Biochimica et Biophysica Acta, 548 (1979) 309—327

BBA 47746

ELECTRON ACCEPTORS OF BACTERIAL PHOTOSYNTHETIC REACTION CENTERS

II. H^+ BINDING COUPLED TO SECONDARY ELECTRON TRANSFER IN THE QUINONE ACCEPTOR COMPLEX *

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(Received October 26th, 1978)
(Revised manuscript received April 20th, 1979)

Key words: Bacterial photosynthesis; Reaction center; Quinone; Electron transfer; Proton transfer

Summary

The photoreduction of ubiquinone in the electron acceptor complex (Q_IQ_{II}) of photosynthetic reaction centers from *Rhodopseudomonas sphaeroides*, R26, was studied in a series of short, saturating flashes. The specific involvement of H^+ in the reduction was revealed by the pH dependence of the electron transfer events and by net H^+ binding during the formation of ubiquinol, which requires two turnovers of the photochemical act. On the first flash Q_{II} receives an electron via Q_I to form a stable ubisemiquinone anion ($\dot{Q}^-_{II}$); the second flash generates $\dot{Q}^-_I$. At low pH the two semiquinones rapidly disproportionate with the uptake of 2 H^+, to produce $Q_{II}H_2$. This yields out-of-phase binary oscillations for the formation of anionic semiquinone and for H^+ uptake. Above pH 6 there is a progressive increase in H^+ binding on the first flash and an equivalent decrease in binding on the second flash until, at about pH 9.5, the extent of H^+ binding is the same on all flashes. The semiquinone oscillations, however, are undiminished up to pH 9. It is suggested that a non-chromophoric, acid-base group undergoes a pK shift in response to the appearance of the anionic semiquinone and that this group is the site of protonation on the first flash. The acid-base group, which may be in the reaction center protein, appears to be subsequently involved in the protonation events leading to fully reduced ubiquinol. The other proton in the two electron reduction of ubiquinone is always

* This work was presented in part at the 22nd Annual Meeting of the Biophysical Society, March 27—30, 1978, Washington, DC.

taken up on the second flash and is bound directly to $\dot{Q}_{II}^{-}$. At pH values above 8.0, it is rate limiting for the disproportionation and the kinetics, which are diffusion controlled, are properly responsive to the prevailing pH. Below pH 8, however, a further step in the reaction mechanism was shown to be rate limiting for both H^+ binding electron transfer following the second flash.

Introduction

The photochemical events of photosynthesis result in a charge separation between the chlorophyllous primary donor and a metastable primary acceptor. In purple photosynthetic bacteria and in Photosystem II of higher plants, this acceptor species was shown to be a quinone [1—6], specialized in some way by its environment; in bacteria this seems to involve association with an iron atom [2,4] which gives rise to a very characteristic ESR signal of the reduced semiquinone form, centered at $g = 1.82$ [7].

Observation of oscillatory phenomena with a period of two in PS II of green plants led to the suggestion that two acceptor species were active in series, such that electrons were accumulated in pairs before transfer to the intermediary electron transport chain [8,9]. Both acceptors appear to be plastoquinones [6,10,11]. In reaction center preparations of the purple, non-sulfur photosynthetic bacterium, *Rhodopseudomonas sphaeroides*, binary oscillations in the appearance and disappearance of an anionic ubisemiquinone were observed directly by both optical and ESR techniques [12,13]. Similar behavior was reported in chromatophores [14,15]. These observations can be accounted for by an electron acceptor complex containing two ubiquinones [10,11]:

1st flash $\quad Q_I Q_{II} \xrightarrow{h\nu} \dot{Q}_I^{-} Q_{II} \rightarrow Q_I \dot{Q}_{II}^{-}$(stable) (a)

2nd flash $\quad Q_I \dot{Q}_{II}^{-} \xrightarrow{h\nu} \dot{Q}_I^{-} \dot{Q}_{II}^{-} \xrightarrow{2\,H^+} Q_I Q_{II} H_2$ (b)

Scheme I

ESR studies have shown that both Q_I and Q_{II} interact with a single iron atom [12] and that the species $\dot{Q}_I^{-} Fe \dot{Q}_{II}^{-}$, which can be formed at low temperature, is diamagnetic, indicating that the two electrons are spin-coupled in some way [16].

Although the two semiquinones, $\dot{Q}_I^{-}$ and $\dot{Q}_{II}^{-}$, have very similar spectra below 500 nm, Vermeglio and Clayton [17] showed that the local electrochromic effects on the bacteriochlorophyll and bacteriopheophytin spectra in the infrared are distinct for the two anionic semiquinones, allowing measurement of the electron transfer from Q_I to Q_{II}. The transfer half-time was about 200 μs following either the first or the second flash indicating it to be independent of the redox state of Q_{II} [17].

This paper reports a more detailed study of the electron transfer events leading to the accumulation of two equivalents in the acceptor complex and, in particular, the protonation events involved in the two electron reduction of Q_{II} to $Q_{II}H_2$. A tentative scheme is proposed in which a protonation step precedes the second electron transfer and, at high pH, is rate limiting. The detailed kinetics lead to the suggestion of an intermediary in a non-limiting protonation

step, which may involve an acid-base group in the protein undergoing an induced p*K* shift in response to the charge on the anionic semiquinones. This group appears to be subsequently involved in proton transfer to the fully reduced quinone.

Materials and Methods

Reaction centers were prepared from *Rp. sphaeroides*, R26, by detergent fractionation of chromatophores in 0.1 M NaCl, 10 mM Tris-HCl (pH 8), with 0.4% lauryl dimethylamine-*N*-oxide (Ammonyx-LO, a gift of the Onyx Corporation, Division of Millimaster, Jersey City, NJ 07023). The inclusion of salt in the detergent treatment was found to give a very clean separation of reaction centers and membrane fragments. Reaction centers were precipitated with 45% satd. $(NH_4)_2SO_4$ and then purified by DEAE-cellulose chromatography (Whatman DE 52). The column was washed extensively with 0.06 M NaCl, 0.1% lauryl dimethylamine-*N*-oxide, 10 mM Tris-HCl (pH 8). Reaction centers were eluted with 0.35 M NaCl, 0.1% lauryl dimethylamine-*N*-oxide, 10 mM Tris-HCl (pH 8) and de-salted and concentrated by ultrafiltration. Exposure to $(NH_4)_2SO_4$ and any prolongation of the chromatographic procedure caused a variable degree of extraction of the ubiquinone content. Thus, after purification and concentration, reaction center stocks were supplemented with ubiquinone-10 (Sigma Chemicals, St. Louis, MO) added a suspension in 30% Triton to a level of about one ubiquinone/reaction center. Reconstitution with ubiquinone-10 (Sigma Chemicals, St. Louis, MO) added as a suspension in ducible and strong oscillatory behavior of the quinone acceptor complex [12].

Kinetic measurements were performed on a single beam spectrophotometer and displayed on a storage oscilloscope (Tektronix D15). Flash excitation was provided either by a rhodamine 6G liquid dye laser (Phase-R, DL 1100) or by a xenon flash lamp of about 8 μs duration. Light saturation was routinely estimated by comparing the extent of reaction center photooxidation (generation of P^+) by a single flash in the absence of exogenous donor with that obtained in a series of closely spaced flashes. It consistently amounted to 92—97%.

