein and it accepts only one electron to form the anionic semiquinone (Q_A^-). Q_B , the secondary electron acceptor is loosely bound and has been shown by the Feher group to occupy different positions in various crystal structures (Stowell et al. 1997). Q_B can be found in three relatively stable redox states: the neutral quinone (Q_B), anionic semiquinone (Q_B^-), and fully reduced and protonated dihydroquinone (Q_BH_2) (Wraight 1979; Okamura et al. 2000; Zhu and Gunner 2005; Gunner et al. 2008). The anionic semiquinone binds more tightly to the Q_B site, while the quinone and dihydroquinone freely exchange with the quinone pool in the membrane (Diner et al. 1984; Madeo and Gunner 2005; Madeo et al. 2011; McPherson et al. 1990). The protein modulates the in situ E_m s so electron transfer from Q_A to Q_B is favorable with a driving force ($-G_{AB}^\circ$) of -60 meV (-1.4 kcal/mol) (pH 5–9) (Kleinfeld et al. 1984; Shopes and Wraight 1987; Zhu and Gunner 2005).

The UQ in the Q_A site can be removed and functionally reconstituted with a large variety of non-native quinones (Okamura et al. 1975; Gunner et al. 1982; Gunner et al. 1985; Gunner and Dutton 1989) and quinonoid compounds (Warncke and Dutton 1993b). This was shown first by Clayton (Cogdell et al. 1974), while Okamura and Feher worked out a milder treatment for Q_A removal, which became the standard procedure (Okamura et al. 1975).

With the availability of quinone-depleted RCs it became possible to investigate how these proteins discriminate between different quinones in the binding site and to use quinone replacement to modify the electrochemistry of the quinone in the Q_A and Q_B sites (Gunner et al. 1986b; Woodbury et al. 1986; Gunner and Dutton 1989; Graige et al. 1998; Graige et al. 1998; Li et al. 1998; Li et al. 2000b). It was found that quinones other than UQ and close analogues (Wraight et al. 2008) added to quinone-depleted RCs show only Q_A activity. Electron transfer from the introduced, reduced quinone at the Q_A site to the same quinone bound at Q_B is never seen (Giangiacomo and Dutton 1989). Thus, it appears that only ubiquinone can support homologous electron transfer from reduced UQ at the Q_A site (UQ_A⁻) to UQ at the Q_B site (UQ_B) (Baccarini-Melandri et al. 1980; Kleinfeld et al. 1984; McComb et al. 1990).

There are a number of possible reasons for the lack of activity with quinones other than UQ in the Q_B site. The native UQ₁₀ has a 10-isoprene tail with 50 carbons and most quinones used for reconstitution do not have a hydrocarbon tail. However, the tailless analogue of UQ₁₀, 2,3-dimethoxy, 5-methyl-benzoquinone (UQ₀) will function in the Q_B site (Baccarini-Melandri et al. 1980; Wraight and Stein 1983). RC structures show quinones bound in an inner, proximal site that is assumed to be active and several more distal sites that are likely to be inactive (Stowell et al. 1997; Lancaster 1998). Crystal structures show herbicides that inhibit Q_B binding in *Bl. viridis* bind near but not in the active Q_B site (Sinning et al. 1990). Non-native replacements could bind weakly or could occupy an inactive, distal, Q_B site. Lastly, RCs need to modulate the electrochemistry of the quinones in the different binding sites so the free energy of forward electron transfer from the semiquinone UQ_A⁻ to UQ_B (G°_{AB}) is favorable. G°_{AB} favors reduction of UQ_B by UQ_A⁻ by ≈ 60 meV (Kleinfeld et al. 1984; Shopes and Wraight 1987; Zhu and Gunner 2005). Non-native quinones can be reduced at the Q_B site, if the reaction is made more favorable by replacing UQ_A with an analogue at lower potential (Giangiacomo and Dutton 1989; Graige et al. 1998; Li et al. 1998; Schmid and Labahn 2000). This suggests that with the same replacement quinones in both sites electron transfer is uphill. One hypothesis is that the UQ methoxys are a required substituent since they are electron donating into the ring when they are in plane, lowering the quinone E_m and electron withdrawing when out of plane raising the E_m (Prince et al. 1983; Prince et al. 1988; Nonella et al. 1999, Remy et al. 2003). Differences in the UQ methoxy positions in the Q_A and Q_B sites could give Q_B a more positive redox potential allowing for forward electron transfer. Wraight has shown that 2-methoxy-3,5-methyl-6-isoprenyl-BQ will reconstitute function but the isomer that moves the methoxy from the 2 into the 3 position will not (Wraight et al. 2008). A comparison of the quinones in the over 30 RC crystal structures found prior to 2007 show the Q_A 2-methoxy angle is $-77 \pm 8^{\circ}$ while the 3-methoxy angle is $139 \pm 25^{\circ}$. For 11 structures with a proximal

Q_B the methoxys are out of plane at angles of $-90\pm 9^\circ$ and $88\pm 20^\circ$ for the 2 and 3-methoxy respectively (Wraight and Gunner 2009).

In the work presented here the affinity of 9 quinones are measured at the Q_A and Q_B sites and compared to earlier studies of quinone binding (McComb et al. 1990; Warncke et al. 1994). As all quinones can reconstitute Q_A function their binding is monitored by the formation of the semiquinone following light activation of the RCs. The Q_B site K_d s are determined by their competitive inhibition of UQ_1 function when UQ_{10} is in the Q_A site. In addition, 1,4-naphthoquinone (NQ) is characterized as a functional Q_B replacement when paired with a low potential quinone at the Q_A site. The E_m of NQ in the Q_B site is estimated and compared with its E_m in the Q_A site to determine how the protein modulates the electrochemistry of this non-native quinone in the two binding sites.

Methods

Protein purification and quinone preparation

Rb. sphaeroides polyhistidine-tagged RCs are purified on nitrilotriacetic acid (Ni-NTA) resin with 0.05% LDAO in 10mM Tris buffer (pH 7.8) (Goldsmith and Boxer 1996). The RCs are eluted with 40 mM imidazole giving about 90% yield. Q_A and Q_B are removed by washing the RCs on a DEAE-cellulose column. The column is pretreated with bovine serum albumin to saturate non-specific hydrophobic binding sites, while keeping the ion binding sites exposed. Reaction centers bound to the column are washed with 4% LDAO, 10 mM 1,10-phenanthroline and 10 mM Tris at 28°C for 2 hours to remove Q_A (Okamura et al. 1975; Woodbury et al. 1986). The RCs have 90–95% of the native ubiquinone-10 at Q_A and all of the Q_B removed. To remove only Q_B , the same buffer is used but the temperature is set at 25°C . In the Q_B -deleted RCs, Q_A retains $\approx 90\%$ of the native UQ_{10} and there is no Q_B . Careful addition of UQ_{10} to Q_A -deleted RCs is used to produce RCs with 100% UQ_{10} at the Q_A site (UQ_A) and 10–15% UQ_{10} at the Q_B site (UQ_B). The protein is stored in 10mM Tris, pH 7.8, 0.05% LDAO at -80°C . The concentration of RCs is determined assuming $A_{802} = 0.288 \text{ M}^{-1}\text{cm}^{-1}$ (Dutton et al. 1975).

The quinone 2-methyl, 3-dimethylamino-1,4-naphthoquinone (Me-diMeAm-NQ) is synthesized (Salmon-Chemin et al. 2001) starting with 2-methyl-1,4-naphthoquinone (200mg, 1.16 mmol) diluted in dichloromethane (0.5 ml). Dimethylamine (5.82 mmol) mixed with ethanol (2 ml) is added in a 10 ml sealed round-bottom flask and kept in an ice-water bath for two hours. An analytical silica gel TLC plate (Analtech, 250 microns) is used to monitor the reaction. The product is purified off of silica gel either on a preparative TLC plate (Analtech, #02013, 1000 micron) or packed into a column with cyclohexane. The eluting solvent is 50:50 ethyl acetate:methanol. The quinone purity was determined to be $>90\%$ by NMR. The quinone 2-decyl, 3-dimethylamino-1,4-naphthoquinone (decyl-diMeAm-NQ) is synthesized using the same method starting with 2-decyl-1,4-naphthoquinone (Ashnagar et al. 1984).

