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Unstable semiquinone in photosynthetic reaction center

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ABSTRACT Ubiquinone can take up two electrons and two protons upon reduction and serves as essential redox cofactor in several proteins. Out of its 9 possible redox states, only Q and Q^- are seen in the Q_A site and Q, Q^- and QH_2 in the Q_B site of the reaction center of photosynthetic bacterium *Rhodobacter sphaeroides*. The focus of our interest was the investigation of kinetic and energetic aspects of Q^- stability in the Q_B binding site. Under physiological conditions, the semiquinone anion is very stable and it binds more tightly than Q or QH_2. At high light intensity of continuous excitation, however, it binds poorly and favors release to the solution. We attribute the decrease of semiquinone affinity to conformational changes in the Q_B binding site upon repetitive and frequent charge separation (and subsequent very fast recombination) in the photochemically closed reaction center. **Acta Biol Szeged 49(1-2):187-190 (2005)**

KEY WORDS

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The ultimate function of all photosynthetic reaction centers (RC) is to export metastable oxidizing and reducing equivalents which will drive energy consuming reactions as ion transport and ATP synthesis (Wraight 2004). In RC of purple bacteria and in Photosystem II of oxygenic organisms, the reducing agent is dihydroquinones (QH_2), the fully reduced form of quinone (Q) that is established by light-induced intraprotein electron transfer. In bacterial RC, the electron transfer proceeds from the excited singlet state of the bacteriochlorophyll dimer (P*), through a bacteriochlorophyll monomer (B) and a bacteriopheophytine monomer (Bpheo) to the acceptor quinone complex constituted of two quinones (Q_A and Q_B), the central iron atom and the ligand field of four histidines and a glutamate. Q_A and Q_B are both bound with the C1 carbonyl hydrogen bonded to the backbone NH, and the C4 carbonyl hydrogen bonded to the $N_\delta H$ of one of the four histidines liganded to the iron atom. Although both quinones are ubiquinones in RC from *Rhodobacter sphaeroides,* their physico-chemical properties are different due to difference in their environments (Zhu and Gunner 2005). While Q_A makes one-electron chemistry and is tightly bound in both of its oxidized (Q) and semi-reduced (Q^-) states, Q_B makes two-electron chemistry and is in tight binding equilibrium when semi-reduced (Q^-) but in weak binding equilibrium when fully oxidized (Q) or reduced (QH_2).

The reduction of the acceptor quinone system in the presence of external electron donor (cytochrome c^{2+}) is organized in a cycle of light and dark reactions (Okamura and Feher 1995; Gerencsér et al. 2000). After the first (flash) excitation of the RC, $PQ_AQ_B^-$+cyt c^{3+}, after a second (flash) excitation, $PQ_A^-Q_B^-$+cyt c^{3+} are formed because cytochrome c^{2+} can reduce P^+ in both cases. Before and after the second reduction of Q_B, 1-1 protons are taken up yielding Q_BH_2 which exchanges for a new oxidized quinone from the (cell membrane) quinone pool. Overall, two photoreactions oxidize two cytochromes in expense of one (fully) reduced ubiquinone. In case of large (unlimited) pool of the reactants (oxidized quinone and reduced cytochrome), continuous excitation drives the quinone reduction cycle with constant rate.

By measurement of the steady-state rate of turnover of the inhibited acceptor side, we will indicate that the RC at high light intensity can get into a conformational state where the anionic semiquinone in the Q_B pocket is higher in energy than in the catalytic (proximal) site or in solution; therefore, it binds poorly and favors the release.

Materials and Methods

The RC protein from photosynthetic purple bacterium *Rhodobacter sphaeroides* was isolated and purified as described earlier (Maróti and Wraight 1988; Gerencsér et al. 2000). The absorption changes induced Xe flash (FX-200, EG/G) or laser diode were measured by a home-made kinetic absorption spectrometer. The RC concentration was determined from the kinetics of flash-induced charge recombination at 865 nm and the rate of turnover of the RC (induced by continuous illumination in the presence of large quinone and cytochrome pools) from the amount of photo-oxidized cytochrome at 551 nm (Gerencsér et al. 2000).

