CHARGE RECOMBINATION KINETICS AS A PROBE OF PROTONATION OF THE PRIMARY ACCEPTOR IN PHOTOSYNTHETIC REACTION CENTERS

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ABSTRACT The kinetics of the charge recombination $D^+Q_A^- \rightarrow DQ_A$ was used to probe the protonation of the primary acceptor in reaction centers from *Rhodopseudomonas sphaeroides*, in which the native ubiquinone was replaced by anthraquinone. We found that Q_A^- is stabilized by the rapid ($t < 10^{-2}$ s) binding of a proton, with a pK of 9.8. The distance between Q_A^- and the proton binding site was estimated to be larger than ~5 Å.

The absorption of light by photosynthetic reaction centers (RCs) leads to a separation of charge between the electron donor, D(a bacteriochlorophyll dimer), and the primary (quinone) acceptor, Q_A, forming $D^+Q_A^-$ (for review, see references 1 and 2). Here we examine the protonation events accompanying the charge separation. Redox titrations of the Q_A/Q_A^- couple (3–5; for review, see reference 6) indicate that Q_A^- associates with a proton ($pK_A = 9.8$). The same conclusion was reached from studies on the kinetics of electron transfer between Q_A^- and the secondary acceptor, Q_B (7). Spectroscopic measurements, however, indicate that the proton is not directly bound to Q_A^-. The optical absorption spectrum of Q_A^- at neutral pH (8–10) resembles that of the unprotonated semiquinone anion (11, 12). Similarly, the EPR spectrum of Q_A^- (13–16) is characteristic of the unprotonated anion (16).

To probe for a protonation site in the vicinity of Q_A, we studied the charge recombination kinetics $D^+Q_A^- \rightarrow DQ_A$, with anthraquinone (AQ) replacing the native ubiquinone (UQ). With AQ, as opposed to UQ, the charge recombination rate, k_{obs}, is sensitive to small perturbations in the free energy of Q_A^- (17, 18). Thus k_{obs} should be sensitive to a shift in the energy of Q_A^- caused by the electrostatic interaction with a nearby proton. A preliminary account of this work has been presented (19).

The charge recombination process of $D^+Q_A^-$ is schematically illustrated in Fig. 1; $k_{H^+}^{on}$ and $k_{H^+}^{off}$ are the rate for proton binding and release, and $k_{obs}^{H^+}$ and k_{obs}^{o} are the recombination rates in the presence and absence of a proton, respectively. The electron on Q_A^- recombines with D^+ via thermal repopulation of the state $D^+I^-Q_A$, where I is the intermediate acceptor (2). The recombination rate depends on the free energy difference, ΔG^0, between $D^+I^-Q_A$ and $D^+IQ_A^-$ (18). The shift in this energy difference, δG^0, caused by the binding of a proton, results in a change in the rate given by (18):

$$k_{obs}^{H^+} = k_{obs}^{0}\, e^{-\delta G^0/k_b T}, \qquad (1)$$

where k_b is Boltzmann's constant and T the absolute temperature.

The time dependence of the recombination kinetics, k_{obs}, will depend on whether the proton equilibration rate, $k_{on}^{H^+} + k_{off}^{H^+}$ (Fig. 1), is fast or slow with respect to the charge recombination rates $k_{obs}^{H^+}$ and k_{obs}^{0}. If the proton equilibration rates is fast, the states[1] $(D^+Q_A^-)H^+$ and $D^+Q_A^-$ are in equilibrium on the time scale of the charge recombination; the observed kinetics will follow a single exponential decay, with k_{obs} given by the sum of $k_{obs}^{H^+}$ and k_{obs}^{0} weighted by the fraction of unprotonated and protonated RCs, respectively, i.e.,

$$k_{obs} = \frac{k_{obs}^{0} + 10^{(pK_A - pH)}\, k_{obs}^{H^+}}{1 + 10^{(pK_A - pH)}}, \qquad (2)$$

where

$$(pK_A - pH) = \log \frac{[(D^+Q_A^-)H^+]}{[D^+Q_A^-]} = \log \frac{k_{on}^{H^+}}{k_{off}^{H^+}}. \qquad (3)$$

When the proton equilibration rate is slow compared with $k_{obs}^{H^+}$ and k_{obs}^{0}, the states $(D^+Q_A^-)H^+$ and $D^+Q_A^-$ do not interconvert on the time scale of the charge recombina-

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[1]These states contain the intermediate state, *I*, which we have omitted for the sake of simplicity.