All other quinones were purchased commercially. Ubiquinone-1 (UQ_1 , 2,3-dimethoxy, 5-methyl, 6-prenyl-benzoquinone), ubiquinone-10 (UQ_{10}), 2,6-dimethyl-benzoquinone (diMe-BQ), tetramethyl-BQ (duroquinone (DQ)), 1,4-naphthoquinone (NQ), 2-methyl-1,4-

naphthoquinone (Me-NQ), 2,3-dimethyl-1,4-naphthoquinone (diMe-NQ) and 9,10-anthraquinone (AQ); were obtained from Sigma and 2,3,5-trimethyl-benzoquinone (triMe-BQ) from Pfaltz & Bauer, Inc. All quinones are dissolved in ethanol for addition to the RCs. Stock solutions are stable for months when stored in the dark at -20°C .

Measuring the quinone-dependent RC activity

Quinone dependent RC activity is measured by time-resolved optical spectroscopy as described previously (Li et al. 1998; Xu and Gunner 2000). A 10 μs xenon flash lamp with a long wavelength, low pass filter with a 750 nm cutoff provides the actinic light to excite the ground state RCs. The concentration of $\text{P}^+\text{Q}_\text{A}^-$ generated by a flash is determined from the amplitude change at 430 nm (A_{430}) 50–100 μs after the flash monitored with a photomultiplier tube (Thorn EMI9798QB). The signal is electronically filtered and amplified before being sent to a LeCroy 9310M (300 MHz) or 9310A (400 MHz) digital oscilloscope. For some experiments RCs are reconstituted with two different quinones, which results in a sample where charge recombination occurs at quite different rates. Here the photomultiplier output is simultaneously fed into two oscilloscopes set at different acquisition time scales. The Levenberg-Marquardt algorithm, a nonlinear least-square fitting routine in IGOR Pro (WaveMetrics) is used to analyze the kinetic traces by fitting to one, two or three exponentials plus a constant. Any residual UQ_{10} is determined from a small signal seen prior to addition of other quinones. This is assumed to represent a constant background activity, which is subtracted from all kinetic traces before analysis.

Determining the occupancy of the Q_A and Q_B site

Concentrated, quinone-depleted RCs stored in 10 mM Tris, pH 7.8, 0.05% LDAO are diluted into Tris buffer with no additional detergent to a final concentration of 1 μM RCs and $\approx 0.005\%$ LDAO. Quinone K_d s are dependent on the detergent concentration, especially above the critical micelle concentration (CMC) (McComb et al. 1990). All experiments are kept well below the LDAO CMC of 0.023% (Herrmann 1962). One or two quinones dissolved in ethanol are added to the RCs (Li et al. 2000b). The system of quinones and protein are at equilibrium as neutral, tailless quinones and short-tailed UQ_1 exchange with the binding sites in milliseconds (Madeo and Gunner 2005). Thus, the Q_A and Q_B binding affinity (K_d) and quinone and RC concentrations determine the site occupancies. The quinone occupying each binding site is identified from the charge recombination kinetics since the $\text{P}^+\text{Q}_\text{A}^-$ or $\text{P}^+\text{Q}_\text{B}^-$ decay rates are different with each quinone (Woodbury et al. 1986; Gunner et al. 1986b; Li et al. 2000b; Xu and Gunner 2000). The A_{430} is followed, monitoring the oxidation and rereduction of P (Slooten 1972; Li et al. 1998). With two added quinones (denoted XQ and YQ) there are 7 types of RCs that can be divided into 4 observable classes (Fig. 1): (1) In RCs with no Q_A the P^+H^- formed by a flash decays with a half time of 10 ns (Kirmaier et al. 1985; Woodbury et al. 1985). These RCs show no A_{430} here since the optical measurements start $\approx 50 \mu\text{s}$ after the flash. (2) All quinones tested reconstitute Q_A function. With only the Q_A site occupied charge recombination from $\text{P}^+\text{Q}_\text{A}^-$ occurs with a nearly single exponential rate constant (k_AP^obs) characteristic of the quinone. If both Q_A and Q_B sites are occupied but there is no forward reaction from Q_A^- to Q_B the same k_AP^obs will be seen in RCs with a subsaturating amount of Q_A or those with both Q_A and Q_B sites filled. (3) With one quinone that can function at the Q_A and Q_B site

(XQ_A:XQ_B) electron transfer from XQ_A⁻ to XQ_B to form P⁺XQ_B⁻, is seen by the appearance of a slower charge recombination rate (k_{BP}^{obs}) with increasing concentration of XQ. Charge recombination from XQ_B⁻ is always slower than from XQ_A⁻. The amplitude of the slow phase increases with concentration. The simplifying assumption (McComb et al. 1990) is made that k_{BP}^{obs} itself is concentration independent to minimize the number of free parameters in the analysis. A two-exponential fit distinguishes between RCs with no Q_A (showing reduced total amplitude), and those where charge recombination is from XQ_A⁻ or XQ_A:XQ_B⁻. (4) In a mixture of 2 quinones, if electron transfer occurs from XQ_A⁻ to YQ_B a distinctive slow phase is observed in a narrow range of XQ and YQ concentration (Giangiacomo and Dutton 1989; Li et al. 2000b). In the mixtures of quinones used here one of the quinones (XQ) has a very low affinity for the Q_B site so there are no RCs with a quinone complement of YQ_A:XQ_B. A three-exponential fit is used to determine the concentration of empty sites, XQ_A⁻ or YQ_A⁻, and XQ_A:YQ_B⁻ RCs formed by a flash. In all data analysis any contribution from the residual UQ₁₀ is subtracted from the signal and k_{AP}^{obs} for each quinone is fixed at the value determined with a sub-saturating concentration of one quinone. A constant is included in all analysis, which is always <5% of the total amplitude.

Quinone affinity for Q_A or Q_B sites

The quinone equilibrium dissociation constant (K_d) is:

$$K_d = \frac{[RC_{Free}][Q_{Free}]}{[RC \cdot Q]} \quad (1)$$

Where [RC_{Free}] is the concentration of RCs without a quinone bound and [Q_{Free}] is the unbound quinone, while [RC Q] is the concentration of RCs with a quinone in the appropriate binding site. The concentration of P⁺Q_A⁻ and P⁺Q_B⁻ is proportional to the initial A₄₃₀ that returns to the ground state at the characteristic rate for that quinone (A). For binding either Q_A or Q_B:

$$[RC \cdot Q] = \frac{[P^+Q^-]}{\Phi} = \left(\frac{\Delta A^k - \Delta A_{min}^k}{\Delta A_{max}^k - \Delta A_{min}^k} \right) \cdot [RC_T] \quad (2)$$

Where RC_T is the total RC concentration, A_{min} is the flash induced amplitude change from the residual UQ_A or UQ_B found before quinone addition, A_{max} is the amplitude when the binding site has been saturated with added quinone. UQ_A has a quantum yield of 102.4% (Loach and Sekura 1968; Wraight and Clayton 1973), indicating that essentially all RCs that are excited go on to form P⁺UQ_A⁻. A_{max} for a particular quinone can be diminished by a quantum yield, Φ, that is less than 100% as described below (Gunner and Dutton 1989).

