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FACTORS CONTROLLING THE BINDING OF TWO PROTONS PER ELECTRON TRANSFERRED THROUGH THE UBIQUINONE AND CYTOCHROME b/c_2 SEGMENT OF *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

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Summary

1. On every turnover, 2.0 protons can be bound by the membrane for each single electron moving through the Q-b/c_2 oxidoreductase.

2. One proton (H^+_{II}) binding reaction is, and one (H^+_I) is not, sensitive to antimycin.

3. The redox states of electron transfer components other than the proton binding agents can affect both the rate of proton uptake and the apparent pK values on the agents binding the protons.

4. The presence of valinomycin under certain well-defined conditions can strongly influence the value of the measured pK on the H^+_{II} binding agent.

Introduction

The proton as a vehicle for energy transduction between electron transfer systems and the ATPase is the subject to a large volume of literature. However, experimental details remain few as to the precise physical chemistry of proton binding and release in these processes. In an attempt to supply such details, we have used membranes (chromatophores) from photosynthetic bacteria where reactions can be followed spectrometrically as they occur from about 10 ps onwards (see ref. 1).

The main advantages of chromatophores over mitochondrial preparations include the following: (a). The electron and proton transfer reactions may be

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initiated as single turnover events by light pulses. (b). They have only one light reaction which occurs in a well-defined reaction center protein (see ref. 1), (cf. the more complicated situation in chloroplasts). (c). Experiments are carried out under anaerobic conditions with no requirement for addition of substrates, and electron flux from endogenous substrates is negligible. The flash-induced processes are cyclic and the behavior of the reaction center-driven ubiquinone-cytochrome b/c_2 (Q-b/c_2) oxidoreductase with respect to electrons and protons depends on its state of reduction prior to flash activation. (d). With *Rhodopseudomonas sphaeroides*, an in situ extinction coefficient is known for the reaction center bacteriochlorphyll dimer, $(BChl)_2$ [2], and this means that absorbance changes induced by short near-saturating single turnover light flashes may be converted precisely into numbers of electrons delivered to the Q-b/c_2 oxidoreductase. This can then be related to the number of protons bound as monitored by absorbance changes in externally added pH indicator dyes, an obvious advantage over the mitochondrial and chloroplasts systems where a major controversy exists as to the number of protons bound at each 'site' [3—7].

In *Rps. sphaeroides* the light-activated reaction center protein which spans the membrane [8,9], operates to drive an electron to the secondary acceptor of the reaction center, a molecule of ubquinone [10,11] at the low potential end of the cycle, and to remove an electron from cytochrome c_2 (two cytochrome c_2 molecules/reaction center) [2] at the high potential end (c_2/c_2^+, $E_{m\,7.0}$ = 295 mV; n = 1). There are 25.3 ± 2.6 quinones/reaction center [12] and the sole quinone species of *Rps. sphaeroides* appears to be ubiquinone-10 [13,14]. The oxidized cytochrome c_2 which is found on the inside of the membrane [15] is itself reduced by the redox center designated Z [35—37] (ZH_2/Z, $E_{m\,7.0}$ = 155 mV; n = 2) [16]. It is becoming apparent [16—18] that Z exerts considerable control over the cycle since rapid electron transfer through the Q-b/c_2 oxidoreductase appears possible only when Z is reduced prior to flash activation.

Microsecond proton uptake by chromatophore membranes was first revealed by Chance et al. [19]. Cogdell et al. [10] established that the reaction is coupled to redox reactions and identified, although only in the presence of valinomycin and K^+, a second, antimycin-sensitive proton binding with a half-time measured to be approx. 2 ms. In a previous paper [20] we identified some of the physical and chemical factors influencing the rapid, antimycin-insensitive proton binding (H_I^+) found in chromatophores of *Rps. sphaeroides* and established that 1.0 ± 0.1 proton is bound/electron delivered to the outer side of the chromatophore membrane by the reaction center protein. A pK at pH 8.5 was apparent for the H_I^+ binding reaction, which was considered to involve the ubisemiquinone ($Q^{\cdot}H/Q^{\cdot-}$). In addition, we have recently shown [18] that in the absence of antimycin, close to 2.0 protons may be bound by each electron and have demonstrated that the rate of binding of the antimycin-sensitive proton (H_{II}^+) appears to be strongly influenced by the state of reduction of the carrier Z prior to the flash. Flash-induced H_{II}^+ binding has a $t_{1/2}$ of about 200 μs when Z is chemically oxidized and about 1.5 ms when Z is reduced before activation.

The behavior of the proton in chemical systems in solution has been the

subject of intense research for over a century and can now be fairly accurately predicted under particular conditions (see ref. 21). The work presented here is a systematic attempt to understand the forces which might govern proton binding during electron transfer in biological membranes. Although substantial progress is made and some guidelines are drawn, it is evident from the results presented here, that we are still at a primitive stage when attempts are made to predict H^+ interactions with membrane-redox proteins at the mechanistic level.