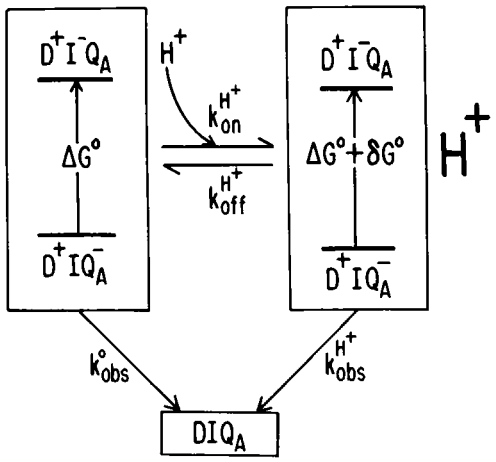

FIGURE 1 Schematic representation of the change in energy levels of D^+I^-Q and $D^+IQ_A^-$ upon protonation. When the primary quinone is anthraquinone, the charge recombination $D^+IQ_A^- \xrightarrow{k_{obs}} DIQ_A$ proceeds via the thermally excited state $D^+I^-Q_A$ and depends, therefore, exponentially on the energy difference $\Delta G^0 + \delta G^0$. (18)

tion, and the observed kinetics will follow two expoential processes; RCs in the state $D^+Q_A^-$ recombine with rate k_{obs}^0, while those in the state $(D^+Q_A^-)H^+$ recombine with rate $k_{obs}^{H^+}$.

The recombination kinetics were measured by monitoring the optical absorption change ΔA^{865}; this change corresponds to the formation and subsequent decay of D^+ (1). Experiments were performed using RCs isolated from *R. sphaeroides* R-26 (1), depleted of the native UQ (20), and reconstituted with either AQ or UQ (18, 20). Flash-induced charge separation was accomplished with a pulsed dye laser (λ_0 = 584 nm, 0.4 μs pulse width, 0.2 J/pulse). Changes in optical absorption were recorded with a spectrophotometer of local design (7). Experimental conditions were as described (7).

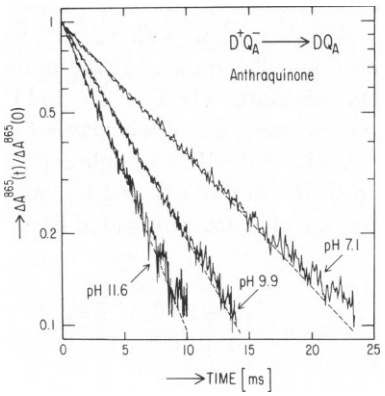

FIGURE 2 Semilogarithmic plot of the change in optical absorption at 865 nm after a single, saturating flash of light (0.4 μs pulse width, 0.2 J/pulse) for different values of pH. The contribution to the kinetics from a residual (~10%) population of RCs containing UQ was measured before the addition of AQ and was subtracted in the data shown. The solid lines represent the best fit of the function $\Delta A^{865}(t)/\Delta A^{865}(0) = e^{-k_{obs}t}$ to the data. The observed kinetics were independent of the light intensity for 10^5 W/cm^2 < I < 10^6 W/cm^2. Conditions: To 1.8 μM RCs depleted of UQ were added, 20 μM AQ in either 10 mM PIPES (piperazine-*N*, *N*'-bis-[2-ethanesulfonic acid]) (pH 7.1), 10 mM CHES (cyclohexylamino-ethanesulfonic acid) (pH 9.9) or 10 mM CAPS [3-(cyclohexylamino)-propanesulfonic acid] (pH 11.6), and 0.025% (wt/vol) LDAO (lauryl dimethylamine-*N*-oxide) at T = 21°C.