The K_d is determined from the dependence of A₄₃₀ on the added quinone, [Q_T], using the Levenberg-Marquardt fitting program in IGOR Pro (WaveMetrics):

$$[RC \bullet Q] = \frac{1}{2} \left\{ \left(1 + \frac{[Q_T] + K_d}{[RC_T]} \right) - \sqrt{\left(1 + \frac{[Q_T] + K_d}{[RC_T]} \right)^2 - 4 \frac{[Q_T]}{[RC_T]}} \right\} \quad (3)$$

Competitive inhibition as a measure of the affinity of non-native quinones for the Q_B site

Not all quinones function at the Q_B site and none other than UQ accepts electrons from UQ_A⁻. Each inactive quinone replacement is used as a competitive inhibitor of ubiquinone with a single isoprene unit tail (UQ₁) binding to the Q_B site in RCs with UQ₁₀ as Q_A. RCs with 100% UQ_A and 15–20% UQ_B were prepared by addition of UQ₁₀ to quinone depleted RCs. Charge recombination is well characterized with ubiquinone as UQ_A and UQ_B (Baccarini-Melandri et al. 1980; Kleinfeld et al. 1984; McComb et al. 1990). The K_d of UQ₁ at the Q_B site is determined from the concentration dependence of the A₄₃₀ with a k_{BP}^{obs} of 1 s⁻¹ (Eqn. 3). This treatment is a simplification as k_{BP}^{obs} becomes dependent on the quinone concentration since free quinone can bind to the Q_B site during the lifetime of P⁺Q_A⁻ (McComb et al. 1990). The kinetic signal from residual UQ₁₀ in the Q_B site, determined prior to any quinone additions, is subtracted from each kinetic trace before analysis. There is no indication that the weakly bound quinones used here can displace UQ₁₀ at the Q_B site. With UQ_A, k_{AP}^{obs} is fixed at 10 s⁻¹. The inhibitor dissociation constant, K_i, for each quinone is determined from the apparent K_d, K_d^{app}, with a single addition of inhibitor, XQ, made prior to the UQ₁ titration given:

$$K_i = \frac{[XQ_T]}{(K_d^{app}/K_d - 1)} \quad (4)$$

K_d for UQ₁ is 0.8 ± 0.2 μM determined with no added XQ inhibitor. Equation 4 assumes the free and total concentrations of XQ are the same. This is appropriate when the amount of XQ added ([XQ_T]) is much greater than the concentration of RCs, so the amount of bound inhibitor is negligible as is the case here.

Given the K_d of a quinone for the Q_A and Q_B sites the distribution of quinone in either site can be determined at any concentration of one or two quinones (designated XQ and YQ) (Fig. 2) (Li et al. 2000b). For modeling the site occupancy the assumptions are that: a) The system is at equilibrium; b) XQ binds to the Q_A site but has a negligible affinity for the Q_B site; c) YQ binds to both sites but will bind to the Q_B site first in the presence of XQ in the Q_A site. As described above with two quinones added there are seven types of RCs ranging from one state with no quinone, two states with one quinone at Q_A and four with both quinone binding sites occupied. Four of these, E_A:E_B (both sites empty), XQ_A:E_B, YQ_A:E_B and XQ_A:YQ_B, can be distinguished by their back reaction kinetics. Since YQ and XQ compete for the Q_A site adding more XQ shifts the YQ_A binding curve to higher YQ concentration, increasing the amount of the desired XQ_A:YQ_B RCs (shaded region in figure 2). In contrast, YQ_B binding is independent of the XQ concentration since it is assumed that XQ does not bind to the Q_B site. Unfortunately, with the range of K_ds and solubility of the

quinones used as XQ and YQ it is not possible get all of the RCs into the XQ_A:YQ_B configuration.

Quantum Yield

Multi-flash technique—The quantum yield, Φ_{HA} , for $P^+Q_A^-$ measures how much of the P^+H^- formed by the actinic flash goes forward to the $P^+Q_A^-$ state (at k_{HA}) rather than returns to the ground state (at k_{HP}) (Fig. 1). The absolute quantum yield of native reaction centers with UQ at Q_A has previously been measured to be 102.4% (Loach and Sekura 1968; Wraight and Clayton 1973). Given the values of k_{HA} and k_{HP} , Φ_{HA} for UQ_A should be 98.4% at room temperature and is assumed to be 100% here (Gunner and Dutton 1989).

Φ_{HA} is measured here by a multiple flash method when the rate of electron transfer from Q_A^- to P^+ (k_{AP}) is slow enough that a second flash can be administered before the protein has returned to the ground state. The fraction of RCs that form $P^+Q_A^-$ after the first flash is Φ_{HA} while $(1 - \Phi_{HA})$ returns to the ground state. A subsequent flash adds an additional $\Phi_{HA}[PQ_A]$ of the reaction centers to the $P^+Q_A^-$ population, where $[PQ_A]$ is the concentration in the ground state at the time of a flash. The multi-flash method is used to estimate the quantum yield of UQ₁, DQ and 1,4-NQ using flashes spaced 50 ms apart. RCs with UQ₁₀ as Q_A are used as a standard to determine the fraction of RCs that do not form $P^+Q_A^-$ on the first flash because the actinic flash is not sufficiently bright to ensure that 100% of the RCs absorb a photon.

Light titration—The quantum yield is also measured by a light titration method (Gunner and Dutton 1989). This is especially useful when $P^+Q_A^-$ is too short lived for a second flash to be given prior to the decay to the ground state. Kodak neutral density filters (0.1 – 2.5 N.D.) placed over the normal long-pass red filter with a cutoff at 750 nm are used to attenuate the actinic light intensity. Given the Poisson statistics for light absorption:

$$[P^+Q_A^-] = [P^+Q_A^-]_{\max} \cdot (1 - \Phi_{HA}(cI')) \quad (5)$$

The concentration of $P^+Q_A^-$ is obtained from the A_{430} . I' is the relative intensity of the flash with a filter, estimated using $I' = (2 \times \text{Tr}_{804} + \text{Tr}_{865})/3$. Tr_{804} and Tr_{865} are the percent transmission of the filters at the wavelength of peak RC absorbance in the near IR determined with a JASCO 530 UV/VIS spectrometer. The RC extinction coefficient at 804 is approximately twice that at 865. A more accurate value could be determined considering the integrated area under each of the three RC absorbance bands in the near IR. I' without a neutral density filter is defined as 1.0. c is a constant that depends on the lifetime of the flash. UQ_A serves as an internal control in a light saturation measurement carried out on a RC sample with a mixture of Q_A s (Gunner and Dutton 1989). For example, k_{AP}^{obs} is 10 s^{-1} with UQ_A and 3000 s^{-1} with Me-diMeAm-NQ_A. It is thus easy to determine the amount of $P^+Q_A^-$ at a give I' with either quinone in a mixture from observing the fraction of A_{430} decaying with each rate constant.

The quantum yield for formation of $P^+Q_B^-$, Φ_{AB} , is the fraction of reaction centers that form $P^+Q_B^-$ from $P^+Q_A^-$. Φ_{AB} is estimated from the initial amplitude of the slow (A_S at k_{BP}^{obs})

and fast (A_F at k_{AP}^{obs}) phases of the P^+ charge recombination (Xu and Gunner 2000). Where:

$$\Phi_{AB} = \frac{A_s}{A_s + A_F} = \frac{[P^+Q_B^-]}{[P^+Q_B^-] + [P^+Q_A^-]} \quad (6)$$

Determination of Free Energy level from thermal back reaction

When the charge recombination in RCs in the $P^+Q_A^-$ or $P^+Q_B^-$ state proceeds via an uphill back reaction, the observed kinetics can be used to determine the equilibrium reaction free energy (supplemental material eqn. s2–4) (Kleinfeld et al. 1984; Woodbury et al. 1986). Starting in the $P^+Q_A^-$ or $P^+Q_B^-$ state, the system can return to the ground state by direct electron tunneling downhill to P^+ (at k_{AP} or k_{BP}) or via a thermal back reaction (at k_{AHP} or k_{BAP} or k_{BAHP}) (Fig. 1). Thus, the observed rate of charge recombination from $P^+Q_A^-$, k_{AP}^{obs} is:

$$k_{AP}^{obs} = k_{AP} + k_{AHP} = k_{AP} + k_{HP} / (K_{AH} + 1) \quad (7a)$$

Where K_{AH} is the equilibrium constant between the reactant state $P^+Q_A^-$ and product state P^+H^- . $G^\circ_{AH} = -RT \ln K_{AH}$. For $P^+Q_A^-$, thermal charge recombination is via reformation of P^+H^- which decays at $k_{HP} + k_{HA}$ (Xu and Gunner 2000). Similarly for $P^+Q_B^-$, thermal charge recombination is via $P^+Q_A^-$ which decays at $k_{AP}^{obs} + k_{AB}$ (Li et al. 2000b).

$$k_{BP}^{obs} = k_{BP} + k_{BAP} = k_{BP} + k_{AP} / (K_{BA} + 1) \quad (7b)$$

In RCs where charge recombination from $P^+XQ_A^-$ is via the thermal route, $P^+XQ_A^-YQ_B^-$ will also return to the ground state via formation of $P^+S^-H^-$ since here the indirect rate k_{AHP} is faster than the direct rate k_{AP} .