Results and Discussion

The rate of quinone reduction cycle driven by rectangular shape of laser diode illumination is determined by the slowest step in the turnover. In most cases of physiological conditions, the light intensity is the rate limiting step. Using very high light intensity (characterized by the rate constant of the primary photochemistry, $k_I > 1{,}000\ s^{-1}$) and RC treated by transition metal ions, the protonation of the anionic semiquinone (k_H = 7 s^{-1} at pH 8.4 with Ni^{2+}) becomes the slowest process. The measured rate of cytochrome photo-oxidation, k_{cyt} would be

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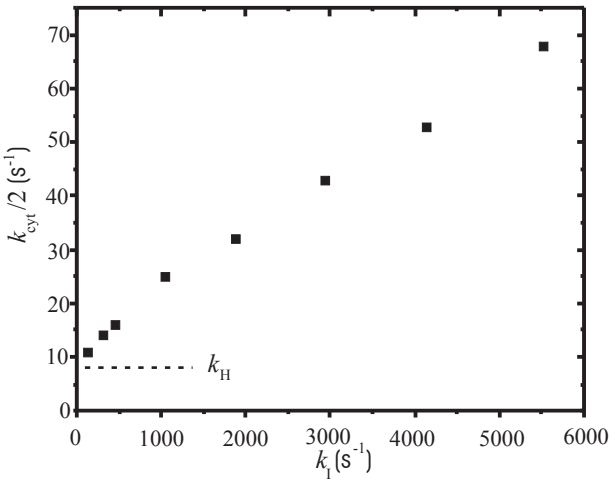

Figure 1. Connection between rates of cytochrome turnover and primary photochemistry (light intensity). Conditions: 1 μM RC, 55 μM cyt c, 70 μM UQ_6, 75 mM NaCl, 100 μM Ni^{2+}, 0.03% Triton X-100 and pH 8.4.

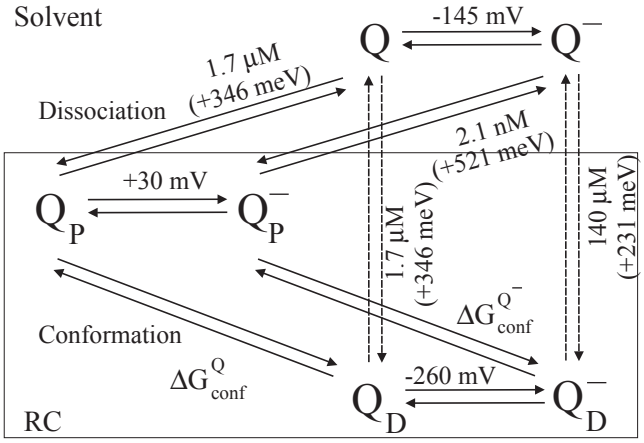

Figure 3. Energetics of the dissociation and conformational change related to the stability of Q and Q^-. The quinone can undergo redox change ($Q \leftrightarrow Q^-$) in solution and in two (distal and proximal) binding sites of the Q_B pocket in the protein at redox midpoint potentials (pH 7) indicated on horizontal arrows. The quinone can dissociate the RC with dissociation constants (dissociation energies) shown on the arrows. The free energy changes of the internal conformation ($\Delta G^{Q^-}_{conf}$ and ΔG^{Q}_{conf}) leading to shift of the quinone position can be calculated from the thermodynamic box.

twice of that of the proton uptake: $k_{cyt} = 2 \cdot k_H$, and would not depend on light intensity at all. In contrast to this expectation, the observed rate of cytochrome photo-oxidation does depend on light intensity and shows significant increase in the light intensity range used in our experiments (Fig. 1).

Our observation cannot be explained by a quinone reduction cycle with widely accepted properties of the intermediates. As the slowest step of the cycle was adjusted to the proton uptake of the unprotonated semiquinone, we can argue for modified kinetic and thermodynamic properties of the semiquinone under high light conditions.