Materials and Methods

Chromatophores free of externally added buffer were prepared from *Rps. sphaeroides* strain Ga as previously described [2,20]. This method of preparation usually produces chromatophores with >60% of the total cytochrome c_2 still attached to the reaction center (see [22]) and this has proved to have a significant influence on the number of protons bound, as will be discussed later. The total amount of bacteriochlorophyll present was estimated using the extinction coefficient at 850 nm of 95 $mM^{-1} \cdot cm^{-1}$ as given by Clayton [23]. Reaction center protein content was provided by the extent of oxidized bacteriochlorophyll dimer, $(BChl)_2^+$, which was assayed following a train of eight, near saturating 6-μs flashes 25 ms apart in the presence of 2 μM antimycin [2,20]. The redox potential prior to flash activation was usually set between 340 and 380 mV so that the $(BChl)_2$ was >90% reduced but cytochrome c_2 and other redox centers of the system were mainly oxidized. In this way, with little $(BChl)_2^+$ re-reduction between flashes, the full extent of $(BChl)_2^+$ could be achieved with minimum complications; to obtain the total reaction center content, the small amount of $(BChl)_2$ already chemically oxidized at the high potential (e.g., 10% at 380 mV) used for the assay was added on to the amount measured during the flash train.

Redox potentiometry in combination with spectrophotometry and flash activation under controlled conditions of pH, E_h and temperature was carried out as previously described [2,24,25]. The redox mediator dyes used, all in 5 μM amounts except where indicated in the figure legends, were: ferro/ferricyanide ($E_{m\,7.0}$; +430 mV); 2,3,5,6-tetramethylphenylenediamine, ($E_{m\,7.0}$; +220 mV); *N*-methylphenazonium methosulphate, ($E_{m7.0}$; +80 mV); *N*-ethylphenazonium ethosulphate, ($E_{m7.0}$; +55 mV), pyocyanine ($E_{m7.0}$; −34 mV) and 2-hydroxyl-1,4-naphthoquinone ($E_{m7.0}$; −145 mV).

Determinations of the extent of proton uptake have also been described before [10,19,20]. The dyes used to monitor the pH of the external medium (chlorophenol red, pH 5.2—6.8; cresol red, pH 7.2—9.0, and phenol violet, pH 8—10) do not bind significantly to the chromatophore membrane [20]: i.e., <5% of the dye added (50 μM) binds to 25 times the chromatophore concentration used in the experiments.

Proton binding was assayed spectrophotometrically at an 'isosbestic' point for the chormatophore optical changes at about 586 nm which is a suitable wavelength to obtain color changes associated with acid-base transitions in the externally added pH indicator dyes. In all cases the 'isosbestic' point was determined at the appropriate pH and redox potential before addition of the pH indicator dye. The baseline was re-checked following addition of ionophores or inhibitors at the end of an experiment after the addition of buffer. It was

similarly confirmed that the selected wavelength was itself free of absorbance changes at all redox potentials considered during the course of the experiment.

Antimycin was present in some experiments to prevent the uptake of H^+_{II} and to prevent electron transport between cytochromes *b* and c_2. Antimycin has been shown to be an effective inhibitor over the entire pH range studied.

Results

Resolution of protons incorporated during a single turnover of the reaction center Q-b/c_2 oxidoreductase

Fig. 1 shows the number of protons incorporated into chormatophores poised under different conditions over a wide range of redox potentials before activation. In essence, the redox poise (see [27,28]) established using redox potentiometry is a quantitative way of adjusting the chromatophore redox centers to a specified state of reduction before the flash so that reactions after the flash can be more readily understood. The attenuation of H^+ uptake/reaction center at high and low redox potentials is due to the familiar description of the equilibrium oxidation of the reaction center bacteriochlorophyll dimer * or reduction of the reaction center primary quinone **. Reaction centers with either $(BChl)_2$ oxidized or the primary quinone (Q(Fe)) reduced are not capable of performing useful photochemistry and so the attenuations express the inactivation of the reaction center.

Fig. 1 shows the extent of H^+ binding under four different states of coupling and inhibition: coupled with no addition (○); plus valinomycin (●); plus antimycin to inhibit electron in the Q-*b*/c_2 oxidoreductase (□); and plus antimycin and valinomycin (■). Clearly under the different conditions of coupling, inhibition and poised redox state established before activation, several constraints are revealed which determine the extent of H^+ binding following a single turnover.

H^+ binding by chromatophores inhibited by antimycin. The open squares of Fig. 1 shows the binding of the antimycin-insensitive H^+_I as a function of the redox state of the chromatophore redox centers. It was previously established that H^+_I is bound with a half-time of 80 μs at pH 6.0 to the extent of 1.0 ± 0.1 H^+_I/e^- [20]. The extent of H^+ binding is independent of the redox state of cytochrome c_2, Rieske Fe-S protein and Z at the time of the flash. However, over the approximate E_h range in which cytochrome b_{50} (E_{m6} = 110 mV) and the main Q complement composed of approx. 19 quinones (E_{m6} = 150 mV) [12] become reduced, the H^+_I/e^- ratio is attenuated following what appears to be an n = 1 Nernst curve with an E_{m6} at approx. 140 mV.