Each sample contained 1—2 μM reaction centers in 6 ml 0.1 M NaCl in an anaerobic cuvette equipped with both platinum and pH electrodes. Lauryl dimethylamine-*N*-oxide, carried over from the reaction center stock solution did not exceed 0.002% in the cuvette. At the relatively oxidising potentials used in this study it did not interfere with the establishment of reproducible and steady redox potentials. 10 mM Tris-HCl buffer was present in some experiments. Ubiquinone, in 30% Triton, was routinely added to a concentration of 10 -20 μM. The final concentration of Triton, therefore, was 0.03 -0.06% which was sufficient to maintain the reaction centers in solution. Diaminodurene (2,3,5,6-tetramethyl-*p*-phenylene diamine), 1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, duroquinone (2,3,5,6-tetramethyl benzoquinone),5-hydroxy-1,4-naphthoquinone,anthraquinone-1-sulfonate and indigo tri- and tetrasulfonates were added, when required for redox poising and titrations, to final concentrations of 5—20 μM.

For spectroscopic measurements of the semiquinone kinetics, both diaminodurene (20—40 μM) and reduced, mammalian cytochrome *c* (15—20 μM) were

used as secondary donors. Diaminodurene was routinely used as it permitted measurement at the visible maximum of the anionic semiquinone absorption at 450 nm. At high pH, however, a faster donation rate was needed to separate P^+ reduction from the progressively slowing semiquinone disproportionation and cytochrome *c* was used. Even at the high ionic strength used in this work, cytochrome *c* donated in less than 2 ms. Cytochrome *c* undergoes a reversible redox midpoint potential shift with an apparent p*K* at pH 9.3 [18—21]. Consequently, donation to P^+ became very slow at high pH and diaminodurene was again used above pH 10.25. Measurements of the semiquinone with cytochrome *c* as secondary donor were performed at the isosbestic point for cytochrome *c* oxidation-reduction near 432 nm, which is a shoulder on the semiquinone absorption spectrum [22].

Electron transfer from $\dot{Q}_I^-$ to Q_{II} was observed at about 750 nm where the electrochromic absorbance contributions of $\dot{Q}_I^-$ and $\dot{Q}_{II}^-$ are different [17]. These measurements required some care in avoiding artifacts in response to the actinic flash. Flash artifacts were reduced to a small transient, complete in 50 μs, by recessing the filter combination (750 nm interference filter plus Wratten 88A) and masking off all except the aperture necessary for the measuring beam. Resolution of fast kinetics at 750 nm was still limited by this residual artifact, however.

Flash-induced H^+ binding was measured by the absorbance changes of the pH indicator dyes chlorophenol red, bromocresol purple, phenol red, cresol red, and thymol blue (50 μM) and calibrated by addition of aliquots of standard HCl. H^+ binding measurements were performed using cytochrome *c* (15—20 μM) as an exogenous electron donor to the flash-generated P^+. Potassium ferrocyanide (0.4—0.8 mM), which maintained the ambient redox potential at about +250 mV, rereduced the flash-oxidized cytochrome *c* with a half-time of about 30 ms. In a few experiments, performed at lower potentials, ferrous/ferric EDTA was also added. The flash-induced responses of the pH indicators were measured at the isosbestic point for cytochrome *c* oxidation at about 557 –559 nm. The precise wavelength was set for zero net absorbance change before addition of indicator. Very small oscillatory changes, however, were still evident and were subtracted from the indicator responses. Except at very high pH, where net H^+ binding was small, these residual changes in absorption were insignificant. At the upper extremes of the pH range of each dye, when the solutions were strongly colored, a dilution artifact contributed significantly to the calibration response but in all cases was readily corrected for. Results obtained from the different dyes in the regions of overlap of their pH ranges agreed well. For H^+ binding kinetics, 10 mM NaCl was used rather than 100 mM since high ionic strength limits the rate of cytochrome *c* oxidation [23]. H^+ binding was also determined in the absence of exogenous donor, measuring at 589 nm which is isosbestic for P^+ absorbance changes.

All measurements with donors present were preceded by 15 min dark adaptation of the sample.

Results

Electron transfer from Q_I to Q_{II}

The anionic ubisemiquinone absorbs characteristically at 450 nm, as does the

oxidized primary donor, P^+. Fig. 1 shows the kinetics observed at this wavelength during a series of saturating, single turnover flashes. The slow decay ($t_{1/2} \approx 100$ ms) seen after every flash is P^+ rereduction by the exogenous donor, diaminodurene. After the first flash, a stable absorption change due to the semiquinone remains when P^+ has been fully reduced and is seen as the elevated baseline for the second flash [12,13]. The rapidly decaying component seen predominantly after even flashes is the loss of semiquinone. Since the fast phase was cleanly monophasic, both semiquinones (Q_I^- and Q_{II}^-) disappear with the same kinetics, consistent with the overall disproportionation reaction of Scheme 1b [12,13]. The H^+ uptake processes involved in this reaction sequence were studied via the pH dependence of the rapid kinetics after the second flash.

Electron transfer from Q_I to Q_{II} was also studied by means of the distinctive electrochromism of the two semiquinones in the near infrared [17]. 750 nm is isosbestic for electrochromism due to Q_{II}^- while that due to Q_I^- is cancelled by the P^+ change. The precise wavelength was set for zero flash-induced absorbance change in the presence of 2 mM *o*-phenanthroline to block electron transfer to Q_{II}. In the absence of *o*-phenanthroline, therefore, and since P^+ rereduction was rather slow, this wavelength should reveal the loss of the Q_I^- electrochromic absorbance change as the electron transfers to either Q_{II} or Q_{II}^- following a

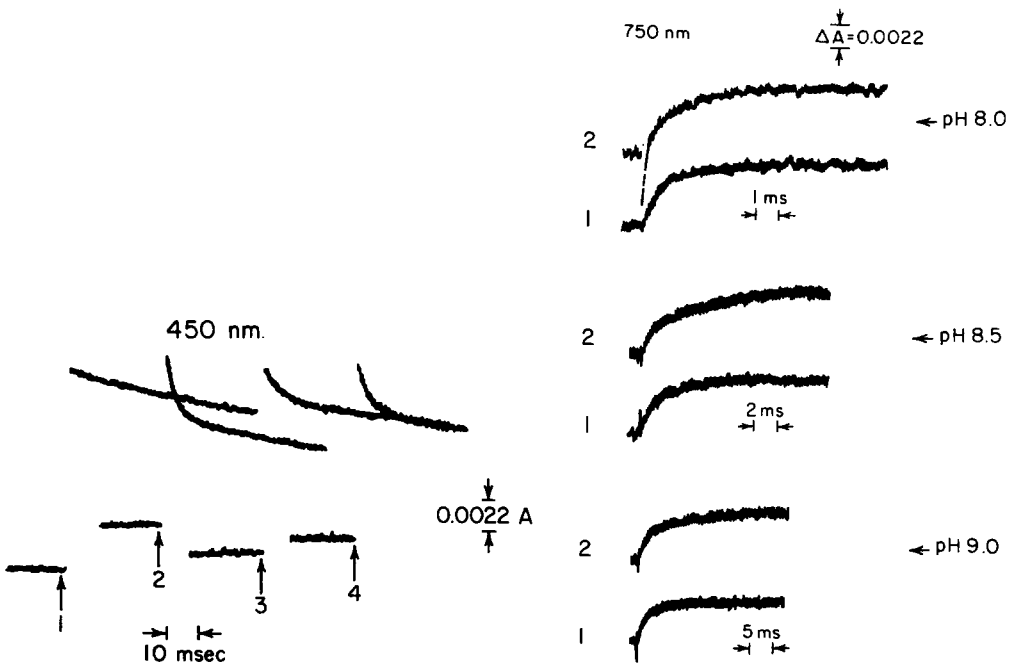


Fig. 1. Flash-induced absorbance kinetics at 450 nm. Approximately 2 μM reaction centers, 40 μM diaminodurene, 20 μM ubiquinone-10 and 0.06% Triton X-100 in 100 mM NaCl (pH 8.2). Flash period, 0.5 s; each flash trace is offset horizontally from the previous one to show the initial decay kinetics more clearly.