The recovery kinetics $D^+ \rightarrow D$ are shown in Fig. 2. The decrease in the recovery rate with decreasing pH implies that $D^+Q_A^-$ is stabilized by the presence of a proton. The kinetics followed a single-exponential decay at all pH values (Fig. 2), implying that the proton binds rapidly compared with k_{obs}, i.e.,

$$k_{on}^{H^+} + k_{off}^{H^+} \gg k_{obs} \sim 10^2 s^{-1} . \quad (4)$$

This limit is consistent with the results of proton uptake measurements (21–23); the stoichiometry of the measured uptake is, however, controversial (21–24).

The pH dependence of k_{obs} is shown in Fig. 3. The value of k_{obs} at low pH is in agreement with previous findings (17, 18, 25). To verify that the increase in k_{obs} at high pH was not an artifact of the quinone removal and reconstitution process, measurements were performed with RCs reconstituted with UQ. The rate k_{obs} changed by <25% over the range 6 < pH < 11, in agreement with results for native RCs (7). The solid line in Fig. 3 represents the best fit of the kinetics model (Eq. 2) to the data with $k_{obs}^{H^+}$ = 97 s^{-1}, k_{obs}^0 = 230 s^{-1} and pK_A = 9.8. Note that the value of pK_A matches that found from both redox titrations (3–5) and electron transfer (7).

The interaction energy of Q_A^- with a nearby proton depends on $k_{obs}^{H^+}$ and k_{obs}^0 (see Eq. 1). The energy splitting δG^0 is given by (Eq. 1 with T = 21°C):

$$\delta G^0 = k_b T \ln \frac{k_{obs}^0}{k_{obs}^{H^+}} = 22 \text{ meV} . \quad (5)$$

The magnitude of δG^0 is approximately two orders of magnitude smaller than the optical transition energy of

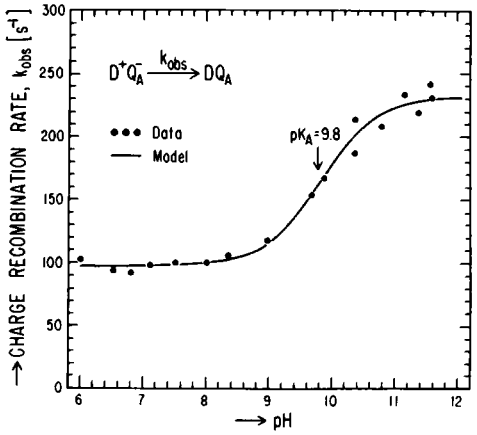

FIGURE 3 The pH dependence of the charge recombination rate k_{obs}. The solid line (Model) was calculated using Eqs. 2 and 3 with pK_A = 9.8, $k_{obs}^{H^+}$ = 97 s^{-1} and k_{obs}^0 = 230 s^{-1}. Conditions as in Fig. 2, except for varying buffers and pH.

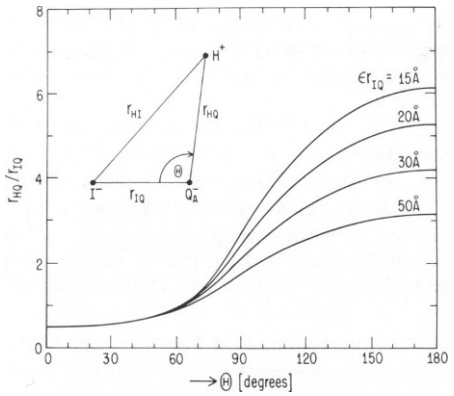

FIGURE 4 The normalized proton to Q_A^- distance, (r_{HQ}/r_{IQ}), as a function of the angle, Θ, between the $I^- - Q_A^-$ and the $H^+ - Q_A^-$ axes, for different values of ϵr_{IQ}. The curves were calculated using Eqs. 5 and 6. Inset shows the geometrical arrangement of the reactants.