H^+ binding in coupled chromatophores with no addition. The open circles of Fig. 1 shows that with all redox centers oxidized except $(BChl)_2$ only 1 H^+/e^- is bound. As the E_h is lowered to reduce cytochrome c_2 before activation, the H^+/e^- ratio rises to as much as 1.7 before falling off, partly (1 H^+/e^-) over the same potential range described in the previous section (cytochrome

* $(BChl)_2/(BChl)^+_2$, E_{m6} = 450 mV, n = 1; no H^+ involved in the redox reaction [29,30].

** A quinone-iron complex Q·H (Fe)/Q(Fe), E_{m6} = 45 mV, n = 1; 1 H^+/e^- involved in the redox reaction at equilibrium from pH <5 to 9.8 where there is a pK on the reduced form [Q^-H(Fe)/Q^-(Fe)]; see ref. 31 for further details.

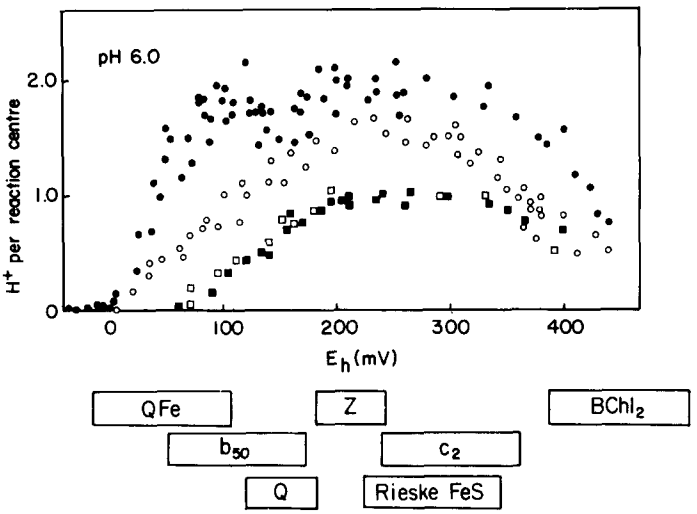

Fig. 1. Titration of the extent of proton uptake under different conditions. Chromatophores (reaction center concentration 0.2 μM) were suspended in the anaerobic cuvette in 100 mM KCl at pH 6.0. 5 μM 2,3,5,6-tetramethylphenylenediamine, 5 μM *N*-methylphenazonium methosulfate, 5 μM *N*-ethylphenazonium ethosulphate and 5 μM pyocyanine were present together with 50 μM chlorophenol red. The measuring wavelength was 586 nm. Absorbance changes in the pH indicator dye were calibrated by addition of 2.5 μM HCl. Titrations were carried out under different conditions: (○) no addition; (□) plus 2 μM antimycin; (●) plus 0.5 μM valinomycin; (■) plus 2 μM antimycin and 0.5 μM valinomycin. Each point represents the average of at least 32 single-turnover flashes spaced 40 s apart, with stirring of the 10 ml suspension between flashes. At the extremes of the titration where absorbance changes were smaller, additional signals were averaged in order to improve the resolution. The points are derived from several different chromatophore preparations. The blocks surrounding the different redox centers in this figure limit those redox potentials between which the centers more from 91% oxidized to 91% reduced. The E_m values of $(BChl)_2$ the redox components are taken from the following references: cytochrome c_2 and cytochrome b_{50}, [2,20]; Q(Fe), [31–33]; the Rieske Fe-S center, [34]; Z, [16]; and Q of the main 19 complement, [12].

b_{50} and Q reduced), and partly with the reduction of the reaction center primary Q(Fe). The half-point of attenuation at the low potential end is about 80 mV; the overall *n*-value is less than 1 and clearly this results from more than one process. Cogdell et al. [10] previously obtained a similar half-point for H^+ binding in chromatophores under similar conditions. If we accept that the H^+ uptake is a combination of H_I^+ and H_{II}^+ then subtracting the antimycin-insensitive H_I^+ from the total extent we may tentatively suggest that the binding of H_{II}^+ is governed at the high potential end by the redox state of cytochrome c_2 (or the Rieske Fe-S protein). At the low potential end it is limited only by photochemical inhibition due to a pre-existing reduced state of the reaction center primary Q(Fe).

The effect of valinomycin on H_{II}^+. Cogdell et al. [10] have reported a stimulation of the extent of proton binding in the presence of valinomycin and potassium ions. The uppermost redox profile in Fig. 1 (●) demonstrates that the maximal extent of binding of the antimycin-sensitive proton, H_{II}^+, is increased from 0.7 H_{II}^+/e^- to 1.0 H_{II}^+/e^- (±0.3 H_{II}^+). Both the extent and shape of the redox profile are altered. In the presence of valinomycin, H_{II}^+ binding can be observed at high potentials where all redox centers except $(BChl)_2$ are chemically oxidized before the flash. At the lower potential end over the E_h

range where we have seen H_I^+ attenuate, there is now only a shallow depression in the extent of proton binding (E_h = 150 mV at pH 6). Final attenuation as expected comes with the reduction of the primary Q(Fe) and cessation of photochemistry.

