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pH-dependence of the free energy gap between DQ_A and $D^+Q_A^-$ determined from delayed fluorescence in reaction centers from *Rhodobacter sphaeroides* R-26

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The pH dependence of the redox midpoint potential of the Q_A/Q_A^- couple in isolated reaction centers from *Rb. sphaeroides* (Maroti, P. and Wraight, C.A. (1988) Biochim. Biophys. Acta 934, 329–347) is about 3-times larger than predicted from the proton uptake by DQ_A^- (preceding reference and McPherson, P.H., Okamura, M.Y and Feher, G. (1988) Biochim. Biophys. Acta 934, 348–368). To investigate the cause of this discrepancy, we have determined from the delayed fluorescence of reaction centers the pH dependence ($7.0 < pH < 10.0$) of a free energy gap, ΔG_1° between DQ_A and $D^+Q_A^-$, that is related to the midpoint potential. The change in ΔG_1° between pH 7.0 and 10.0 was found to be within experimental error the same as that determined from the proton uptake by $D^+Q_A^-$. This agreement validates the proton uptake method.

The absorption of light by photosynthetic reaction centers results in a charge separation between the primary electron donor D (bacteriochlorophyll dimer) and the primary acceptor quinone, Q_A (ubiquinone-50), forming $D^+Q_A^-$ (reviewed in Ref. 1). The electron is subsequently transferred to a secondary quinone and eventually to exogenous acceptors. The free energy change (redox midpoint potential) associated with the reduction of Q_A partly determines the direction and kinetics of electron flow in the reaction center and therefore must be known in detail for a complete understanding of this process. In this work, we focus on the pH dependence of this free energy change.

The pH dependence of the redox midpoint potential of Q_A/Q_A^- in isolated reaction centers has been determined previously from redox titrations [2] and measurements of the proton uptake by DQ_A^- [2,3]. The results of these two methods are in serious disagreement: the redox titrations indicate that the midpoint potential decreases by about 60 mV per pH ($6 < pH < 10$), whereas the proton uptake indicates a slope of about −20 mV per pH unit *. A measurement of the midpoint potential by a third independent method would, therefore, be helpful in resolving this discrepancy. Unfortunately, there is no good alternative method of measuring the midpoint potential directly; however, its value can be checked indirectly by determining the value of a free energy gap, ΔG_1° between DQ_A and $D^+Q_A^-$ (see Fig. 1), that is related to the midpoint potential.

The free energy gap ΔG_1° has been determined previously at pH 8 from the delayed fluorescence of $D^+Q_A^-$ (see for example, Refs. 4,5). In this work we have measured the delayed fluorescence as a function of pH and from it determined the pH dependence of ΔG_1°. We compare this result for ΔG_1° with that determined from the proton uptake by $D^+Q_A^-$ [3]. An agreement between the results determined from the delayed fluorescence and proton uptake would validate the proton uptake

Abbreviations: D, primary donor; Q_A, primary acceptor.

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* A slope of −60 mV/pH at 300 K for the midpoint potential corresponds to a proton uptake by DQ_A^- of 1 H^+/e^- and that of −20 mV/pH corresponds to about 0.3 H^+/e^- (see Eqn. 5). The semiquinone Q_A^- does not protonate directly; the observed proton uptake that causes the pH dependence of the midpoint potential is due to shifts in the pK values of amino acid residues that interact with Q_A^- [2,3]. The proton uptake can, therefore, be any fraction of a proton depending on the number of interacting residues and the magnitude of their pK shifts (for a more detailed discussion see Ref. 3).

method. The midpoint potential of Q_A/Q_A^- and the free energy gap ΔG_1° are related, since they involve the same interactions between Q_A^- and the protonatable residues (see previous footnote). The advantage of the delayed fluorescence method is that it avoids the use of redox dyes, whose slow equilibration with Q_A [6] could affect the accuracy of the redox titrations. A preliminary account of this work has been presented [7].