Charge recombination by direct electron tunneling at k_{HP} , k_{AP} and k_{BP} can be distinguished by the reaction being essentially temperature independent over the temperature range studied here (Gunner et al. 1986b; Shopes and Wraight 1987; Labahn et al. 1995; Xu and Gunner 2000). In contrast, charge recombination via the thermal back reactions at either k_{AHP} or k_{BAP} is strongly temperature dependent (Woodbury et al. 1986; Labahn et al. 1995; Xu and Gunner 2000). As these uphill rates are proportional to the reaction free energy gap (Eqn. 7), the thermodynamic parameters for the reaction can be obtained from the temperature dependence of k_{AP}^{obs} and k_{BP}^{obs} . For k_{AP}^{obs} :

$$\log(k_{AHP} - k_{AP}) = \log k_{HP} + (\Delta S_{AH}^\circ / 2.3k_B) - \Delta H_{AH}^\circ / 2.3k_B T \quad (8)$$

Where k_B is the Boltzmann constant, T is temperature. The direct, downhill electron transfer rate, k_{AP} changes little down to cryogenic temperatures (McElroy et al. 1969).

The analysis of the reaction kinetics and affinity described here does not consider quinone binding to the Q_B site during the lifetime of Q_A^- (McComb et al. 1990). Thus, while Q_B is

in rapid equilibrium with the binding site, Q_B^- dissociates slowly. In regimes of a titration when there is sufficient free Q_B in solution, more will be bound during the lifetime of Q_A^- leading to formation of extra $P^+Q_B^-$. This makes the apparent quinone K_d smaller than the true value. This is not a problem for measurements of the Q_A affinity as the P^+H^- state only lives for 10 ns so there is no time for additional quinone to bind to the Q_A site. The additional $P^+Q_B^-$ also extends the lifetime of the charge separated state so the apparent G°_{AB} will be more negative than the value that would be found if there were no additional Q_B bound during the lifetime of Q_A^- . However, the assumption that no additional Q_B is bound during the lifetime of $P^+Q_A^-$ reduces the number of free parameters in the analysis.

Results

1. Characterization of replacement quinones in the Q_A site

Affinity for the Q_A site—The activity of 4 BQs, 4 NQs and AQ were determined at the Q_A sites of *Rb. sphaeroides* RCs (Table 1). All of these compounds show a time-dependent absorbance change at 430 nm (A_{430}) that is stable for $>100\ \mu\text{s}$ showing that $P^+Q_A^-$ is formed. The affinity of each of these compounds for the Q_A site was obtained from the dependence of the A_{430} on the concentration of added quinone (eqn. 3, Table 2). The Q_A K_d s vary by $\approx 3 \times 10^3$ fold, ranging from 30 nM to 80 μM . As found previously the affinity increases as the number of rings increase from one (1,4-benzoquinone, BQ) to two (1,4-naphthoquinone, NQ) to three (9,10-anthraquinone, AQ) (Gunner et al. 1985; Warncke et al. 1987). AQ binds 2.2 Kcal/mol more tightly than NQ and 4.6 Kcal/mol tighter than previously found for BQ (Warncke and Dutton 1993a). Ortho-methyl groups increase the affinity of both BQs and NQs. The newly synthesized 2-dimethyl-amino, 3-methyl-NQ (Me-diMeAm-NQ) and 2-decyl, 3-dimethyl-amino-NQ (decyl-diMeAmino-NQ) have K_d s of 60 and 300 nM. Increasing the tail length from a methyl to a decyl group had been expected to make the quinone bind more tightly, but instead it weakens the affinity 5 fold. It has been previously established that quinones with two tails bind weakly (Warncke et al. 1994). It may be that both the decyl and bulky dimethyl-amino groups of the decyl, diMeAmino-NQ compete for the tail-binding site, lowering the affinity.

The rate of charge recombination and the in situ Q_A E_m —The nine different quinones compared here have different E_m s in solution. In addition, the protein environment can substantially modify the Q_A redox potential (Woodbury et al. 1986; Ishikita and Knapp 2003; Rinyu et al. 2004; Zhu and Gunner 2005). The free energy difference between $P^+Q_A^-$ and P^+H^- (G°_{AH}) was determined by the measurements of the amplitude of delayed fluorescence in these proteins (Woodbury and Parson 1984) to be 520 meV. G°_{AH} can also be determined from the observed back reaction rate (k_{AP}^{obs}) as long as the rate of charge recombination involving rereduction of H^- (k_{AHP}) is comparable to, or faster than, the rate via direct electron tunneling from Q_A^- to P^+ at k_{AP} (eqn 7a) (Woodbury et al. 1986; Franzen and Boxer 1993; Takahashi et al. 1998; Xu and Gunner 2000). With a reconstituted Q_A (XQ_A) the rate constants for charge recombination from $P^+XQ_A^-$ vary from 1.5 to 3000 s^{-1} (Table 1). If XQ_A has an in situ redox potential at least 100 meV lower than UQ at the Q_A site (UQ_A), k_{AP}^{obs} becomes fast and temperature dependent (Fig 1). It is now proportional to the equilibrium concentration of P^+H^- (Eqn. 7b). The sub-millisecond charge recombination

rates found with AQ, Me-DiMeAm-NQ and decyl-DiMeAm-NQ are used to determine G°_{AH} from the equilibrium constant between $P^+XQ_A^-$ and P^+H^- (Eqn. 7a). Given the k_{AP}^{obs} of 175 s^{-1} G°_{AH} is 330 meV in RCs with $P^+AQ_A^-$, 190 meV higher in energy than $P^+UQ_A^-$, in reasonable agreement with earlier results (Gunner and Dutton 1989; Xu and Gunner 2000). The Me-DiMeAm-NQ k_{AP}^{obs} of 3000 s^{-1} indicates that G°_{AH} is only 260 meV, 260 meV higher than in RCs with UQ_A (Table 2 & 3). Substitution of the methyl in decyl-diMeAm-NQ slows k_{AP}^{obs} indicating $P^+Q_A^-$ is stabilized by 40 meV relative to that found with methyl-diMeAm-NQ.

The thermal back reaction via P^+H^- can be distinguished from direct tunneling from Q_A^- to P^+ by the temperature dependence of k_{AP}^{obs} . The derived enthalpy and free energy change characterize the equilibrium connecting P^+H^- and $P^+Q_A^-$ (Table 3). With Me-DiMeAm-NQ at the Q_A site, H°_{AH} is 244 meV and $T S^{\circ}_{AH}$ is 19 meV at 298K (Eqn. 8). The enthalpy change, which is close to the reaction free energy, dominates and the entropy change is small, as has been found previously with other quinones in the Q_A site (Woodbury et al. 1986; Xu and Gunner 2000).

Quantum yield for formation of $P^+Q_A^-$ —The rate of electron transfer from H^- to UQ_A (k_{HA}) has been determined to be $6.7 \times 10^9\text{ s}^{-1}$ from direct observation of the reaction kinetics (Kirmaier et al. 1985). As the forward driving force decreases k_{HA} becomes comparable to the rate of charge recombination from H^- to P^+ (k_{HP}) (Eqn. 6). Under these conditions Φ_{HA} can be used to estimate k_{HA} . Since the quantum yield for reduction of UQ_A by H^- , Φ_{HA} , is $\approx 100\%$ (Loach and Sekura 1968; Wraight and Clayton 1973), the measured A_{430} in RCs saturated with UQ_1 represents 100% of the RCs that absorb a photon forming long-lived P^+ . Four quinone replacements show a smaller amplitude change (Table 1). The maximum

A_{430} with AQ as Q_A is 90% of that found with UQ_A , in agreement with earlier measurements (Gunner and Dutton 1989; Liang et al. 1981). The quantum yield of Me-DiMeAm-NQ was measured by light titration to be 60%. This value is similar to that found for 2-dimethylamino-NQ and 1-amino-AQ which have a similar G°_{HA} (Gunner and Dutton 1989). The maximum XQ_A in a binding titration can be smaller than Φ_{HA} if the quinone can bind in a competing but inactive manner or if the quinone affinity is low so it reaches its solubility limit before the binding site is saturated.