Q_A^- (9–11). This may explain why the optical spectrum of Q_A^- is essentially unaffected by the binding of a proton.[2]

From the value of δG^0, one can make a rough estimate of the location of the proton binding site relative to Q_A^-. We assume that the interaction of the proton with both Q_A^- and I^- is electrostatic in origin, as has been done to explain the effects of point charges in other photosynthetic systems (26–29). The change in energy δG^0 is caused by the difference in the distance of the proton to Q_A^- (i.e., r_{HQ}) compared with the distance to I^- (i.e., r_{HI}). From Coulomb's law we obtain[3]

$$\delta G^0 = \frac{-e^2}{\epsilon r_{HI}} - \frac{-e^2}{\epsilon r_{HQ}}$$

$$= \frac{-e^2}{\epsilon r_{IQ}}\left[\frac{1}{[1 + (r_{HQ}/r_{IQ})^2 - 2(r_{HQ}/r_{IQ})\cos\theta]^{1/2}} - \frac{1}{(r_{HQ}/r_{IQ})}\right], \quad (6)$$

where e is the electronic charge, ϵ is the effective dielectric constant, r_{IQ} is the distance between the I^- and Q_A^- and Θ is the angle between the $I^- - Q_A^-$ and $H^+ - Q_A^-$ axes (see insert in Fig. 4). The distance r_{IQ} has been estimated from spectroscopic measurements to be 8–12 Å (30,31). The value of ϵ is difficult to estimate when one is dealing with distances on the scale of atomic dimensions[4] (see references 32–35). Consequently, we have not assumed a specific value of ϵ, but have calculated distances in terms of ϵr_{IQ}. By equating Eqs. 5 and 6, permissible combinations of (r_{HQ}/r_{IQ}) and θ were determined for the range of 15 Å $\leq \epsilon r_{IQ} \leq$ 50 Å (Fig. 4). The minimum distance of the proton to Q_A^- occurs when the proton is situated about halfway between I^- and Q_A^-, i.e., $\theta = 0$. At this location, $r_{HQ} \simeq 0.5\, r_{IQ} \simeq 5$ Å; the ratio r_{HQ}/r_{IQ} is essentially independent of ϵr_{IQ}.

In conclusion, we have shown that a proton is associated with Q_A^- at a distance $\gtrsim 5$ Å. This relatively large distance may explain why the protonation has not been observed in either the optical or EPR spectrum of $(Q_A^-)H^+$. The pK that we determined (9.8) is in agreement with the values found from redox (3–5) and electron transfer (7) studies.

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[2]Although δG^0 represents the change in energy difference between two states (Fig. 1), the shifts in the individual energies are expected to be also small with respect to the optical transition energy.

[3]We assume that the relative distances between the reactants D^+, I^-, Q_A^- do not change upon protonation.

[4]It will be of interest to obtain the value of ϵ when the distances will have been determined from crystallographic and/or electron nuclear double resonance measurements.