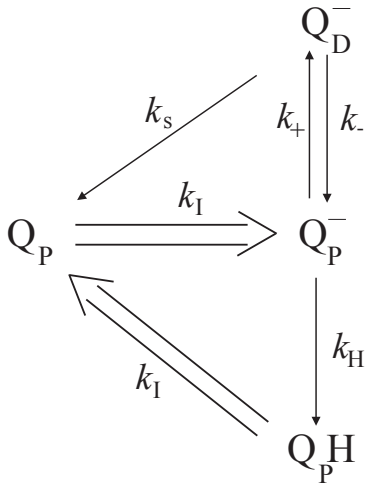

Figure 2. Extended kinetic model of the quinone reduction cycle. The rate limiting step is the uptake of the first proton (rate k_H). Before proton binding, the anionic semiquinone at the proximal (P) site can be shifted to a distal (D) binding site with light intensity-dependent equilibrium constant (k_+/k_-) and from here it can dissociate with rate constant k_s.

The $Q_A^- Q_B^-$ state generated by two successive flashes is stable and (in the dark and without major loss) can wait for the slow protonation of Q_B^-. If, however, the $Q_A^- Q_B^-$ state is exposed to high light excitation, Q_B^- in the binding pocket can be shifted to a new (distal) position where it can be exchanged for Q from the pool much easier than in the catalitic (proximal) site. Although the physical interactions leading to this conformational change in the RC are not yet revealed, the repetitive (5 kHz) and short-lived (10 ns) charge separations (P^+Bpheo^-) by continuous light excitation can certainly contribute to this effect. The conformational transition shortcuts the first half of the regular quinone reduction cycle generating more photo-oxidized cytochomes without significant decrease of the turnover (quinol production). The mechanism can be expressed in coherent kinetic and thermodynamic models.

Kinetic model

The minimum four states model of quinone reduction cycle for large quinone and cytochrome pools and for RC where the proton uptake by the semiquinone is inhibited can be seen in Figure 2. The semiquinone is allowed to equilibrate rapidly between the proximal (P) and the distal (D) positions from where it is exchanged by a pool quinone with rate constant k_s. The expression of steady state rate of cytochrome photo-oxidation normalized to concentration of RC is split into two

terms ($k_I >> k_+, k_- >> k_s, k_H$)

$$k_{cyt} = \frac{2 \cdot k_H}{1+K} + \frac{K}{1+K} \cdot k_S,$$

where K ($= k_+/k_-$) is the equilibrium constant. If $K = 0$ (the normal cycle is not extended by conformational change), the rate of cytochrome oxidation is $2 \cdot k_H$, as expected. By increase of K, however, the second term starts to dominate and saturates at k_s. As K increases with light intensity (the higher is the light intensity, the greater is the shift), our observation of acceleration of cytochrome photo-oxidation upon increasing light intensity (Fig. 1) is qualitatively supported by the model if $k_s >> k_H$.

Thermodynamic model

The free energy changes associated to reduction of quinone $\Delta G^{Q/Q^-} = -F(E_h - E_m)$ in solution, and in proximal and distal positions within the Q_B binding pocket of the RC can be related to free energy changes of dissociation $\Delta G_d = -60$ meV$\cdot \log K_d$ and conformation ΔG_{conf} (Fig. 3). Here F is the Faraday constant (used to convert units of electric potential to units of energy), E_h is the actual redox potential, E_m is the midpoint redox potential and K_d is the dissociation constant. Walking around a thermodynamic box, the sum of the free energies should be 0. Taking values from experiments ($E_{m,P} = +30$ mV, Rutherford and Evans 1980; $K^Q_{d,D} = 1.7$ μM, McComb et al. 1990), and electrostatic calculations ($E_{m,sol} = -145$ mV, $E_{m,D} = -260$ mV, Zhu and Gunner 2005) with reasonable assumption ($K^Q_{d,P} = 1.7$ μM), we can calculate the free energy changes of all transitions (Fig. 3). Here, we call the attention to two conclusions: 1) The dissociation constant of the semiquinone increased (the binding affinity decreased) by about 5 orders of magnitude upon conformational change from P to D positions (from 2.1 nM to 140 μM), and 2) this shift required 290 meV more free energy for semiquinone than for quinone:

$$\Delta G^{Q^-}_{conf} = \Delta G^{Q}_{conf} + 290 \text{ meV},$$

That amount of free energy (6.6 kcal/mol) can cover the energy cost to break H-bonds of semiquinone to His L190 and Glu L212. After invasion of several water molecules to the bottom of the binding pocket, Q^- leaves the catalytic site (P position) and moves to a more distal (D) site.