The delayed fluorescence is emitted by the small fraction (about 10^{-15}) of reaction centers in the excited state D^*Q_A in equilibrium with $D^+Q_A^-$ following charge separation (see Fig. 1.) The intensity, F_d, of the delayed fluorescence is determined by the radiative rate constant k_f and the concentration of the species D^*Q_A:

$$F_d = k_f \cdot [D^*Q_A]. \tag{1}$$

Since the species D^*Q_A and $D^+Q_A^-$ are in thermal equilibrium, their concentrations are related by the Boltzmann factor determined by the free energy difference $\Delta G_2^\circ - \Delta G_1^\circ$ (see Fig. 1):

$$F_d = k_f \cdot [D^+Q_A^-] e^{-(\Delta G_2^\circ - \Delta G_1^\circ)/kT} \tag{2}$$

where ΔG_2° is the free energy gap between DQ_A and D^*Q_A, k is Boltzmann's constant, and T is the absolute temperature. The species $D^+Q_A^-$ decays by charge recombination at a rate k_{AD} (see Fig. 1). The value of F_d immediately after charge separation depends on the initial concentration of $D^+Q_A^-$, i.e., $[D^+Q_A^-]_0$:

$$F_d = k_f \cdot [D^+Q_A^-]_0 \, e^{-(\Delta G_2^\circ - \Delta G_1^\circ)/kT} \qquad (t = 0). \tag{3}$$

The pH dependence of ΔG_1° can be obtained in terms of F_d by rearranging Eqn. 3 and cancelling the pH-independent ($7.0 < pH < 10.0$) parameters: k_f, ΔG_2° and $[D^+Q_A^-]_0$ **:

$$\Delta G_1^\circ(pH_2) - \Delta G_1^\circ(pH_1) = kT \ln\left\{\frac{F_d(pH_2)}{F_d(pH_1)}\right\} \qquad (7.0 < pH < 10.0,\ t = 0). \tag{4}$$

The pH-dependence of ΔG_1° obtained independently from the proton uptake (H^+/e^-) by $D^+Q_A^-$ is given by the thermodynamic relation [3]:

$$\Delta G_1^\circ(pH_2) - \Delta G_1^\circ(pH_1) = 2.3kT \int_{pH_1}^{pH_2} (H^+/e^-)\, dpH. \tag{5}$$

In this work we compare the results obtained from Eqns. 4 and 5.

Reaction centers were isolated from *Rb. sphaeroides* R-26 as described [9]. The concentration of reaction centers was determined from the absorption at 802 nm, and the extinction coefficient $\epsilon^{802} = 288\ mM^{-1} \cdot cm^{-1}$ [10]. The reaction centers were depleted of the secondary acceptor quinone Q_B [11] to obtain 0.7 quinones per reaction center as determined by a cytochrome *c* photooxidation assay [12] (significantly less than 1 quinone per reaction center was chosen to insure the absence of Q_B). During the delayed fluorescence experiments the pH was adjusted by adding 1 M KOH or 1 M potassium acetate (pH 4.5). The delayed fluorescence was detected at a right angle to the incident laser flash by a photomultiplier (Hamamatsu R632) that was protected by a shutter and a filter (Schott RG-830) to block wavelengths less than 830 nm. The data were digitized (Biomation model 802 transient recorder) and transferred to a computer for analysis (see Fig. 2 for other experimental details.)

The effect of pH on the delayed fluorescence is shown in Fig. 2. The state $D^+Q_A^-$ in Q_B-depleted reaction centers was formed by a laser flash. The photomultiplier was protected from the intense prompt fluorescence (emitted prior to charge separation) by a

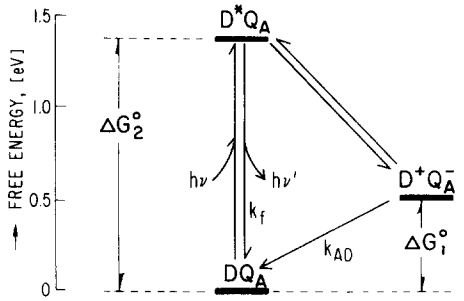

Fig. 1. Simplified energy level scheme showing the photon absorption/emission and electron transfer reactions discussed in this work. Following absorption of a photon, charge separation occurs at a rate of about $10^{10}\ s^{-1}$. Most of the reaction centers ($\gg 99\%$) decay to the ground state DQ_A by charge recombination ($k_{AD} \cong 10\ s^{-1}$). A small fraction decays by fluorescence (delayed fluorescence) from the state D^*Q_A which is in equilibrium with $D^+Q_A^-$. This fraction, and, therefore, the intensity of the fluorescence, depends on the free energy difference $\Delta G_2^\circ - \Delta G_1^\circ$.