The effect of antimycin in the presence of valinomycin. Antimycin is able to override all the effects supported by valinomycin, returning the system to that seen with antimycin alone.

Further details on the redox properties of the antimycin-insensitive proton binding (H_I^+)

The E_m/pH relatioship on the first turnover. In order to investigate the E_m/pH relationship of the agent binding or controlling the binding of H_I^+, redox titrations of the kind typified in Fig. 1 were done in the presence of antimycin at a variety of pH values. These are shown in Fig. 2. As already indicated from Fig. 1 under these conditions, the course of oxidation or reduction follows a curve that approximates a Nernst curve of *n*-value 1, although the scatter in the points makes this an uncertain conclusion; several other possibilities are shown in the top right of the figure which we shall discuss later. The E_m/pH relationship however, seems more certain.

The E_m of the redox agent responsible for H_I^+ binding at every pH value shown in Fig. 2 is plotted against pH as shown by the solid circles (●) in Fig. 3. The E_m/pH relationship (−60 mV/pH unit below pH 8.0 and approx. 0 mV above pH 8.0) reveals what appears to be a p*K* on the reduced form of the redox couple at about pH 8. It should be emphasized that this p*K* derived from the E_m/pH plot is a different value from that evident from the H_I^+/e^- ratio which appears at 8.5 [20]; the value of this previously reported p*K* [20] is confirmed in Fig. 2 from the diminution in the H_I^+/e^- ratio (i.e. 0.5 at pH 8.5) as the pH is raised through the pH 8—9 region. The loss of H^+ binding at the higher pH values ultimately limits the E_m/pH determinations; for example, a maximum of only 0.09 H_I^+/e^- is bound at pH 9.5, which approaches the current limits of measurement.

The E_m/pH relationship on the second turnover. The open circles (○) in Fig. 3 represent the E_m values from redox titrations (not shown) of the extent of H^+ binding after a second flash delivered 25 ms after the first. Below pH 8 the E_m/pH relationship is within experimental error the same as that encountered on the first flash. However, in contrast, above pH 8 the second flash E_m/pH relationship maintains a −60 mV/pH unit dependency. Nevertheless we find (not shown) that the p*K* of H_I^+ on the second turnover, provided by the H_I^+/e^- ratio is still at pH 8.5.

H_I^+ binding on the first and subsequent turnovers. The results presented so far enable us to optimize conditions of pH and E_h to present key features of H_I^+ binding. The differences in H_I^+ binding between the first and three subsequent turnovers delivered 25 ms apart are presented in Fig. 4. At pH 6.0 (well below the H_I^+ p*K*) and an E_h of 230 mV (cytochrome b_{560} and Q essentially oxidized; cytochrome c_2 reduced before activation) protons are picked up on each turnover. The amount bound following each flash after the first diminishes, but this is because in the presence of antimycin $(BChl)_2^+$ is not completely reduced between each flash and so becomes increasingly oxidized with