REFERENCES

1. Feher, G., and M. Y. Okamura. 1978. Chemical composition and properties of reaction centers. *In* The Photosynthetic Bacteria. W. R. Sistrom and R. K. Clayton, editors. Plenum Publishing Corp., New York. 349–386.
2. Parson, W. W., and B. Ke. 1982. Primary photochemical reactions. *In* Photosynthesis: Energy Conversion by Plants and Bacteria. Govindjee, editor. Academic Press, New York. 331–385.
3. Prince, R. C., and P. L. Dutton. 1976. The primary acceptor of bacterial photosynthesis: its operating midpoint potential? *Arch. Biochim. Biophys.* 172:329–334.
4. Rutherford, A. W., and M. C. W. Evans. 1980. Direct measurement of the primary and secondary quinone electron acceptors in *Rhodopseudomonas sphaeroides* (wild type) by EPR spectroscopy. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 110:257–261.
5. Wraight, C. A. 1981. Oxidation-reduction physical chemistry of the acceptor quinone complex in bacterial reaction centers: evidence for a new model of herbicide activity. *Isr. J. Chem.* 21:384–354.
6. Prince, R. C., and P. L. Dutton. 1978. Protonation and the reducing potential of the primary electron acceptor. *In* The Photosynthetic Bacteria. W. R. Sistrom and R. K. Clayton, editors. Plenum Publishing Corp., New York. 439–453.
7. Kleinfeld, D., M. Y. Okamura, and G. Feher. 1984. Electron transfer in reaction centers of *Rhodopseudomonas sphaeroides*. I. Determination of the charge recombination pathway of $D^+Q_AQ_B^-$ and free energy and kinetic relations between $Q_A^-Q_B$ and $Q_AQ_A^-$. *Biochim. Biophys. Acta.* 766:126–140.
8. Clayton, R. K., and S. C. Straley. 1972. Photochemical electron transport in photosynthetic reaction centers. IV. Observations related to reduced photoproducts. *Biophys. J.* 12:1221–1234.
9. Slooten, L. 1972. Electron acceptors in reaction acceptor preparations from photosynthetic bacteria. *Biochim. Biophys. Acta.* 275:208–218.
10. Verméglio, A., and R. K. Clayton. 1977. Kinetics of electron transfer between the primary and the secondary electron acceptor in reaction centers from *Rhodopseudomonas sphaeroides*. *Biochim. Biophys. Acta.* 461:159–165.
11. Morrison, L. E., J. E. Schelhorn, T. M. Cotton, C. L. Bering, and P. A. Loach. 1982. Electrochemical and spectral properties of ubiquinone and synthetic analogs: relevance to bacterial photosynthesis. *In* Function of Quinones in Energy Conserving Systems. B. L. Trumpower, editor. Academic Press, New York. 35–58.
12. Benasson, R., and E. J. Land. 1973. Optical and kinetic properties of semireduced plastoquinone and ubiquinone: electron acceptors in photosynthesis. *Biochim. Biophys. Acta.* 325:175–181.
13. Loach, P. A., and R. L. Hall. 1972. The question of the primary

acceptor in bacterial photosynthesis. *Proc. Natl. Acad. Sci. USA.* 69:786–790.