** k_f and ΔG_2° were determined to be independent of pH by measuring the absorption spectrum (dimer band at about 865 nm) as a function of pH. A change in ΔG_2° (the 0-0 transition energy) would cause a shift in the position of the dimer band; we observed no shift between pH 7.0 and 10.0 (detection limit ≈ 1 nm). According to the Strickler-Berg relation [8], k_f is proportional to the integral: $\int(A/\nu)d\nu$, where A is the absorption, ν the frequency, and the integration is over the dimer band; since we observed no shifts in the position, shape or area of the dimer band between pH 7.0 and 10.0 we conclude that k_f does not change significantly in this range. At pH < 7.0 and > 10.0 the position of the maximum absorption varied with pH, e.g., by 12 nm between pH 5.0 and 7.0. We, therefore, limited our analysis to pH 7.0–10.0. The concentration $[D^+Q_A^-]_0$ depends on the occupancy of the Q_A site (70%), the quantum yield of charge separation ($\approx 100\%$), and the extent of light-saturation ($\approx 100\%$). $[D^+Q_A^-]_0$ was determined from the extent of bleaching at 865 nm to be independent of pH ($7 < pH < 10$) at saturating light-intensities.

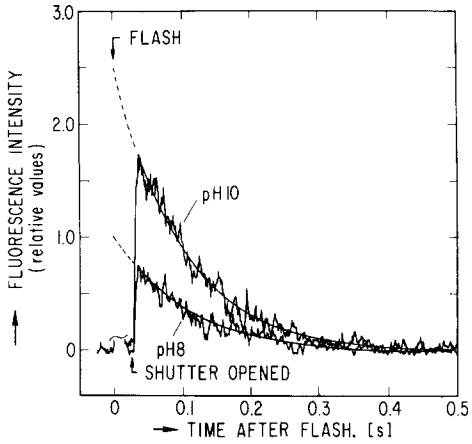

Fig. 2. Effect of pH on the delayed fluorescence from $D^+Q_A^-$. The detector was protected from the intense 'prompt' fluorescence by a shutter which was opened 30 ms after the flash. The increase in fluorescence intensity with pH is caused by an increase in the free energy gap ΔG_1° between DQ_A and $D^+Q_A^-$ (see Fig. 1 and Eqn. 4). The decay of the intensity is due to the charge recombination $D^+Q_A^- \rightarrow DQ_A$. The initial intensity was obtained by fitting the data with a single exponential decay (solid line) and extrapolating to the time of the flash (dashed line). The transient spike at $t = 0$ is an electrical artifact from the laser. 100 traces were averaged. Illumination: laser flash (Phase R DL2100A, 590 nm, 0.5 μs in duration.) Detector: S1 photomultiplier (Hamamatsu R632) protected with a shutter and a filter (Schott RG-830) to block wavelengths less than about 830 nm. Conditions: 2.8 μM reaction centers (0.7 quinones per reaction center), 0.03% lauryldimethylamine *N*-oxide (LDAO), 2 mM 4-morpholineethanesulfonic acid (Mes), 2 mM 3-(cyclohexylamino)-1-propanesulfonic acid (Caps), 2 mM 2-amino-2-hydroxymethylpropane-1,3-diol (Tris), 2 mM cyclohexylaminoethanesulfonic acid (Ches), 2 mM 3-(4-morpholino)propanesulfonic acid (Mops), 50 mM KCl, $T = 23^\circ$C, pH as indicated.