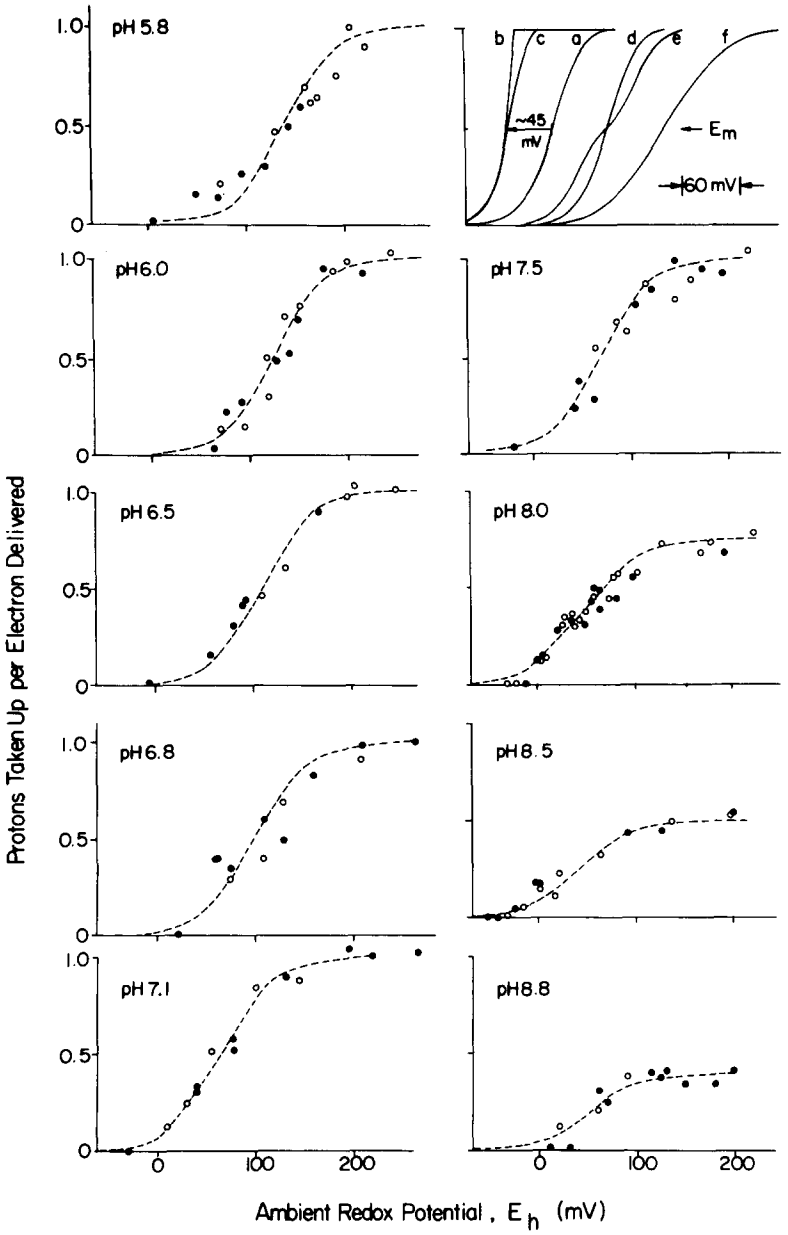

Fig. 2. Titrations to determine the midpoint at different pH values of the H_I^+ binding agent. Conditions as in Fig. 1 except that 2 μM antimycin were present in all cases and different pH indicators were used: pH 5.8—6.8 chlorophenol red; pH 6.8—7.8 phenol red; pH 7.5—8.8 cresol red. •, points taken as the chromatophores were reduced by addition of specks of solid sodium dithionite, and ○, points taken as the potential was raised with dilute potassium ferricyanide. The lack of hysteresis in performing oxidative and reductive titrations is an indication that the system was at equilibrium. Nernst $n = 1$ lines are drawn through the points in all cases; alternative curves are depicted in the top right hand corner. (a) An $n = 2$ Nernst curve; (b) is the course of reduction of the last of the 19 Q complement if any Q can react with any reaction center; (c) is the restricted version of (b) statistically based on a model in which the 19 Q complement is fixed to one reaction center (i.e., the curve is $1 - (1 - x)^{19}$ where x is the fraction of the total 19 quinones oxidized at a prescribed E_h; see ref. 16); (d) and (e) present equal mixes of $n = 2$ curves separated by 30 and 60 mV, respectively, and (f) is a simple $n = 1$ curve.

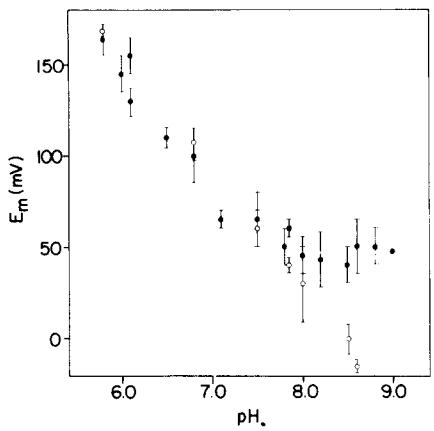

Fig. 3. The E_m/pH profile of the H_I^+ binding agent. The midpoints obtained at each pH value in Fig. 2 are plotted against the appropriate pH. E_m on the first flash (●), E_m when a second flash is given 25 ms after the first (○). Error bars indicate the limits of values obtained in at least 5 separate determinations, but at pH 8.8 only a single experiment was performed.

successive flashes. This causes fewer reaction centers to be active, but nevertheless the H_I^+/e^- ratio under these conditions stays at about 1.0 throughout (see Fig. 5 in ref. 20). At pH 6.0 and at an E_h of 70 mV (cytochrome b_{50} ≈30% reduced; Q ≈90% reduced, and Q(Fe) ≈25% reduced) proton binding is barely observable on any turnover. At pH 8.6 (approximately the H_I^+ pK) and an E_h of 160 mV (cytochrome b_{50} and Q are essentially oxidized) protons are bound on every flash although, because of the closeness of the ambient pH to the pK, the H_I^+/e^- ratio is now close to 0.5. At pH 8.6 and an E_h of −10 mV (cytochrome b_{50} ≈80% reduced; Q ≈50% reduced; Q(Fe) ≈10% reduced) very little H^+ uptake is observed on the first turnover, but it is observed on the second