Possible structural bases of the stabilization of the semiquinone

The observed acceleration of the cytochrome photo-oxidation was attributed to light induced conformational change in the RC which transition lowered the stability of the semiquinone in the Q_B pocket. The stage will be open for characterization of the possible changes if the factors determining the stability of the semiquinone will be surveyed.

The appearance of the negative charge of the anionic semiquinone inside the RC is a rude insult to its dark-adapted equilibrium state and the protein accommodates the new situation. The dielectric response of the RC occurs over an enormous time range from subnanoseconds to seconds. While some responses are very rapid and accompany the appearance of Q_A^- (200 ps), other relaxation processes are slower and follow the interquinone electron transfer (100 μs). The detection of the decay of the delayed fluorescence from re-excited dimer (P*) has proved to be an excellent tool to track the kinetics of the dielectric response of the RC (Filus et al. 2004). Re-distribution of the charges (protons) in the protein is one of the most essential constituents of the dielectric response of the protein. Net proton uptake and internal redistribution of H^+ ions between ionizable residues contribute substantially to the partial shielding, solvation and stabilization of the semiquinones. The appearance of Q_A^- or Q_B^- induces pKa shifts in ionizable groups, equivalent to the electrostatic interaction energy. Surprisingly, but informatively, the stoichiometry of H^+ uptake is quite similar for Q_A^- and Q_B^- and originates from the same residues (Maróti and Wraight 1988). This behavior resides in the structure of the Q_B domain, which features an unusually high density of ionizable residues with a striking excess of acidic groups. The most prominent acidic residues (AspL210, GluL212, AspL213, AspM17 and GluH173) experience significant pK_a changes. Depending on the pH, H^+ ions taken up are mostly distributed among these residues, but also with many small contributions from more weakly coupled residues. The responses to Q_A^- and to Q_B^- largely arise from the same cast of characters in the Q_B domain. This reflects the dearth of ionizable residues around Q_A, which translates into a low effective dielectric that allows the electric field from Q_A^- to spread further.

The stability of Q_B^- depends on the local electric field. Mutations, that cause higher negative electric potential in the Q_B binding pocket, make Q_B^- lifetime smaller (Okamura and Feher 1995). The effect can be conveniently detected by the rate of the $P^+Q_B^- \rightarrow PQ_B$ charge recombination. The AsnM44 → Asp mutant has an extra negative charge which accelerates the back reaction (3 s^{-1}) relative to that measured in wild type (0.7 s^{-1}).

In the semiquinone anion from, Q_B^- is also hydrogen bonded by the OγH of SerL223 that contributes to the stabilization of this species. This proton coupling could be detected by ENDOR signals in the Q_B^- spectrum (Paddock et al. 2005). The ENDOR signal was correlated with the lifetime of the $P^+Q_B^-$ state, as an indicator of the stability of Q_B^-. The results implicate SerL223 in providing much of the stabilization of Q_B^- that also "gates" the interquinone electron transfer. When $P^+Q_B^-$ is prepared at room temperature and frozen in the light, it is stable for at least a year at 100 K ("light bulb" effect, Kleinfeld et al. 1984). When prepared at low temperature, however, it decays in a few seconds - at

least 10^7 times faster. In a mutant with SerL223 replaced by alanine, the charge separated state frozen in the light is much less stable. Tentatively, this is interpreted as indicating that the rotation of SerL223 (OγH) to engage the Q_B carbonyl oxygen is required to stabilize the electron on Q_B^- and is the rate limiting step in the first electron transfer. However, the H-bond switch of SerL223 does not rule out proton transfer (H^+ redistribution) as a factor in this event, since the strongly stabilized Q_B^- state can only be generated in the wild type at room temperature.

Acknowledgments

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