Fig. 2. Flash-induced absorbance kinetics at 750 nm. Conditions as in Fig. 1, except pH as indicated in the figure. Flash one (bottom of each pair) and two (top) are shown.

flash (Fig. 2). After the first flash the kinetics, associated with the $\dot{Q}_I^- \rightarrow Q_{II}$ transfer, were monophasic. After a second flash the kinetics were biphasic with a slower phase in good agreement with the kinetics of disproportionation seen at 450 nm, i.e. the $\dot{Q}_I^- \rightarrow \dot{Q}_{II}^-$ electron transfer. However, the rather small amplitude of this phase and the presence of a significant fast phase, very similar to that seen after one flash, are anomalous and it seems probable that the electrochromic spectral shifts are not simple indicators of the two semiquinones.

Demonstration of two phases at 750 nm after the second flash was only possible in a limited pH range. Below about pH 8 the 'slow' phase became quite fast and could not be separated from the 'fast' component. Above pH 9 the electron transfer on the second flash was sufficiently slow that the rereduction of P^+ interfered with the kinetic resolution.

The pH dependence of $Q_I \rightarrow Q_{II}$ electron transfer rates

The pH dependences of the kinetics measured at 750 nm and 450 nm are shown in Fig. 3. After the first flash, the kinetics at 750 nm exhibited a weak pH dependence of 0.3 decade/pH unit. This is not indicative of any specific protonation events and the process, therefore, appears not to be limited by H^+ binding. This is consistent with the anionic nature of the stable semiquinone formed. Nevertheless, the pH dependence was non-zero and was very similar to that observed by Parson [24] for the electron transfer from primary to secondary acceptor in *Chromatium vinosum*. Some other pH dependences of this anomalous type (i.e. non-integral slope) have been shown [25] or suggested [26] to be due to inadvertant averaging over a pK region, but there was no indication that this was the case in this study.

After the second flash the kinetics at 450 nm, characteristic of the anionic semiquinones, showed a marked pH dependence of 1 decade/pH unit above pH 8 (Fig. 3) indicating that the second electron transfer ($\dot{Q}_I^- \rightarrow \dot{Q}_{II}^-$) was rate limited by the binding of a single proton. It is likely that this rate limitation arises from the need to neutralize the anionic charge of one of the semiquinones, probably $\dot{Q}_{II}^-$ since the midpoint potential (E_m) of Q_I is pH independent in reaction centers [27]. Electron transfer following the second flash would, thus, be as follows:

$$Q_I\dot{Q}_{II}^- \xrightarrow{h\nu} \dot{Q}_I^-\dot{Q}_{II}^- \xrightarrow[\downarrow]{H^+} \dot{Q}_I^-\dot{Q}_{II}H \rightarrow Q_IQ_{II}H^- \xrightarrow[\downarrow]{H^+} Q_IQ_{II}H_2$$

Scheme 2

This interpretation is consistent with known mechanisms of quinone reduction in aqueous solutions which occur by alternate addition of electrons and protons [28].

Below pH 8, the second flash kinetics at 450 nm exhibited the same anomalous pH dependence as the first flash kinetics at 750 nm (0.3 decade/pH unit), suggesting that the electron transfer was no longer limited by H^+ binding but perhaps by a process which may be common to both first and second electron transfers.

Between pH 9 and 10, the second transfer was measured using both diamino-

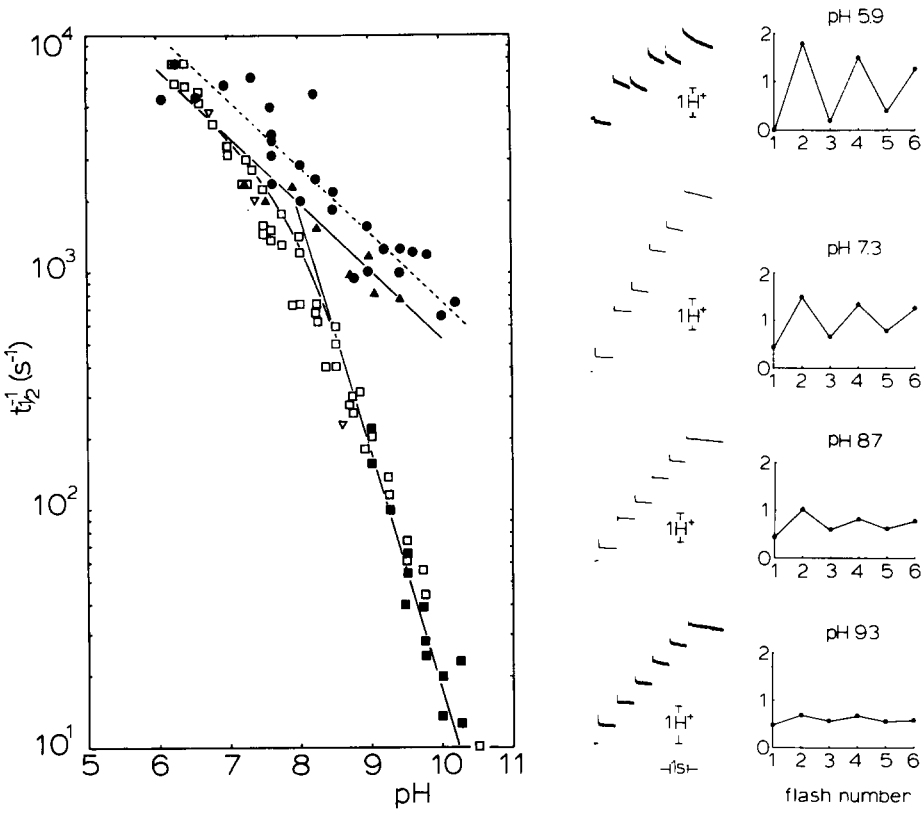


Fig. 3. pH dependence of electron and proton transfer in the quinone acceptor complex. Data points for the electron transfers (●, □, ■) were taken from experiments as shown in Figs. 1 and 2. First flash data (●), obtained at 750 nm both in the presence and absence of secondary donor (diaminodurene or cytochrome *c*). Second flash data, taken at 450 nm, with diaminodurene (□) or cytochrome *c* (■) as secondary donor. H^+-binding kinetics were determined with pH indicators (see legend to Fig. 8). H^+ binding on the first flash (▲) was measured in the absence of secondary donor, at 586 nm. The data for H^+ binding on the second flash (▽), measured at 559 nm, are taken from Fig. 8.

Fig. 4. H^+ binding by reaction centers. 2 μM reaction centers, 15 μM cytochrome *c*, 10 μM ubiquinone-10 and 0.03% Triton X-100 in 100 mM NaCl. Flash period, 1 s. Measurements were made at the isosbestic for cytochrome oxidation-reduction at about 559 nm. pH indicators (50 μM each) were present as follows: pH 5.9, chlorophenol red; pH 7.3, phenol red; pH 8.7, cresol red; pH 9.3, cresol red and thymol blue. At each pH the actual traces are shown (left) together with the corrected H^+/flash (right) after subtraction of small background changes seen in the absence of indicator.

durene and cytochrome *c* as donors and no significant difference was noted in the kinetics of disproportionation at 450 nm (Fig. 3). Since in the one case P^+ rereduction lagged and in the other preceded the disproportionation, it is apparent that the redox state of P did not affect the rate-limiting process which, from the pH dependence, is a protonation event.