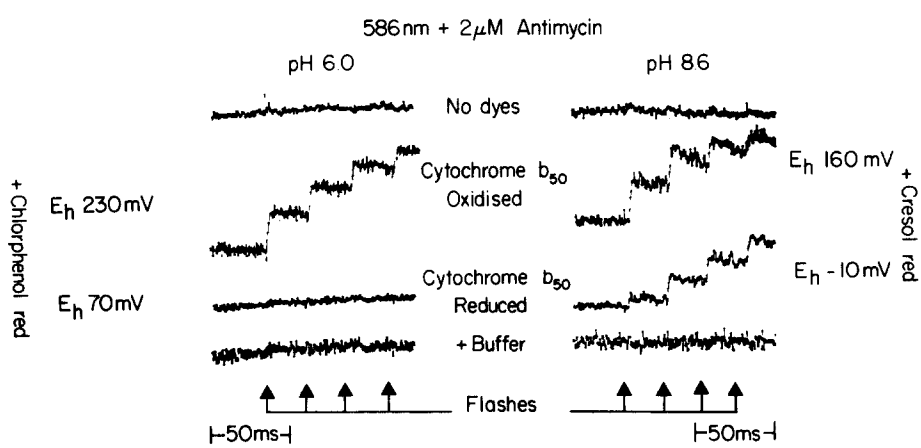

Fig. 4. H_I^+ binding on the first four turnovers. The conditions are as described in the legend to Fig. 2. Proton binding was induced by a train of 4 flashes 25 ms apart at pH 6.0 and pH 8.6 at ambient redox potentials where cytochrome b_{50} and/or Q will be either reduced or oxidized prior to the flash. A full commentary of the experiment, as well as the calibrated H^+/e^- ratios of the flash-induced reactions are given in the text.

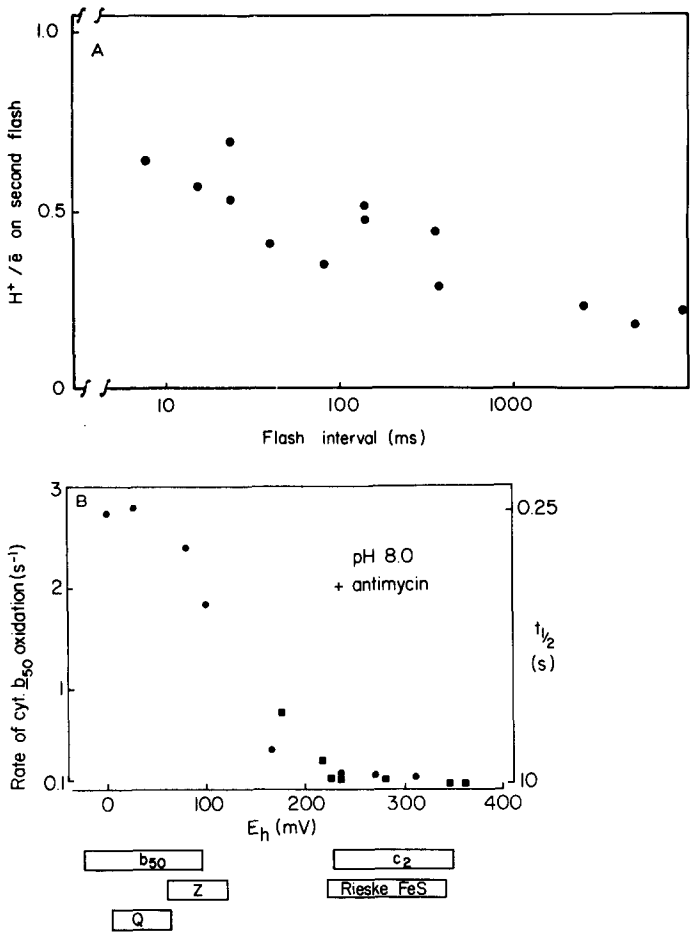

Fig. 5. (A). The H^+/e^- ratio on the second flash delivered at different time intervals after the first. The conditions were as in the legend to Fig. 2, but using the pH indicator dye cresol red (50 μM). The pH was 8.6 and the E_h was 20 mV. (B). A redox titration of the kinetics of flash-reduced cytochrome b_{50} re-oxidation. The experiments were done at pH 8.0 in the presence of 2 μM antimycin. 5 μM each of the redox mediating dyes listed in Fig. 1 were present (•). However, some measurements (▪) were done in the absence of redox mediating dyes with the approximate redox potential estimated from the estent of cytochrome c_2 oxidation measured immediately after the determination on the *b* cytochrome. The re-oxidation of cytochrome b_{50} was approximately first order and so half-times were calculated from semilogarithmic plots, although we are aware that these reactions may ultimately prove to be more complicated (cf. refs. 22 and 43).

and subsequent ones. The H_I^+/e^- ratio here is 0.25 because, in addition to the pK at 8.5 which at pH 8.6 diminishes the maximum H_I^+/e^- ratio to approx. 0.5, the E_h at −10 mV is near the E_m point of Q (see Figs. 3 and 6) and this cuts the value by another 50%. Under these conditions, the confinement of H^+ binding failure to only the first turnover suggests that this is not an binary oscillatory system of the kind reported in the reaction center primary and secondary Q [40—43].