2. Characterization of Q_B activity and affinity

Inability of non-native quinones to support XQ_A^- to XQ_B electron transfer—A flash generated A_{430} shows long lived P^+ if either $P^+Q_A^-$ or $P^+Q_B^-$ is formed. In Q removed RCs addition of Q_A increases the A_{430} while Q_B activity is measured by the appearance of a longer-lived P^+ state. All of the nine quinones tested show a $P^+Q_A^-$ signal that is within 40% of the value seen with UQ (Table 1). However, for all quinones other than UQ_1 the back reaction rate is independent of the quinone concentration. Thus, as has been shown previously (Giangiaco and Dutton 1989), added quinones (XQ) other than UQ do not carry out electron transfer from XQ_A^- to XQ_B . In addition, when the same non-native quinones are added to RCs with UQ_{10} at the Q_A site the back reaction rate changes little from the $10 \pm 0.5\text{ s}^{-1}$ characteristic of $P^+Q_A^-$ RCs at all XQ concentrations. Thus, the

semiquinone UQ_{10}^- in the Q_A site cannot reduce any of the quinones tested here other than UQ_1 or UQ_{10} in the Q_B site.

Affinity of the replacement quinones for the Q_B site determined by competitive inhibition of UQ_1 binding

As none of the quinones studied here except UQ_1 reconstitutes Q_B activity when added to UQ_{10} containing RCs, the affinities of the non-native quinones for the Q_B site were determined by a competitive inhibition assay. The affinity of UQ_1 is determined in RCs with UQ_{10} in the Q_A site from the increase in the amplitude of the component with a charge recombination rate of 1s^{-1} characteristic of $\text{UQ}_\text{A}:\text{UQ}_\text{B}$ RCs as a function of added UQ_1 . Each non-native quinone is added as a competitive inhibitor. For most accurate measurements 10 to 300 μM inhibitor is added, sufficient to change the K_d^app for UQ_1 by 2 to 10 fold (Fig. 3, Table 2). With the exception of Me-diMeAm-NQ, a single addition of quinone (XQ) does not change the amount of P^+ formed or the back reaction rate, k_AP . The diMeAmino binds tightly enough that it can displace some UQ_{10} at the Q_A site and a fast k_AP^obs characteristic of Me-diMeAm-NQ Q_A is seen. Comparison of the apparent K_d for UQ_1 with and without XQ added provides the K_i of XQ at the Q_B site. The results are analyzed with the appropriate and simplifying assumption that the concentration of quinone added (Q_T) is equal to the free quinone (Q_F) (Eqn. 4a, Table 2). An additional non-competitive component to the inhibition of UQ_1 is seen, as the maximum amount of slow phase with the inhibitory quinone added is 75–90% of that seen for UQ_1 alone (Fig. 3).

Relative affinity of quinones for the Q_A and Q_B sites—The affinities of quinones for the Q_A and Q_B sites are compared in Figure 4. For all quinones except the three largest quinones, Me-diMeAm-NQ, diMe-NQ and AQ, the affinity for the Q_B site is 7 ± 3 fold weaker than for the Q_A site. The behavior of the smaller quinones agrees with comparisons of the relative affinities of ubiquinone with different tail lengths in detergent solution or hexane (McComb et al. 1990; Warncke et al. 1994). The tight affinity of the 6 smaller quinones in the competitive inhibition assay shows the reason there is no UQ_A^- to XQ_B or XQ_A^- to XQ_B electron transfer is not because they are not bound to the Q_B site. However, while the three large quinones bind tightly to the Q_A site with $K_\text{ds} < 200\text{ nM}$, they bind at least 2000 times more weakly to the Q_B site with $K_\text{ds} > 100\text{ }\mu\text{M}$. It appears that addition of a second ortho-methyl on the NQ ring exceeds the size of the binding site. Thus, these three large quinones will not reconstitute Q_B function because they do not bind to the Q_B site. These three low affinity quinones have low solubility so the effective concentration may be smaller than expected. This would cause the measurements to underestimate the affinity.

3. Electron transfer from the low potential Q_A to NQ as Q_B

Electron transfer from Me-diMeAm-NQ Q_A to NQ Q_B —Only UQ, or an analog with a single methoxy in the 2 position has been found to be able to carry out electron transfer between the same quinones in the Q_A and Q_B sites (Wraight et al. 2008). This ability is independent of the tail length of the quinone in Q_A and Q_B sites (McComb et al. 1990; Warncke et al. 1994). Small enough non-native quinones will bind as competitive inhibitors of UQ in the Q_B site (Fig. 4), but functional electron transfer from a non-native Q_A to a replacement Q_B has been previously shown to be possible only using a low potential Q_A to

provide greater driving force for Q_B reduction (Giangiacomo and Dutton 1989). Here the low potential, tight binding Me-diMeAm-NQ is added in a mixture with NQ. With only one of these quinones added RCs return to the ground state in a process characterized by single exponential rate constant (Fig. 5). However, with both added, a slow phase of charge recombination is seen, indicating that charge recombination is from $P^+NQ_B^-$. Thus, the reduced Me-diMeAm-NQ in the Q_A site (Me-diMeAm- NQ_A^-) can reduce NQ in the Q_B site (NQ_B).

Hybrid Me-diMeAm- $NQ_A:NQ_B$ RCs were reconstituted with different concentrations of NQ (Fig. 6). The binding curve for reconstituting NQ_B is independent of the concentration of Me-diMeAm-NQ since this has negligible affinity for the Q_B site (Table 1). Adding more Me-diMeAm-NQ does shift the amount of NQ needed to displace the low potential quinone at the Q_A site, however by a smaller amount than that predicted (data not shown). This may indicate that the activity of the NQ is less than its concentration, not unexpected, as the NQ concentration when it binds to Q_A is only ≈ 10 fold below its solubility limit. The maximum fraction of Me-diMeAm- $NQ_A:NQ_B$ RCs is also independent of the concentration of Me-diMeAm- NQ_A . From 2 to 50 μM Me-diMeAm-NQ the maximum fraction of RCs with Me-diMeAm- $NQ_A:NQ_B$ is expected, given the binding constants of the quinones to Q_A and Q_B site, to change from 60% to 95% while the observed value remains $\approx 45\%$. As more NQ is added it displaces Me-diMeAm- NQ_A and the back reaction kinetics characteristic of NQ_A are found. Thus, with two quinones added there are 6 possible types of RCs with one or two quinones bound. Three distinct kinetic phases show that three forms of active RCs are reconstituted (Fig. 5 and 6). These have Me-diMeAm- NQ_A alone at low NQ concentration (Me-diMeAm- NQ_A), NQ_A only at high NQ concentration (NQ_A) and Me-diMeAm- $NQ_A:NQ_B$ at intermediate concentrations. No reaction kinetics suggesting electron transfer from NQ_A^- to NQ_B , from Me-diMeAm- NQ_A^- to Me-diMeAm- NQ_B or from NQ_A^- to Me-diMeAm- NQ_B is seen.

Φ_{AB} , the quantum yield for electron transfer from Me-diMeAm- NQ_A^- to NQ_B —

The slow phase of charge recombination at $0.5s^{-1}$ indicative of charge recombination in P^+ Me-diMeAm- $NQ_A:NQ_B^-$ RCs is seen in 45% of the RCs with 50 μM Me-diMeAm-NQ and 1mM NQ added (Fig. 5 & 6). The low yield can be a measure of a low quantum yield. Thus, only Φ_{HA} of the electrons on H^- make it to Me-diMeAm- NQ_A and only Φ_{AB} of the reduced Me-diMeAm- NQ_A^- reduce NQ_B rather than charge recombining to the ground state at the k_{AP}^{obs} of $3000 s^{-1}$. Φ_{HA} with Me-diMeAmino- NQ_A is 60%. Using the K_d s for the two quinones at their respective binding sites to determine how many RCs have the correct quinones bound, Φ_{AB} for electron transfer from Q_A to Q_B in Me-diMeAmino- $NQ_A^-:NQ_B$ RCs is $\approx 75\%$ (Eqn. 6, Fig. 6).