H^+ binding by reaction centers in a series of flashes

Scheme 1, above, has previously been used to explain binary oscillations in H^+ binding observed in reaction centers [29] and chromatophores [14]. However, Fig. 4 shows that only below pH 6 were the oscillations in strict accor-

dance with the expected zero H^+ on the first flash and two on the second. At pH values higher than 6, the amount of H^+ uptake on the first flash increased progressively while that on the second decreased until, at about pH 9.5, the oscillations were not detectable.

At high pH, net H^+ binding decreased with an apparent pK at pH 9.3 (Fig. 5a). This is very similar to an apparent pK reported for oxidized cytochrome c which is responsible for pH dependence of the midpoint potential of cytochrome c at high pH [18—21]. Cytochrome c oxidation coupled to H^+ release might be expected to obscure the H^+ binding associated with reduction of the quinone acceptor. However, the H^+ release from ferricytochrome c is driven by a very slow conformational change which occurs over tens of seconds [19]. This slow change would not interfere with the H^+ binding measured shortly after each flash (within 0.5 s). Furthermore, ferrocyanide (0.4—0.8 mM) rereduced the flash-oxidized cytochrome c within 100 ms so that there was no possibility of H^+ release even at longer times. The loss of H^+ binding was, therefore, real and the probably source of the decline is shown in Fig. 5b and c.

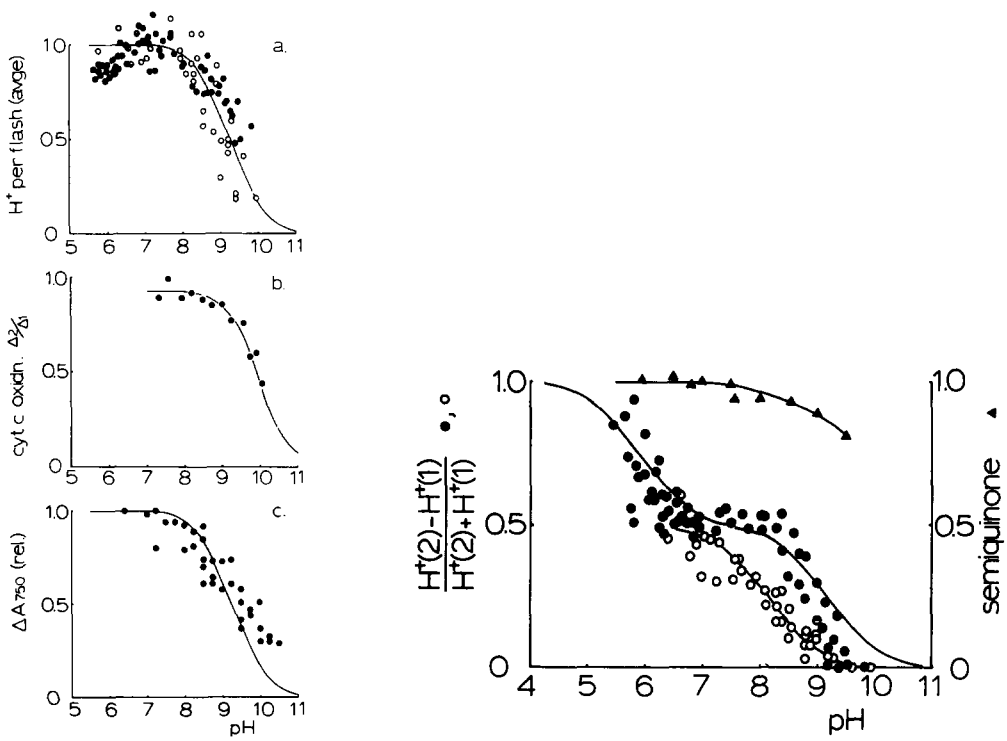


Fig. 5. pH dependence of turnover in the quinone acceptor complex. (A) H^+/flash, averaged over the first four or six flashes. Conditions as for Fig. 4. Data points are grouped in two redox ranges: ○, +120 — +180 mV; ●, 240 — +300 mV. (B) Oxidation of cytochrome c on the second flash relative to that on the first. 0.9 μM reaction centers, 15 μM cytochrome c, 15 μM diaminodurene, 20 μM ubiquinone, 0.06% Triton X-100, 100 mM NaCl. (C) Magnitude of the first-flash 750 nm absorbance change normalized to 1 at low pH. Conditions as for Fig. 2.

Fig. 6. pH dependence of H^+ binding oscillations. Data points are grouped in two redox ranges: ○———○, +120 — +180 mV, ●———●, +240 — +300 mV. $H^+(1)$, proton uptake on first flash; $H^+(2)$, proton uptake on second flash. The relative magnitude of the first-flash semiquinone signal at 432 nm, normalized to 1 at low pH, is also shown (▲). Conditions as for Fig. 4.

Fig. 5b shows that the ratio of cytochrome *c* oxidation on the second flash, relative to that on the first, fell off at high pH, suggesting that reoxidation of Q_I^- was diminished above pH 8.5. Thus, the decline in net proton uptake/flash (Fig. 5a) follows the failure in the reoxidation of Q_I^-, consistent with H^+ binding accompanying the reduction of Q_{II}. The 750 nm absorbance change after the first flash also decreased at high pH (Fig. 5c), consistent with it indicating electron transfer from Q_I^- to Q_{II} or a closely related process. However, in view of their electrochromic origin, it is possible that the infrared absorbance changes are themselves pH dependent.

The observed decrease in net proton uptake affected the H^+ binding stoichiometries for each flash and the pattern of the oscillations. To compensate for this, the H^+-binding oscillations were normalized for net uptake on the first two flashes. The calculated data are shown in Fig. 6. The expression plotted on the ordinate of Fig. 6 is equivalent to the 'strength' of the oscillations. The H^+-binding oscillations titrated out in two waves, the higher apparent p*K* displaying some redox potential dependence. Because of the loss of net H^+ binding in the same pH range (probably not redox potential dependent, Fig. 5a), the high pH wave of the titration curve of H^+ binding was somewhat truncated at the higher redox potentials.

pH dependence of semiquinone formation

Although H^+ binding on the first flash increased progressively as the pH was raised above 6, the amplitude of the stable semiquinone absorbance change was constant up to about pH 9. Fig. 6 shows the amplitude at 432 nm using cytochrome *c* as donor. Since the protonated ubisemiquinone (QH) has a considerably lower extinction coefficient in this wavelength region than the anionic form [30], Fig. 6 demonstrates that the semiquinone maintained its anionic nature throughout the pH range. The slight drop-off above pH 9 can be attributed to the increased failure of electron transfer from Q_I^- to Q_{II} as described above (see Fig. 5) and a slightly lower absorbance at 432 nm for Q_I^- than for Q_{II}^- [17]. This was confirmed using diaminodurene as exogenous donor and measuring at 450 nm, where the difference between Q_I^- and Q_{II}^- is small (unpublished observations). The decrease in the flash-induced absorbance change above pH 9 was barely detectable. The photooxidation products of diaminodurene, however, gave rise to absorbance changes both at low (p$K \approx$ 7.7) and high (p$K \approx$ 9.2) pH, necessitating a correction to the stable absorbance change seen. Furthermore, if the decline at 432 nm were due to protonation of the semiquinone, a rapid decay should be observable after the first flash; none was seen at either 432 nm or 450 nm.