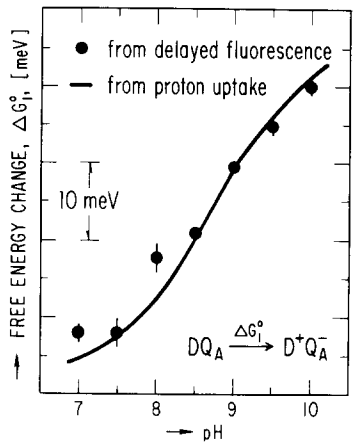

Fig. 3. Comparison of the pH-dependences of ΔG_1° determined by two independent methods. ΔG_1° (filled circles) was determined from the initial amplitude of the delayed fluorescence from $D^+Q_A^-$ (see Fig. 2) using Eqn. 4. Each filled circle represents the average of 2–5 measurements and the error bars indicate the standard deviation of the mean. ΔG_1° (line) was determined from the proton uptake by $D^+Q_A^-$ [3] using Eqn. 5. The uncertainty in the total change in ΔG_1° (line) between pH 7 and 10 was estimated from the uncertainty in the proton uptake data to be about 2 meV. The absolute value of ΔG_1° (e.g., integration constant in Eqn. 5) was not determined in this work; the relative offset of the two curves (filled circle and line) along the ordinate was adjusted to obtain the best agreement between them.

shutter, which was opened 30 ms after the flash. As can be seen (Fig. 2), the pH has a strong effect on the intensity of the delayed fluorescence. The intensity was reversible with pH ($7.0 < pH < 10.0$) within the precision of the measurement (i.e., within about 6% of the intensity at pH 8.0). The intensity decayed at a rate of about 10 s^{-1}, corresponding to the charge recombination $D^+Q_A^- \rightarrow DQ_A$ (see Fig. 1). The initial intensity of the fluorescence (i.e., at $t = 0$) was obtained by fitting the data with a single exponential decay [13] (solid line, Fig. 2) and extrapolating to $t = 0$ (dashed line, Fig 2)***.

The pH dependence of ΔG_1° (filled circles in Fig. 3) was obtained from the initial intensity of the fluorescence (Fig. 2) using Eqn. 4. The change in ΔG_1° between pH 7.0 and 10.0 was found to be 32 ± 2 meV. These data are in agreement with those (line in Fig. 3) obtained by integrating the proton uptake by $D^+Q_A^-$ (obtained from Ref. 3) using Eqn. 5. From the proton uptake we determined the change in ΔG_1° between pH 7.0 and 10.0 to be 36 ± 2 meV.

The agreement between the delayed fluorescence and proton uptake results validates the method used to measure proton uptake [2,3]. The discrepancy between the proton uptake [2,3] and redox titration [2] results remains puzzling. The discrepancy may be due to the different methodologies used to measure the proton uptake and redox potential. One important difference between the two experiments is the method of reduction: in the proton uptake and delayed fluorescence experiments, Q_A was photoreduced (i.e., internally reduced by the dimer) whereas in the redox titrations, Q_A was chemically (externally) reduced. The possibility that the method of reduction affects the protonation is supported by the observation that the EPR linewidth of Q_A^- is different in photo and chemically reduced reaction centers [16]. In photoreduced reaction centers the linewidth was found to be 290 G, independent of pH; in chemically reduced reaction centers, the linewidth in-

*** The recombination rates obtained from the fits to the data are: pH 8.0: $k_{AD} = 9.7 \pm 0.1$ s^{-1}; pH 10.0: $k_{AD} = 10.1 \pm 0.1$ s^{-1}. These values disagree by $\approx 10\%$ with those obtained from optical absorption changes (pH 8.0: $k_{AD} = 9.0 \pm 0.1$ s^{-1}; pH 10.0: $k_{AD} = 11.2 \pm 0.1$ s^{-1} [14]). Woodbury et al. [5] observed a similar discrepancy and suggested that it is due to the presence of two different binding conformations for Q_A^- that exhibit slightly different recombination rates. The delayed fluorescence signal would be weighted in favor of reaction centers with Q_A in the higher energy conformation and would therefore decay at a different observed rate than the absorption changes. The crystal structure of the reaction center from *Rb. sphaeroides* [15] indicates that Q_A could move as much as 2 Å without any steric hindrance. This would not affect significantly the results obtained in this work since the interactions between Q_A^- and the protonatable residues that affect ΔG_1^0 are belived to occur over much larger distances (> 10 Å) [3].

creased from 270 to 340 G from pH 6.0 to pH 8.5. Determination of the proton uptake associated with the chemical reduction of Q_A may help to solve this puzzle.

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