It is of some interest to determine the time period required between the first and second turnovers such that H^+ binding does not occur on the second turnover, and to correlate this with events in the antimycin inhibited Q-b/c_2 oxido-

reductase. These results are shown in Fig. 5. With chromatophores inhibited with antimycin and poised at pH 8.6 and E_h = 20 mV, Fig. 5A shows that with a 10 s or greater period between each single turnover, H^+ uptake on the second turnover is the same as the first; under the slightly higher E_h conditions chosen for this experiment (cytochrome b_{50} 70% reduced; Q 30% reduced) this is about 0.15 H_I^+/e^-. However, as expected, shorter intervals between the flashes permit the H_I^+/e^- ratio to rise above this basal ratio. The time at which the H_I^+/e^- ratio on the second flash is half-maximal is in the range 100—500 ms. Fig. 5B shows an experiment to estimate the re-oxidation time of ferrocytochrome b_{50} under conditions close to those used for the experiment of Fig. 5A. However, it is not possible to measure flash-activated ferrocytochrome b_{50} oxidation starting with the cytochrome reduced prior to activation because no net redox change is observed in the presence of antimycin. Thus the experiment was done starting with cytochrome b_{50} oxidized; the cytochrome was then flash reduced (at E_h values approx. 200 mV this is 1—2 ms half-time) and the course of re-oxidation was measured. To gain some feeling of the variability of this reaction, the measurements were done over a wide range of E_h values from high values down into the Nernst curve of cytochrome b_{50} itself, where obviously the extent of flash-induced reduction and oxidation diminishes and ultimately becomes experimentally limiting. At 20 mV (the same E_h as in Fig. 5A) the half-time for re-oxidation of flash-reduced cytochrome b_{50} is in the 200—300 ms range, which is in reasonable agreement with the dark period for half-maximal second turnover proton binding. (Although not of primary interest to this paper we were surprised to note that at the higher E_h values where the components of the Q-b/c_2 system are more oxidized, the actual course of cytochrome b_{50} oxidation became much slower approaching a half-time of about 10 s.) The E_h dependency roughly followed the Nernst curve of the ZH_2/Z couple, so it appears that the prior reduced state of Z (i.e. ZH_2) promotes more rapid electron transfer through the antimycin block of the Q-b/c_2 oxidoreductase (see ref. 12 for further discussion).

In summary, the results are consistent with the idea that two components (cytochrome b_{50}, and an agent with an E_m and pH dependency (although apparently not an n-value) similar to that of the main 19 Q complement) can influence the binding of H_I^+. The E_m/pH behavior for the first turnover (from Fig. 3) are presented again (□) in Fig. 6 to allow comparison with the E_m/pH relationships of other redox agents associated with the reaction center Q-b/c_2 oxidoreductase. On the first turnover, the prevention of H_I^+ binding as the E_h is lowered follows the component with the higher E_m. Below pH 8.0 this is the Q; above pH 8.0 this is cytochrome b_{50}. The E_m/pH data from Fig. 3 for the second and subsequent turnovers are not presented in Fig. 6 for reasons of congestion, but comparison of Figs. 3 and 6 clearly shows that the influence of cytochrome b_{50} is eliminated on the second turnover, and the control then follows the E_m of Q over the entire pH range. Indeed, the prior state of oxidation of the agent with close similarity to Q is obligatory for H_I^+ binding under all conditions in the presence of antimycin; the influence of cytochrome b_{50} is only encountered at pH values where its E_m is higher than the Q, and only then on the first turnover after a suitable dark period. Under these conditions, the dark period required such that upon the second turnover the influence of cyto-

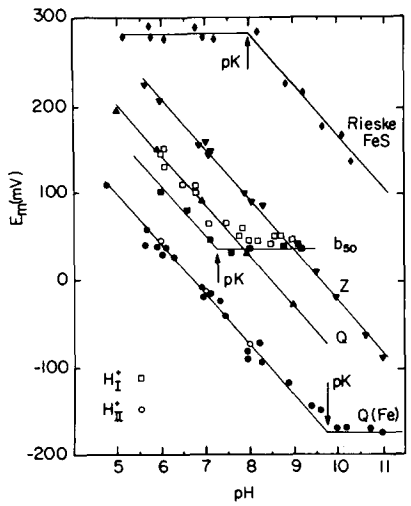

Fig. 6. Plots to demonstrate the similarities between the E_m/pH profiles of the H_I^+ binding agents. The points (□) are taken from Fig. 2. The others are as follows: cytochrome b_{50} (■, ref. 38), the main 19 complement (△, ref. 12), Z (▼, ref. 16) and the Rieske Fe-S (◆, ref. 34). The figure also compares the E_m/pH profile of the H_{II}^+ binding agent (○) and the reaction center Q(Fe) (●, refs. 31—33). The midpoint of the H_{II}^+ binding agent at different pH values was determined in the same was as described in the legend to Fig. 1.

chrome b_{50} is re-instated is similar to the time required for the re-oxidation of ferrocytochrome b_{50}. This may mean that with cytochrome b_{50} already reduced before activation, the first electron emerging from the reaction center cannot bind a H^+. With the arrival of a second, and subsequent electrons, H^+ binding is possible if they arrive before the first has moved from the Q-cytochrome *b* via Z to cytochrome c_2.