The pH dependence of the semiquinone absorbance changes in a series of flashes is shown in Fig. 7. The magnitudes of the absorbance changes are shown relative to those of the previous flash. It is clear that for even flashes a strong pH dependence set in above pH 8, while the odd flashes were unaffected up to pH 9.5.

Kinetics of H^+ binding

The rate of H^+ binding after one or two flashes was studied as a function of pH. Good resolution was limited by the rate of rereduction of P^+ using cyto-

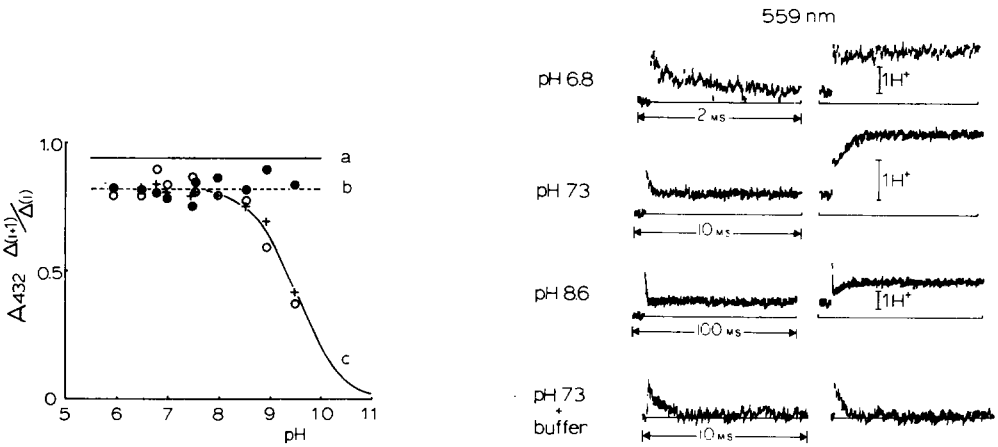


Fig. 7. pH dependence of the semiquinone absorption oscillations. The magnitudes of the odd and even flash, stable semiquinone absorbance changes at 432 nm ($\Delta i + 1$) are shown relative to those on the previous flash (Δi). •, $\Delta 3/\Delta 2$; ○, average of $\Delta 2/\Delta 1$ and $\Delta 4/\Delta 3$. Line a, the value for $\Delta 1$ given by the first flash miss factor as described in the text (first flash hits = $(1 - x) = 0.94$). Line b, the pH-independent value for $\Delta i + 1/\Delta i$ calculated from the overall damping factor at low pH (overall damping factor = $1/2(2x + y) = 0.09$). Line c, a tritation curve drawn through the experimental (○) and calculated (+) points for the total second-flash hits, $1 - (x + y)$. The additional miss factor on even flashes, y, is pH dependent.

Fig. 8. Flash-induced H^+ binding kinetics. Conditions as for Fig. 4 except 10 mM NaCl and 30 μM cytochrome *c*. First flash, left; second flash, right. Indicators (50 μM) were present as follows: pH 6.8, chlorophenol red; pH 7.3, phenol red; pH 8.6, cresol red. Bottom traces. P^+, absorbance change seen in the presence of 4 mM Tris-HCl buffer.

chrome *c* as donor. Several kinetic traces are shown in Fig. 8. The kinetics of H^+ binding can be obtained by subtraction of the P^+ change, measured either before addition of pH indicator or after addition of excess buffer. The half-time of H^+ binding on the second flash was readily measured and it is clear that this uptake included the rate-limiting proton inferred from the pH dependence of the $\dot{Q}_I^- \rightarrow \dot{Q}_{II}^-$ transfer on the second flash above pH 8.0. The rate of H^+ binding was in good agreement with that of electron transfer throughout the pH range covered (see Fig. 3, open triangles). The apparent bimolecular rate constant for this protonation step, calculated from the unit slope region of Fig. 3, is about $10^{11}\ M^{-1} \cdot s^{-1}$. This is in good agreement with accepted values for proton transfer in aqueous solutions [31]. The fact that the kinetics of proton binding match those for the electron transfer on the second flash below pH 8, rather than continuing to accelerate as the pH was decreased, indicates that H^+ binding was not in response simply to the appearance of $\dot{Q}_I^-$ (i.e. an immediate p*K* shift in $\dot{Q}_{II}^-$) but that an additional process exists which was limiting for both H^+ binding and electron transfer at low pH.

The extent of H^+ binding on the first flash increased with increasing ambient pH values above 6 (Figs. 4 and 6). The kinetics of this H^+ uptake were readily measured in the absence of exogenous donor. They were found to be only weakly pH dependent and followed very closely the 750 nm absorption kinetics (Fig. 3). The rate of H^+ binding after the first flash appeared, therefore, not to be diffusion limited. Since it was not controlled by the prevailing pH up to pH 9.5, a true, binding rate constant could not be determined. It is note-

worthy, however, that at pH 9.5 the apparent rate constant was 10^{12} $M^{-1} \cdot s^{-1}$, an order of magnitude greater than that calculated for H^+ binding on the second flash which was diffusion controlled.

Discussion

Electron transfer on first and second flashes

Vermeglio and Clayton [17] concluded from measurements in the infrared that the half-times for the first and second electron transfer at pH 7.5 were the same (200 μs). The present work generally confirms this result for pH values below 8. Although the kinetics at 750 nm consistently appeared to be about twice as fast the second flash kinetics measured at 450 nm, this can be attributed to the higher noise level of the absorption measurements in the infrared. Furthermore, below pH 8, the kinetics of H^+ binding following the first flash were in close agreement with the second flash electron transfer kinetics at 450 nm (Fig. 3).

At pH values above 8, however, the second flash kinetics at 450 nm became progressively slower in a manner which suggested the rate-limiting involvement of a protonation step. Although these slower second flash kinetics could be discerned at 750 nm (Fig. 2), there was a significant contribution from a faster phase similar to that seen after the first flash. The magnitude of the slow phase did not agree well with that expected from the measurements at 450 nm. Discrepancies in the amplitudes of the infrared absorption changes were also reported by Vermeglio and Clayton [17].

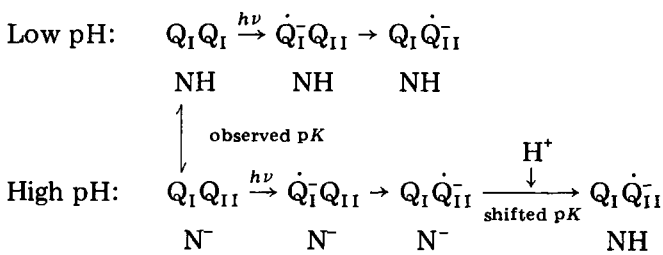
The absorbance changes in the infrared arise, in part, from perturbations of the porphyrin pigment spectra by the anionic semiquinones, e.g. electrochromism [17]. Such effects are likely to be sensitive to events other than electron transfer, including protonation and charge distribution. The absorbance at 450 nm, on the other hand, is an attribute of the semiquinone itself [30] and thus reports directly on the fate of the electrons. The discrepancies, at high pH, between the second flash kinetics measured at 450 nm and 750 nm, strongly suggest that the fast component at 750 nm is not reflecting the electron transfer per se but some other related event which, at pH values below 8, is rate limiting for the electron transfer. The similarity in the kinetics and pH dependences of the fast 750-nm absorbance changes for first and second flashes indicates that this event may be common to both first and second electron transfer processes.