Rate of electron transfer from Me-diMeAm- NQ_A to NQ_B —The estimated Φ_{AB} can provide the electron transfer rate from Me-diMeAm- NQ_A^- to NQ_B (k_{AB}) (Eqn. 6) (Kleinfeld et al. 1984; Xu and Gunner 2002). In $UQ_A:UQ_B$ RCs, the forward rate is 100 μs (Mancino et al. 1984; Tiede et al. 1996; Graige et al. 1998) and k_{AP} is $10 s^{-1}$ ensuring 98% of $UQ_A^-UQ_B$ form $UQ_AUQ_B^-$. In contrast, k_{AP}^{obs} for Me-diMeAm-NQ is $3000 s^{-1}$, thus k_{AB} needs to be proportionally faster to maintain a high quantum yield for Q_B reduction. Given a 75% yield

for Me-diMeAmino-NQ_A⁻ reduction of NQ_B the competition between k_{AB} and k_{AP}^{obs} (Fig. 6) gives a forward electron transfer rate, k_{AB} , of $\approx 9000 \text{ s}^{-1}$ ($\Phi_{AB} \approx 77 \mu\text{s}$). This is the lower limit for k_{AB} as the yield of NQ_B⁻ can be limited by other factors. For example, the yield could be lower if some RCs have NQ binding to competing yet, inactive sites found near the Q_B site (Stowell et al. 1997). Finding a k_{AB} that is similar to that found for UQ_A to UQ_B agrees with earlier studies showing no free energy dependence for the forward electron transfer rate from Q_A to Q_B (Graige et al. 1998). It is slower than expected given a study of various low-potential methyl-NQs used as Q_A to reduce UQ_B where the rate was found to increase as the reaction driving force, G_{AB}° , became more favorable (Li et al. 2000b).

Estimating the NQ_B redox potential—Electron transfer is found to take place from Me-diMeAm-NQ_A⁻ to NQ_B, so NQ can bind in the Q_B site in a position to accept electrons. However, neither NQ_A⁻ nor UQ_A⁻ reduces NQ_B. Thus, the presumption is the energy level of NQ_B lies between that of UQ_A and Me-diMeAm-NQ_A so electron transfer from NQ_A⁻ or UQ_A⁻ is uphill and from Me-diMeAm-NQ_A⁻ downhill.

The back reaction kinetics with the Q_A and Q_B sites occupied can be used to determine the in situ G_{AB}° (Eqn. 7, (Li et al. 1998)). In UQ_A:NQ_B RCs k_{BP}^{obs} changes by less than 10% of the value found with UQ_A alone. Slowing k_{BP}^{obs} from 10 s^{-1} to 9 s^{-1} would place UQ_ANQ_B⁻ 55 meV higher in energy than UQ_A⁻NQ_B (Eqn. 5d). This is the lower limit for G_{AB}° , as a smaller change in k_{BP}^{obs} places UQ_ANQ_B⁻ at even higher in energy. Given a G_{AB}° of -60 meV for electron transfer from UQ_A⁻ to UQ_B (Kleinfeld et al. 1984; Shopes and Wraight 1987; Zhu and Gunner 2005), NQ_B⁻ is at least 115 meV higher in energy than UQ_B⁻.

An upper limit for the energy of the P⁺UQ_A:NQ_B⁻ state can be obtained from the back reaction with the low potential quinone Me-diMeAm-NQ at the Q_A site. Given a k_{AP}^{obs} of 3000 s^{-1} for Me-diMeAm-NQ_A and the k_{BP}^{obs} of 0.5 s^{-1} when NQ is added, NQ_B⁻ is calculated to be 220 meV lower in energy than Me-diMeAm-NQ_A⁻ if $k_{BP}^{obs} = k_{BAP}$ (Eqn. 7b). However, the rate of the thermal back reaction, k_{BAP} , slows as free energy of electron transfer, K_{AB} , favors Q_B reduction. Thus, when G_{AB}° is significantly more than -100 meV downhill, more of the electrons stay on Q_B rather than thermally repopulating Q_A. Now direct electron transfer from Q_B⁻ to P⁺ (at k_{BP}) becomes important (Shopes and Wraight 1987; Sebban and Wraight 1989; Takahashi and Wraight 1990; Labahn et al. 1994; Labahn et al. 1995). Estimates for k_{BP} range from 0.1 to 0.4 s^{-1} (Kleinfeld et al. 1984; Labahn et al. 1994; Takahashi and Wraight 1990). Using these as the limits for the k_{BP} contribution to k_{BP}^{obs} places G_{AB}° in the range -225 to -260 meV in Me-diMeAm-NQ_A:NQ_B⁻ RCs.

Estimates of the in situ E_m for NQ_A⁻—There has been no direct measure of the in situ E_m of NQ_A. If the E_m for formation of the semiquinone were as much as 75 meV lower than found for UQ_A, charge recombination from P⁺NQ_A⁻ would begin to use the thermal back reaction at k_{AHP} . This path yields a faster, temperature dependent k_{AP}^{obs} (Franzen and Boxer 1993, Woodbury, 1986 #1573), which is not seen. In dimethyl formamide (DMF) solution NQ has an E_m 20 mV more positive than UQ (Gunner et al. 1986b). Alkyl NQs such as 2-methyl-NQ have E_ms in the Q_A site that are more positive than predicted from

their values in DMF (Woodbury et al. 1986). Given the measured E_m for other quinone replacements, NQ_A is predicted to have an in situ E_m close to or more positive than UQ_A (Gunner et al. 1986a; Woodbury et al. 1986; Warncke et al. 1987). In contrast, the E_m of NQ_B is shown here to be 100–125 mV lower than UQ_B . Thus, the stabilization of the NQ semiquinone in Q_A and Q_B sites is significantly different.

Temperature dependence of k_{BP}^{obs} in Me-diMeAm- $NQ_A:NQ_B$ RCs and the role of direct electron transfer from NQ_B^- to P^+ —The estimate of G°_{AB} from the back reaction rate assumes k_{BP}^{obs} has a significant contribution of charge recombination occurring via thermal repopulation of $P^+Me-diMeAm-NQ_A^-$. The temperature dependence of k_{BP}^{obs} was measured for RCs with Me-diMeAm- $NQ_A:NQ_B$ from 277 to 303K. In these RCs the modest temperature dependence of k_{BP}^{obs} is consistent with the reaction proceeding by a mixture of k_{BP} and k_{BAP} , with the direct tunneling rate, k_{BP} being only weakly temperature-dependent (Schmid and Labahn 2000). A G°_{HB} of -500 meV and a k_{BP} of 0.2 s $^{-1}$ provide a good match to the limited study of the temperature dependence of k_{BP}^{obs} (Fig. 8, Eqn. s.2–4). For comparison, in $UQ_A:UQ_B$ RCs where $>95\%$ of $P^+Q_B^-$ decay is via $P^+Q_A^-$ (Kleinfeld et al. 1984), G°_{AB} is ≈ 60 meV (Kleinfeld et al. 1984; Shopes and Wraight 1987; Zhu and Gunner 2005), $H^\circ_{AB} = -150$ meV and $S^\circ_{AB} = -0.3$ meV near 300K (Li et al. 2000b; Mancino et al. 1984; Schmid and Labahn 2000).

Conclusions

As found previously (Giangiacomo and Dutton 1989), only UQ and close analogues (Wraight et al. 2008) can participate in electron transfer between the same quinones in the Q_A and Q_B sites. Two hypotheses for the failure to reconstitute Q_B function were explored. One is that the non-native quinones do not bind to the Q_B site. The other is the quinones bind at the Q_B site, but the electron transfer between Q_A and Q_B is unfavorable because of how the reaction center modulates the electrochemistry of the quinones in the different sites.

The affinities of quinones at the Q_B site were determined by a competitive inhibition binding assay and compared to their K_d at the Q_A site. For quinones as large as 2-methyl-naphthoquinone the affinity at the Q_B site is approximately 7 times weaker than at the Q_A site. However, 2,3-dimethyl-naphthoquinone binds >3000 fold more strongly at the Q_A site than it does at the Q_B site. The low affinity of the dimethyl-NQ may provide an explanation for how the photosynthetic bacteria that make both UQ and menaquinone (MQ), 2-methyl-3-isoprenyl-1,4-naphthoquinone, can segregate the correct quinone to each binding site. Even in *Rb. sphaeroides*, an organism which does not make MQ, the 2,3-diMeNQ head group binds 3-fold tighter than UQ_1 to the Q_A site while at the Q_B site it binds more than >100 times weaker. However, while a few quinones are too weakly bound to function at the Q_B site, the others show significant affinity for the binding site in the competitive inhibition assay.