14. Feher, G., M. Y. Okamura, and J. D. McElroy. 1972. Identification of an electron acceptor in reaction centers of *Rhodopseudomonas sphaeroides* by EPR spectroscopy. *Biochim. Biophys. Acta.* 267:222–226.
15. Gast, P., and A. J. Hoff. 1979. Transfer of light-induced electron spin-polarization from the intermediate acceptor to the prereduced primary acceptor in the reaction center of photosynthetic bacteria. *Biochim. Biophys. Acta.* 548:520–535.
16. Hales, B. J., and E. E. Case. 1981. Immobilized radicals. IV. Biological semiquinone anions and neutral semiquinones. *Biochim. Biophys. Acta.* 637:291–302.
17. Gunner, M. R., D. M. Tiede, R. C. Prince, and P. L. Dutton. 1982. Quinones as prosthetic groups in membrane electron-transfer proteins. I. Systematic replacement of the primary ubiquinone of photochemical reaction centers with other quinones. *In* Function of Quinones in Energy Conserving Systems. B. L. Trumpower, editor. Academic Press, New York. 265–269.
18. Gopher, A., Y. Blatt, M. Schönfeld, M. Y. Okamura, G. Feher, and M. Montal. 1985. The effect of an applied electric field on the charge recombination kinetics in reaction centers reconstituted in planar lipid bilayers. *Biophys. J.* 48:311–320.
19. Kleinfeld, D., M. Y. Okamura, and G. Feher. 1984. Charge recombination kinetics in reaction centers with anthraquinone as the primary acceptor: evidence for protonation accompanying the formation of $D^+Q_A^-$. *Biophys. J.* 45(2, Pt.2):256*a*. (Abstr.)
20. Okamura, M. Y., R. A. Isaacson, and G. Feher. 1975. The primary acceptor in bacterial photosynthesis: the obligatory role of ubiquinone in photoactive reaction centers of *Rhodopseudomonas sphaeroides. Proc. Natl. Acad. Sci. USA.* 72:3491–3495.
21. Marinetti, T. 1984. Transient conductivity changes in solutions of photoexcited reaction centers. *Biophys. J.* 45(2, Pt. 2):217*a*. (Abstr.)
22. Marotti, P., and C. A. Wraight. 1985. First flash proton binding by the acceptor quinone complex of reaction centers from *Rhodopseudomonas sphaeroides. Biophys. J.* 47(2, Pt. 2):5*a*. (Abstr.)
23. Cogdell, R. J., R. C. Prince, and A. R. Crofts. 1973. Light induced H^+-uptake catalyzed by photochemical reaction centers from *Rhodopseudomonas sphaeroides. FEBS (Fed. Eur. Biochem. Soc.) Lett.* 35:204–208.
24. Wraight, C. A., R. J. Cogdell, and R. K. Clayton. 1975. Some experiments on the primary acceptor in reaction centers from *Rhodopseudomonas sphaeroides. Biochim. Biophys. Acta.* 396:242–249.
25. Okamura, M. Y., R. J. Debus, D. Kleinfeld, and G. Feher. 1982. Quinone binding sites in reaction centers from photosynthetic bacteria. *In* Function of Quinones in Energy Conserving Systems. B. L. Trumpower, editor. Academic Press, New York. 299–317.
26. Pellin, M. J., C. A. Wraight, and K. Kaufmann. 1978. Modulation of the primary electron transfer rate in photosynthetic reaction centers by reduction of a secondary acceptor. *Biophys. J.* 24:351–369.
27. Davis, R. C., S. L. Ditson, A. F. Fentiman, and R. M. Pearlstein. 1981. Reversible wavelength shifts of chlorophyll induced by a point charge. *J. Am. Chem. Soc.* 103:6823–6826.
28. Rackovsky, S., and H. Scher. 1982. Effect of neighboring charges and electric fields on photosynthetic electron transfer. *Biochim. Biophys. Acta.* 681:152–160.
29. Eccles, J., and B. Honig. 1983. Charged amino acids as spectroscopic determinants for chlorophyll *in vivo. Proc. Natl. Acad. Sci. USA.* 80:4959–4962.
30. Peters, K., Ph. Avouris, and P. M. Rentzepis. 1978. Picosecond dynamics of primary electron transfer processes in bacterial reaction centers. *Biophys. J.* 23:207–217.
31. Okamura, M. Y., R. A. Isaacson, and G. Feher. 1979. Spectroscopic and kinetic properties of the transient intermediate acceptor in reaction centers of *Rhodopseudomonas sphaeroides. Biochim. Biophys. Acta.* 546:394–417.
32. Pethig, R. 1979. Dielectric and Electrical Properties of Biological Materials. John Wiley & Sons, New York.
33. Warshel, A., S. T. Russell, and A. K. Churg. 1984. Macroscopic models for studies of electrostatic interactions in proteins: limitations and applicability. *Proc. Natl. Acad. Sci. USA.* 81:4785–4789.
34. Honig, B. H., and W. L. Hubbell. 1984. Stability of "salt bridges" in membrane proteins. *Proc. Natl. Acad. Sci. USA.* 81:5412–5416.
35. Warshel, A., and S. T. Russell. 1984. Calculations of electrostatic interactions in biological systems and in solutions. *Q. Rev. Biophys.* 17:283–422.