The similar rates of electron transfer for first and second flashes observed at low pH values is somewhat surprising in view of the different redox state of Q_{II} in the two cases. It should not be construed, however, that the electron transfer is indifferent to the state of Q_{II} since the pH dependence of the second flash kinetics above pH 8 shows the transfer to be highly sensitive to the charge, i.e. the protonation state of $\dot{Q}_{II}^-/\dot{Q}_{II}H$. Furthermore, it is suggested here that even at low pH the electron transfer per se is not rate limiting and that some other event, which also perturbs the infrared spectra, controls the transfer rate.

H^+ binding on the first flash

It is apparent from Figs. 4 and 6 that at high pH a proton is bound on the

first flash and the H^+-binding oscillations disappear, but the yield of anionic semiquinone signal is undiminished. The attenuation of H^+ binding on the first flash displays a complex dependence on pH, with two apparent pK values, and a possible dependence on redox potential. A simple scheme cannot account for this but the general phenomenon of enhanced H^+ binding on the first flash at high pH, with no concomitant effect on the semiquinone absorbance, can be readily accounted for by the following scheme:



Scheme 3

Here, N^-/NH (or N/NH^+) is a general acid-base group, perhaps associated with the reaction center protein, which undergoes a pK shift in response to the charge on the semiquinone, causing N^- to protonate following a flash at high pH. The semiquinone itself remains in the anionic form. First flash H^+ binding thus titrates in with a pK appropriate for the dark-adapted, acid-base equilibrium of N^-/NH ('observed pK'). Since this H^+ uptake was observed at the highest pH values used, the pK must shift to at least 9.5 ('shifted pK'). It is not clear whether this rather large pK is in response to $\dot{Q}_I^-$ or $\dot{Q}_{II}^-$ or both but *o*-phenanthroline, which inhibits electron transfer from Q_I to Q_{II}, abolishes H^+ binding on first and subsequent flashes in reaction centers (unpublished data), consistent with the reprotonation of N^- as a response to $\dot{Q}_{II}^-$ rather than $\dot{Q}_I^-$. However, *o*-phenanthroline does strongly affect the physical chemistry of Q_I in chromatophores [25,27].

There is, currently, no evidence for formation of $Q_{II}H$ after the first flash at any pH, and the pK for this protonation reaction must, therefore, be low (less than pH 6). The possibility exists that the lack of protonation of $\dot{Q}_{II}^-$ after the first flash is due to a kinetic limitation, but the stability of the anionic semiquinone for many minutes [3,12,13,22] rather strains the notion of a kinetic barrier.

Accessibility as a factor in H^+ binding kinetics

Remarkably, the rate of H^+ binding on the first flash is almost independent of pH and, at pH values above 8, is considerably faster than expected for diffusion-controlled protonation. This can be seen clearly in Fig. 8 by comparison of the first flash H^+ binding with the diffusion-controlled uptake on the second flash. This kinetic anomaly cannot be accounted for by electrostatic arguments, i.e. the net negative charge of the reaction center causing a locally low pH, or by reduction of dimensionality [32], since the rate should still be fully pH dependent. As an alternative for the anomalous, first flash H^+-binding kinetics, it is possible that the fast protonation reaction occurs directly

from the protonated indicator which is present at relatively high concentration (5—50 μM). The site of protonation (N^- according to the suggestion above) would, therefore, be 'accessible' to the pH indicators and located at a point exposed to the external phase of the protein. Binding of the pH indicator to the reaction center cannot be excluded at this time.

Since the fast protonation is not strictly pH dependent, even though the proportion of protonated indicator varies with pH in a logarithmic fashion, it must be assumed that over the useful pH range of any indicator used, the concentration of protonated species (5—50 μM) is sufficient for H^+ transfer not to be rate limiting.

A very similar situation is observed in chromatophores of both *Chr. vinosum* [33] and *Rp. sphaeroides* [37] where H^+ binding also exhibits a pH dependence of about 0.3 decade/pH unit and is anomalously fast. By analogy with the reaction center system described here, it is suggested that the in vivo protonation process may involve a similarly 'accessible' intermediary group.

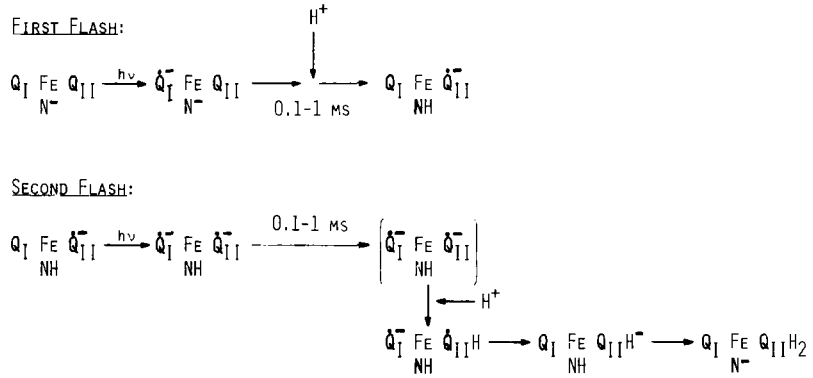
Electron and proton transfer in the production of $Q_{II}H_2$

The events leading to the two electron reduction of Q_{II} are summarized in Fig. 9 which also shows the iron atom (Fe), known to interact with both Q_I and Q_{II} [16]. The first electron transfer from $\dot{Q}_I^-$ to Q_{II} and the protolytic reactions of the group N^-/NH have already been discussed but the results reported here also reveal several details of the electron and proton transfer events following the second flash.

Because of the induced shift in the pK of N^-/NH, the state of the acceptor complex prior to the second flash is $Q_I\dot{Q}_{II}^-$ at any pH and the immediate photoproduct is $\dot{Q}_I^-\dot{Q}_{II}^-$ (NH). The disproportionation of the two semiquinones involves at least two steps. The first is revealed by the pH dependence of the electron transfer after the second flash at high pH, implying that uptake of a single proton is rate limiting for the electron transfer from $\dot{Q}_I^-$ to $\dot{Q}_{II}^-$. Electron transfer follows H^+ binding immediately and these processes, therefore, cannot be separated in the kinetics at 450 nm. It could be considered that the restriction on electron transfer prior to the proton uptake arises from electrostatic repulsion between the two semiquinone anions, or, equivalently, from a prohibitively low redox midpoint potential for $\dot{Q}_{II}^-/Q_{II}^{2-}$ as expected from electrochemical studies [28]. H^+ binding, to give $Q_{II}H$, releases this restriction. The dependence on bulk pH means that this rate-limiting protonation does not occur from NH, either because the pK of $\dot{Q}_{II}^-$ is not high enough or for kinetic reasons.