1,4-Naphthoquinone (NQ) binds tight enough that the Q_B site can be saturated at accessible quinone concentrations. Various measurements show that the NQ_B E_m is at least 100 mV more negative than UQ_B so electron transfer from UQ_A^- to NQ_B is unfavorable. In hybrid reaction centers with a low potential Me-diMeAm-NQ quinone as Q_A , electron transfer

between the low potential Me-diMeAm-NQ_A and NQ_B was detected. Thus, electron transfer can be seen, but it requires added driving force (Giangiacomo and Dutton 1989). While there is no measured E_m for NQ_A, comparison with other quinones suggests it should be similar to or more positive than UQ_A. Thus, the Q_B site destabilizes the non-native NQ_B[−] while the Q_A binding site generally stabilizes non-native Q_As (Woodbury et al. 1986). This differential interaction of the NQ with the different binding sites makes electron transfer from NQ_A[−] to NQ_B uphill. In contrast, UQ[−] is stabilized in the Q_B site more than in the Q_A site allowing forward electron transfer from UQ_A[−] to UQ_B.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

RCs	bacterial photosynthetic reaction centers
diMe-BQ	2,6-dimethyl-benzoquinone
DQ	tetramethyl-BQ (duroquinone)
triMe-BQ	2,3,5-trimethyl-benzoquinone
UQ₁	Ubiquinone-1 (2,3-dimethoxy, 5-methyl, 6-prenyl-benzoquinone)
UQ₁₀	ubiquinone-10
NQ	1,4-naphthoquinone
Me-NQ	2-methyl-1,4-naphthoquinone
diMe-NQ	2,3-dimethyl-1,4-naphthoquinone
Me-diMeAm-NQ	2-methyl, 3-dimethylamino-1,4-naphthoquinone
decyl-diMeAm-NQ	2-decyl, 3-dimethylamino-1,4-naphthoquinone
AQ	9,10-anthraquinone

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Figure 1.

Electron transfer pathways in *Rb. sphaeroides* reaction centers with no external electron donor. P is the bacteriochlorophyll dimer, H is the bacteriopheophytin. When P absorbs a photon, the excited P^* reduces H in 3 ps. With UQ as Q_A and Q_B , the electron is transferred to Q_A in 200 ps (k_{HA}) and then to Q_B in 100 μ s (k_{AB}). At each stage in the reaction cycle there are competing charge recombination reactions that transfer the electron back to P^+ either by direct electron tunneling or via repopulation of a higher energy states (dashed lines). P^+H^- decays to the ground state in ≈ 10 ns (k_{HP} is 7×10^7 s $^{-1}$) if there is no Q_A . Once the electron reaches the quinones it can return to P^+ by either direct tunneling (k_{AP} in $P^+Q_A^-$ or k_{BP} in $P^+Q_B^-$ RCs) or via a thermal back reaction. k_{AHP} is the rate of $P^+Q_A^-$ charge recombination via $P^+H^-Q_A$, while k_{BAP} is the back reaction rate from $P^+Q_AQ_B^-$ via $P^+Q_A^-Q_B$. The observed rates k_{AP}^{obs} or k_{BP}^{obs} are the sum of the rates by the two routes. In native RCs, if there is no Q_B , $k_{AP}^{obs} \approx k_{AP}$ is 10 s $^{-1}$ via direct electron tunneling from Q_A^- to P^+ , while the back reaction from UQ_B^- goes via reformation of $P^+Q_A^-$ at $k_{BP}^{obs} \approx k_{BAP}$ which is 1 s $^{-1}$.

Figure 2.

Occupancy of Q_A and Q_B sites in a mixture of 2 quinones $[RC_T]=1$ M; $K_d XQ_A = 0.06$ M; $K_d YQ_A = 8$ M; $K_d YQ_B = 80$ M. The concentration of XQ is 10 M (light lines): or 50 M (darker lines). The fraction of RCs as $XQ_A:E_B$ (Dotted-Dashed lines); $YQ_A:E_B + YQ_A:YQ_B$ (Dashed lines); $XQ_A:YQ_B$ (Solid lines). E_B represents an empty Q_B site. XQ does not bind to the Q_B site so there is no $XQ_A:XQ_B$. Where there is the same quinone in Q_A and Q_B sites ($YQ_A:YQ_B$) there is no forward electron transfer from the semiquinone YQ_A^- to YQ_B . Desired $XQ_A:YQ_B$ with 10 μ M XQ_T (shaded darker region) or with 50 μ M XQ_T (shaded lighter region). Mathematica equations used to find distribution of quinones bound to each site given in Supplementary Material (Eqn. s1).

Figure 3.

Reconstitution of Q_B function with UQ_1 following addition of non-native quinones as competitive inhibitors. (○): UQ_1 alone; (▲): NQ; (○): Me-NQ; (▽): diMe-NQ. Titration carried out with 1 μ M RCs, 0.005% LDAO, 10mM Tris, pH 7.8 at 298.2 K. Theory lines show the best fit K_i derived from K_d^{app} and the fraction of $P^+Q_B^-$ at saturating UQ_1 using values given in Table 2 in equation 4.

Figure 4.

Comparison of quinone binding affinity at Q_A and Q_B sites. (■) This work (Table 1).

Ubiquinone with different tail lengths in (○): water-saturated hexane (Warncke et al. 1994);

(●): 10mM Tris, pH= 8.0, 0.1% LDAO, 298 K (McComb et al. 1990). Small numbers refer to the number of isoprenyl units in the UQ tail for measurements in either water or hexane.

Straight line: $K_d^{Q_B} / K_d^{Q_A} = 7$.

Figure 5.

The rate of decay of P^+ , monitored at 430 nm after an actinic flash shows which quinone is in the Q_A and Q_B binding sites. The kinetic traces are normalized to give the same initial amplitude (A_{430}) to facilitate comparison. The signal from RCs retaining residual UQ_A is subtracted. Residuals from the analysis of the kinetic traces are shown in the two lowest traces. 1 μ M RCs, 0.005% LDAO, 10 mM Tris, pH 7.8 at 298 ± 2 K. *Top: faster trace:* 300 μ M NQ: $k_1 = k_{AP} = 7.8 \text{ s}^{-1}$, $A_1 = 95\%$; constant $A_0 = 5\%$; *slower trace:* 2 μ M Me-diMeAm-NQ and 300 μ M NQ: $k_1 = k_{AP} = 7.8 \text{ s}^{-1}$, $A_1 = 60\%$; $k_2 = k_{BP}^{\text{obs}} = 0.50 \text{ s}^{-1}$, $A_2 = 36\%$. $A_0 = 4\%$. $A_{430} 1.7$ shown. *Insert:* Me-diMeAm-NQ at Q_A at 2000 fold faster data acquisition rate: $k_1 = k_{AP}^{\text{obs}} = 3000 \text{ s}^{-1}$, $A_1 = 95\%$, $A_0 = 5\%$. No NQ is added here.

Figure 6.

Fraction of Me-diMeAm-NQ in Q_A and NQ in Q_A or Q_B sites as a function of NQ concentration. The fraction of $NQ_A \cdot E_B$, Me-diMeAm- $Q_A \cdot E_B$ and Me-diMeAm- $Q_A \cdot NQ_B$ RCs obtained from a three exponential fit of the back reaction kinetics with fixed rate constants (Fig. 5). (●) RCs with Me-diMeAm-N Q_A and an empty Q_B site is derived from the initial amplitude of A_{430} decaying at $k_1 = 3000 \text{ s}^{-1}$ normalized using A_{430}/Φ_{HA} where Φ_{HA} is 60%; (▽) RCs with N Q_A and no Q_B from the initial amplitude of A_{430} decaying at $k_2 = 7.8 \text{ s}^{-1}$; Φ_{HA} for NQ is 100%. (▲) RCs with Me-diMeAm-N $Q_A \cdot NQ_B$ obtained from the initial amplitude of A_{430} decaying at $k_3 = 0.5 \text{ s}^{-1}$ normalized using A_{430}/Φ_{AB} , with Φ_{AB}

of 75%. Theoretical lines generated with Mathematica using Eqn. s1 in supplementary material with 1 M RCs and 50 M Me-diMeAm-NQ. K_d of Me-diMeAm-NQ at Q_A is 0.06 M. K_d^{app} of NQ at Q_A is 1640 μ M when it is competing with 50 μ M Me-diMeAm-NQ. The K_d for NQ is 175 μ M in the Q_B site with either NQ_A or Me-diMeAm- NQ_A . There is negligible Me-diMeAm- Q_B since its K_d in the Q_B site is >100 M. The dashed line tracks the amplitude of the fast k_{AP}^{obs} indicative of RCs with Me-diMeAm- NQ_A without NQ_B . The dotted curve represents NQ_A . The solid line represents the hybrid Me-diMeAm- $NQ_A:NQ_B$ RCs. The shaded region assumes the quantum yield for electron transfer from Me-diMeAm- NQ_A^- to NQ_B is 75%. The maximum NQ added represents the limit of its solubility.