A second step in the dismutation of the semiquinones is revealed at low pH. Thus, both H^+ binding and electron transfer are limited by the same rapid event at low pH when H^+ binding itself is not limiting. This process could drive H^+ binding either by following or preceding the protonation step. The latter is shown in Fig. 9, but no distinction between the two can be made at the present time. It is parsimonious to suppose that this event is the same as that inferred from the 750 nm absorbance kinetics to limit electron transfer at low pH. An attractive possibility for this event is a conformational change, or charge

A. High pH



B. Low pH

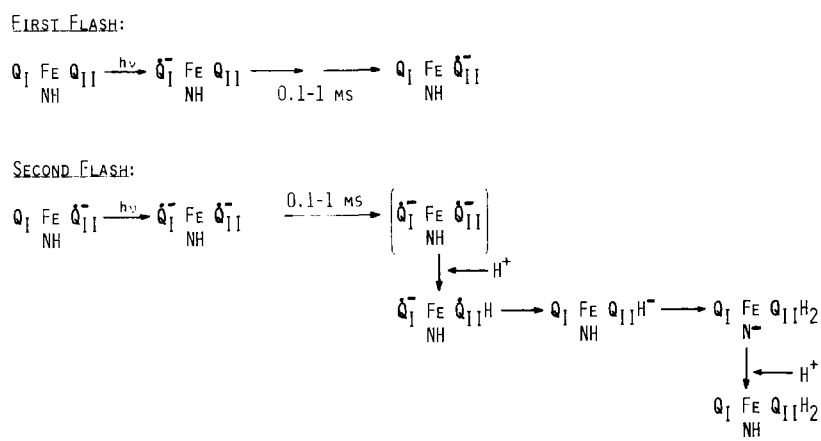


Fig. 9. A scheme for electron transfer and proton binding in the quinone acceptor complex of photosynthetic reaction centers. (A) At high pH the proton taken up on the first flash is not initially bound to a semiquinone but to a non-chromatophoric group (N^-/NH) in the reaction center complex. After the second flash, the disproportionation of the two semiquinones is rate limited by the uptake of a single H^+. This is the first proton onto the quinone (Q_{II}). The subsequent electron transfer produces fully reduced quinol which can obtain its second proton from the group NH, protonated on the first flash. (B) At low pH (<7) it has not been possible to kinetically distinguish the two protons taken up on the second flash but the model derived at high pH can be readily extended to low pH. In this case, the second proton bound regenerates NH. The two protons would indeed be kinetically indistinguishable if the intervening steps, including electron transfer, were sufficiently fast.

redistribution, in the acceptor complex making $\dot{Q}_{II}^-$ subject to protonation.

Following protonation of $\dot{Q}_{II}^-$ the electron transfer from Q_I^- to $\dot{Q}_{II}H$ occurs in an unrestricted fashion to generate $Q_{II}H^-$. At high pH (Fig. 9A), only one proton is taken up on the second flash and this is seen in the rate-limiting role of neutralizing $\dot{Q}_{II}^-$. Thus, the second proton for the formation of $Q_{II}H_2$ must come from NH, leaving N^- as the dark-adapted equilibrium species. In Fig. 9B, the same series of events is envisaged at low pH except that following proton transfer from NH, N^- is reprotonated from the medium by the second of the two H^+ taken up on the second flash. So far, no kinetic distinction between the

two second-flash protons has been detected, implying that electron transfer from $\dot{Q}_I^-$ to $Q_{II}H$ is, indeed, fast as suggested above.

In order to complete the photoredox cycle necessary for multiple turnover of the reaction center, oxidised Q_{II} must be regenerated. No kinetic limitation due to this process was observed with the fastest flash repetition rates available (120 Hz; unpublished data). Restoration of Q_{II} could be accomplished either by reoxidation of $Q_{II}H_2$ or by exchanging it for an oxidised quinone from the bulk quinone pool. The latter possibility would be consistent with the observation that extraction of quinone from reaction centers is facilitated under reducing conditions [4].

Kinetic and thermodynamic implications

Whichever order is assumed for the conformational and protonation events immediately following the second flash, the proposed proton binding by $\dot{Q}_{II}^-$ implies a large shift in effective pK for $\dot{Q}_{II}^-/\dot{Q}_{II}^-H$. The free energy which is manifested as a shift in the pK of $\dot{Q}_{II}^-$ must come from the photoact and, since the rereduction of P^+ does not affect the kinetics of disproportionation, is most likely associated with the appearance of $\dot{Q}_I^-$. The pK shift could, most simply, arise from the electrostatic interactions between $\dot{Q}_I^-$ and $\dot{Q}_{II}^-$. If this were the case, the change in pK should be reflected in an equivalent change in the midpoint potential for the $Q_{II}/\dot{Q}_{II}^-$ couple above the pK. Thus if, as appears to be the case, the pK shifts from less than 6 to greater than 10 then, prior to the H^+ binding to give $\dot{Q}_{II}H$, the effective E_m of $Q_{II}/\dot{Q}_{II}^-$ following the second flash, i.e. as $\dot{Q}_I^-(Q_{II}/\dot{Q}_{II}^-)$, would be at least 240 mV lower than that for the long-lived semiquinone generated by the first flash $[Q_I(Q_{II}/\dot{Q}_{II}^-)]$. Conversely, The $Q_I/\dot{Q}_I^-$ couple will be similarly affected by the charge on $\dot{Q}_{II}^-$ and the E_m for Q_I on the second flash will be at least 240 mV lower than that on the first. Similar effects, depending on the distances involved, will be felt between all the charged redox couples of the reaction center.

Although electrostatic interaction between the two anionic semiquinones is inevitable, H^+ binding at low pH is not an immediate (i.e. diffusion-controlled) response to the appearance of $\dot{Q}_I^-$ and, as discussed above, another reaction step is indicated. If the pK shift were conformationally driven, rather than a direct electrostatic response, then the effect on the E_m could be manifested either above or below the pK and could, therefore, raise the E_m of the Q_{II} semiquinone to that of the $Q_{II}/Q_{II}H$ couple without affecting the anionic couple.

Equilibrium between Q_I and Q_{II}

The anionic semiquinone of Q_{II} is stable for many minutes, depending on the ambient conditions [3,12,13,22]. Nevertheless, since the net product of successive illuminations is the fully reduced quinone, the semiquinone is seemingly metastable rather than stable. This is borne out by redox potentiometric studies (unpublished data). Equilibration with external quinoid mediators is extremely slow, but titrations exhibit $n = 2$ character with a pH dependent midpoint potential of about +40 mV at pH 7. A rough calculation of the one electron equilibrium constant between Q_I and Q_{II} can be made using the forward rate constant, obtained directly by measurement at 750 nm, and rate constants for the back reaction between P^+ and $\dot{Q}_I^-$ or $\dot{Q}_{II}^-$, obtained from the

decay of P^+ in the absence of secondary donor (unpublished data). At pH 7, one obtains a value of 10—20 indicating a redox midpoint potential span of 60—80 mV. Blankenship and Parson [37] have independently arrived at a similar conclusion.

At pH values above 8.5, oxidation of cytochrome *c* on the second flash (Fig. 5b) and the magnitude of the 750 nm absorbance change (Fig. 5c) diminish and the back reaction speeds up (unpublished data). These effects could arise from a pH-dependent midpoint potential for $Q_{II}/\dot{Q}_{II}^-$ due to the coupled protonation of the group N^-/NH. This would diminish the redox potential gap between Q_I and Q_{II} and displace the equilibrium in favor of $\dot{Q}_I^-$. These effects could also arise from loss of Q_{II} from the reaction center, perhaps due to the titration of a binding site, and at present no distinction can be made between these possibilities.