Figure 7.

The estimated energy for charge separated states in RCs with UQ, NQ and Me-diMeAm-NQ. Energies for UQ containing RCs from (Arata and Parson 1981; Gunner et al. 1982; Woodbury et al. 1986). Values for NQ and Me-diMeAm-NQ (abbreviated in the figure as dMA-NQ_A) containing RCs from tables 1 and 3. The lower limit for P⁺NQ_B⁻ of 55 meV above P⁺UQ_A⁻ (115 meV above P⁺UQ_BUQ_B⁻) is obtained from the negligible slowing of the back reaction rate k_{AP}^{obs} when NQ is added to UQ_A RCs. The G_{AH}° of 260 meV with Me-diMeAmQ_A is obtained from the k_{AP}^{obs} of 3000 s⁻¹ with this quinone. Charge recombination from Me-diMeAm-NQ_A⁻ proceeds through the thermal back reaction via reformation of P⁺H⁻, so charge recombination from Me-diMeAm-NQ_A:NQ_B⁻ will also use this route (Eqn. 7). Using k_{HP} of 7×10⁷ s⁻¹ G_{HB}° is from 480 (k_{BP} =0) to 520 (k_{BP} =0.4 s⁻¹) meV (see Fig. 8). G_{AH}° is 520 meV for UQ_A (Arata and Parson 1981; Gunner et al. 1982; Woodbury et al. 1986), and G_{BH}° is 580 meV with UQ_B. Thus, P⁺NQ_B⁻ is estimated to be 225–260 meV lower in energy than P⁺Me-diMeAm-NQ_A⁻ and 100–125 meV higher in energy than P⁺UQ_B⁻ (see equations 7 and s2-s4 in supplementary material).

Figure 8.

Van't Hoff plot for temperature dependence of the k_{BP}^{obs} in $P^+Me-diMeAm-NQ_A:NQ_B^-$ RCs assuming charge recombination occurs via both the direct route at k_{BP} and the thermal route at k_{BHP} . 1 μ M RC, 2 μ M Me-diMeAm-NQ, 300 μ M NQ, 10 mM Tris pH=7.8, LDAO =0.005%. The solid lines represent the fit to Eqn. 8 with G°_{BH} of 506 meV and k_{BP} varying from 0 to 0.4 s⁻¹. Temperature from 277K to 303K.

Table 1Characterization of replacement quinones at the Q_A site.

Quinone	$\frac{\Delta A_{\max}^{XQ_A}}{\Delta A_{\max}^{UQ_A}}$	$\Phi_{\text{HA}}(\%)$	$k_{\text{AP}}^{\text{obs}} (\text{s}^{-1})$	$G^{\circ}_{\text{HA}} (\text{meV})$
UQ ₁	1	100	10	−520 ^d
diMe-BQ	0.4	70 ^a	1.75	--
triMe-BQ	1	100	1.47	--
DQ	1	100 ^c	10	−550 ^d
NQ	1	100 ^c	7.8	−540 ^d
Me-NQ	1	--	7.4	--
diMe-NQ	1	--	9.5	−500 ^d
Me-diMeAm-NQ	0.5	60 ^b	3000	−257
Decyl-diMeAm-NQ	0.4	ND	550	−300
AQ	0.9	89 ^c	175	−330

All measurements made at 298.2 K, with 1 μM RCs, 10 mM Tris, pH 7.8; 0.005% LDAO. $\Delta A_{\max}^{XQ_A} / \Delta A_{\max}^{UQ_A}$: Maximum A₄₃₀ compares RCs saturated with replacement quinone, XQ_A, and those with UQ_A (eqn. 6).

^a Φ_{HA} : The quantum yield for electron transfer from H[−] to XQ_A measured by the multi-flash technique that monitors the additional P⁺Q_A[−] formed on additional flashes before full charge recombination;

^b measured by light titration (eqn. 5);

^c measured previously (Gunner and Dutton 1989). G°_{HA} , the free energy for the electron transfer from P⁺H[−] to P⁺XQ_A[−] determined from $k_{\text{AP}}^{\text{obs}}$ with Eqn. 7a assuming $k_{\text{HP}} = 7.7 \cdot 10^7 \text{ s}^{-1}$; $k_{\text{AP}} = 10 \text{ s}^{-1}$ under conditions when $k_{\text{AP}}^{\text{obs}} > k_{\text{AP}}$.

^d G°_{AH} obtained previously from delayed fluorescence measurements (Woodbury et al. 1986). Abbreviations for quinones: UQ₁: ubiquinone-1 (2,3-dimethoxy, 5-methyl-BQ); BQ: 1,4-Benzoquinone; diMe-BQ: 2,6-dimethyl-BQ; triMe-BQ: 2,3,5-trimethyl-BQ; DQ: duroquinone (2,3,5,6-tetramethyl-BQ); NQ: 1,4-naphthoquinone; Me-NQ: 2-methyl-NQ; diMe-NQ: 2,3-dimethyl-NQ; Me-diMeAm-NQ: 2-methyl, 3-dimethylamino-NQ; Decyl-diMeAm-NQ: 2-decyl-3-dimethylamino-1,4-naphthoquinone; AQ: 9,10-Anthraquinone.

Table 2

Quinone binding affinity at the Q_B site determined by competitive inhibition of UQ₁ binding with UQ₁₀ at the Q_A site

Quinone	K _d at Q _A (M)	[XQ] (M)	$K_d^{app} U_{Q_B} U_{Q_B}$ (M)	XQ _B K _d (M)	Maximum P ⁺ UQ _B ⁻	$\frac{K_d^{Q_B}}{K_d^{Q_A}}$
UQ ₁	0.1	none	0.8	0.8 0.2	>0.9	8
diMe-BQ	88	42	0.86	>500	0.8	>6
triMe-BQ	16	120	1.3	>180	0.9	>11
DQ	0.8	48	7.1	6.1 2.4	0.5	9
NQ	8	133	4.2	32	0.8	4
Me-NQ	1.4	57	6.8	5.6	0.6	4
diMe-NQ	0.03	300	3.8	100	0.6	3300
Me-diMeAm-NQ	0.06	2	0.82	>100	>0.9	>1700
AQ	0.2	10	0.85	160	>0.9	1000

[XQ]: Concentration of the single addition of XQ added as a competitive inhibitor prior to the UQ₁ titration. K_d^{app}UQ_B: apparent K_d for UQ₁ with XQ present. K_i is determined with equation 4. All measurements made at 298.2 K, with 1 μM RCs, 10 mM Tris, pH 7.8; 0.005% LDAO.

Table 3

The energy level of the quinones at Q_A and Q_B

Q_A	Q_B	$k_{\text{back}} \text{ s}^{-1} \text{ 298K}$	$G^{\circ}_{\text{HA}} \text{ (meV)}$	$G^{\circ}_{\text{HB}} \text{ (meV)}$	$G^{\circ}_{\text{AB}} \text{ (meV)}$	H°	$-T \Delta S^{\circ} \text{ 298K}$
Me-diMeAm-NQ	--	3000	-263			-244	-19
UQ ₁₀	UQ _I	1.0	-520	-581	-61	-193	+132
Me-diMeAm-NQ	NQ	0.5	-257	-477	-220	-188	-300

k_{back} is the apparent rate of charge recombination with only Q_A ($k_{\text{AP}}^{\text{obs}}$), or with Q_A and Q_B ($k_{\text{BP}}^{\text{obs}}$). The G°_{HA} with Me-diMeAm-NQ_A is obtained with equation 7a; G°_{HA} with UQA obtained from delayed fluorescence measurements (Blankenship and Parson 1979; Wraight and Stein 1980; Arata and Parson 1981; Woodbury et al. 1986); The H°_{HA} and S°_{HA} with Me-diMeAm-NQ_A and H°_{AB} and S°_{AB} with UQA:UQB obtained from the temperature dependence of the back reaction (Eqn. 7). The rationale for the thermodynamic parameters for the Me-diMeAm-NQ_A:NQB reaction is given in Figure 7.