The midpoint potential of $Q_{II}/\dot{Q}_{II}^-$ at pH 7 is thus known relative to that of $Q_I/\dot{Q}_I^-$. In reaction centers the latter is about —50 mV and is pH independent [27]. This would place the E_m for Q_{II} at +10—+30 mV, barely more negative than the $n = 2$ midpoint potential for $Q_{II}/Q_{II}H_2$. This rather positive value for the first one electron reduction step may explain the inability of $\dot{Q}_{II}^-$ to reduce the pool quinone, which is likely to have a much more negative one electron potential for semiquinone formation [28,35]. An approximate equivalence of the two reduction potentials also has the attraction of requiring about the same driving force for $\dot{Q}_I^- \rightarrow Q_{II}$ transfer on first and second flashes, i.e. the reduction potential of $\dot{Q}_I^-$ is equally sufficient in both cases.

The situation is complicated, however, by the fact that in chromatophores the E_m of the primary quinone is pH dependent, although the protonation event is probably too slow to actually occur during the turnover of $\dot{Q}_I^-$ [26,41]. The possibility that the pH dependence of Q_I observed in chromatophores by equilibrium titrations is related, or coupled, to that for Q_{II} is currently under investigation.

Damping in the acceptor quinone complex

Below about pH 8, the binary oscillations in semiquinone formation and H^+ binding could be quantitatively described by a constant damping (miss) factor of 0.08 ± 0.03. There was no indication of double hits. A substantial portion of these misses can be accounted for by the fraction of reaction centers not activated in a single flash. This was routinely determined to be 3—8%. Below pH 8, there were no detectable indications that the reconstitution of active Q_{II} was incomplete. This would give rise to asymmetrical oscillations.

At pH values above 8, the damping increased markedly and unevenly with flash number (Fig. 7). Thus, the semiquinone absorbance change elicited by even flashes declined precipitously with increasing pH. This additional damping titrated in with an apparent $pK \approx 9.5$. The one electron equilibrium between Q_I and Q_{II} or a pH-dependent binding of Q_{II}, described above, are major candidates for this source of damping as the reaction centers in the $\dot{Q}_I^-$ state would be blocked and register as misses on the next flash. In either case the additional damping seen at high pH is attributed to the one electron reduction state of the acceptor quinone complex which is active on even flashes. The equilibrium estimated above would suggest a miss factor (y) of 0.05—0.1 from

this source, even at pH values below 8. Using a value of 0.06 for these additional misses on even flashes at low pH, and an overall damping factor of 0.09 taken from the data of Fig. 7, one may calculate that the constant fraction of misses (x) is also 0.06. This value is consistent with the percentage activation of reaction centers determined for a single flash (92—97%). Finally, taking this value of 0.06 for the constant miss factor, the pH dependence of the even flash misses can be determined. This is shown in Fig. 7. It agrees well with the observed pH dependence of the even-flash semiquinone absorbance change.

The effect of iron and H^+ binding on the Q_{II} semiquinone

The role of iron in the acceptor complex is currently not understood but it is known to interact almost equally with both quinones [12,16]. Removal of the iron has been shown to modify the redox properties of Q_I [36] as well as inhibit electron transfer from Q_I to Q_{II} [37]. It has been suggested that the iron may be directly involved in this transfer [16,38], but a more general function could be to modify the oxidation-reduction and acid-base properties of the quinones [16,36] or to participate in the binding of Q_{II}.

Semiquinones usually exhibit rather low pK values [28] leading to pH-independent midpoint potentials at high pH. A low pK will generally contribute to the stability of the semiquinone relative to the quinol. The events described here, in which pH dependence of the Q_{II} redox properties is imposed by the coupled protonation of the group N^-, therefore, serve to extend the pH dependence and thus should continue to destabilize the semiquinone. The redox properties of the quinone are ultimately determined by the binding site and by a balance between the hydrophobic and polar contributions of the environment of the quinone, including those of the iron atom and other charged groups.

The function of the acceptor complex

The two quinone acceptor complex acts as a two electron gate between the essentially one electron photochemistry of the reaction center and the components of the subsequent electron transport chain. A central role for quinones in coupled electron transport reactions is increasingly apparent from recent work and several related hypotheses have been proposed which involve a subtle interplay between one and two-electron reduction processes of quinones [39,42]. Nevertheless, there seems to be no obvious reason for a specialized mechanism of electron pairing in the reaction center. On the other hand, restricting the two electron reduction to the reaction center in situ could ensure that the accompanying protons are taken up from one side of the membrane, a necessary refinement for maximal energy conservation by a chemiosmotic mechanism. Free mobility of the semiquinone forms, with their relatively low pK values [28], could otherwise lead to random protonation on subsequent disproportionation.

The proposal of the group, N^-/NH, as an intermediary in the protolytic reactions accompanying quinone reduction is obviously related to the suggestion by Chance et al. [33] of a membrane-Bohr effect in H^+ binding by *Chr. vinosum*. The earlier proposal, however, lacked any apparent capability for turnover and was thus not suitable for adoption in an overall scheme of transmembrane

proton pumping. The mechanism proposed here, on the other hand, is cyclical since the binding site is regenerated on transfer of the H^+ to fully reduced quinone and can be envisaged as an integral part of the process leading to proton translocation.

Concluding remarks

The complex pH dependence (i.e. two apparent pK values) for H^+ binding is currently not understood. The two waves of the titration each represent half a proton/reaction center for which no physical significance is apparent. A possible source of this behavior is heterogeneity of the reaction centers, half with a pK at about pH 6 and half at pH 9 or so. This same behavior has been observed in at least three different reaction center preparations so it is unlikely to be purely artifactual or the result of partial degradation but such possibilities are under further scrutiny. The possible redox potential dependence of the higher pK is also unaccounted for but it is noteworthy that the potential range involved is similar to that found by de Grooth et al. [15] to regulate the occurrence of semiquinone oscillations in vivo. The significance of semiquinone oscillations in vivo is obscure at the present time since they have been observed so far, only under relatively exotic conditions [14,15]. Furthermore, although binary oscillations in H^+ uptake [14] and H^+ release [43] have been reported for chromatophore preparations the vast majority of the literature on this subject reports no indication of oscillatory behavior [26,33,34,44–47]. If oscillations are, indeed, normally absent in vivo, rather than, for example, requiring very thorough dark adaptation, a mechanism may exist in vivo to circumvent them. An involvement of cytochrome *b* has been suggested as one possibility [15]. Cooperation between reaction centers at the level of Q_{II} would also achieve the same result and could lead to heterogeneity of reaction centers on isolation. This possibility is currently under investigation. Cooperation at the level of Q_I has been proposed for reaction centers in *Rhodospirillum rubrum* [48] but no evidence supports such a model in other species.

In spinach chloroplasts, Mathis and Haveman [11] have reported that the kinetics of disappearance of the plastoquinone absorption signal, following the second flash, are slower at pH 5.5 than at pH 7.6. This is in contrast to the result reported here for the otherwise similar, bacterial acceptor system. However, the pH dependences in both systems are quite small below 8.0 and the discrepancy is unlikely to reflect a fundamental difference. Mathis and Haveman also reported the first and second flash electron transfer rates to be very similar for chloroplasts in the rather narrow pH range studied. This result might be surprising if the two transfer processes were $\dot{Q}_I^- \rightarrow Q_{II}$ and $\dot{Q}_I^- \rightarrow \dot{Q}_{II}^-$, as they believed [11]. Analogy with the bacterial system, however, suggests that at relatively low pH the rate-limiting step of the second transfer is, in fact, $\dot{Q}_I^- \rightarrow Q_{II}H$.

Acknowledgement

This work was supported by a grant from the National Science Foundation, PCM 77-25725.

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