Under all the conditions mentioned above, the p*K* of the H_I^+, as given by the H^+/e^- ratio, seems to be at 8.5.

Further details of the properties of the antimycin sensitive proton (H_{II}^+) binding

The E_m/pH relationship on the first turnover. Titrations of the extent of proton binding in the absence of antimycin of the kind shown in Fig. 1 (● and ○) at different pH values show that from pH 6 to 8, the midpoint of the agent controlling the binding of H_{II}^+ appears to be the same as that of the reaction center primary quinone Q(Fe); E_m values at different pH values are plotted (○) in Fig. 6. Above pH 8 it becomes very difficult to study the effect of redox potential on the extent of proton binding, since, as will be discussed in the next paragraph, there is an apparent p*K* on the agent binding H_{II}^+ at pH 7.5, so at pH values higher than the p*K*, the same experimental limitations exist for E_m measurements using H_{II}^+ that were mentioned above for H_I^+.

The pK apparent for H_{II}^+ binding. This was measured in two ways as shown in Fig. 7. The flash-induced proton uptake shown at each pH value is uniquely that for H_{II}^+, because the contribution from H_I^+ to the overall experimentally observed H^+ binding has been subtracted. In Fig. 7A the ambient E_h was altered by −60 mV/pH unit of change in the suspending medium. This combined

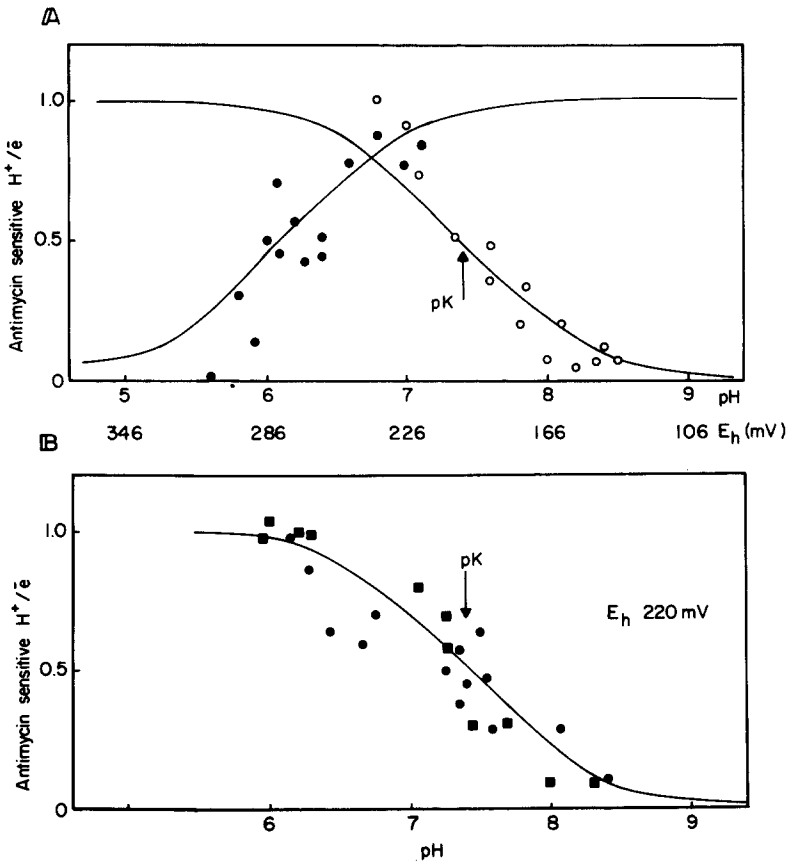

Fig. 7. Determination of the pK on the H^+_{II} binding agent. (A). The conditions were as in the legend to Fig. 1 without antimycin and valinomycin. Measurements were made with 50 μM cresol red (○) or 50 μM chlorophenol red (●). The contribution to the total proton uptake which would theoretically be expected at each potential for H^+_I (pK = 8.5) has been subtracted from each point so that the data is uniquely H^+_{II}. In this determination the E_h was altered together with the pH by —60 mV/pH unit. (B). The conditions were as described in (A) except that a constant E_h of +220 mV was used (●) and valinomycin was present in some cases (■). The pH indicator dyes were as in (A), i.e., cresol red above pH 7, chlorophenol red below pH 7.

change of E_h with pH was to permit examination of flash-induced H^+_{II} binding while maintaining the same relative position in E_h with respect to the redox components of the Q-b/c_2 oxidoreductase which have pH-dependent E_m values. At high pH the H^+_{II}/e^- ratio diminishes suggesting that H^+_{II} has a pK at 7.5. At lower pH values, the increasing E_h (as was seen in Fig. 1) clearly eliminated H^+_{II} binding. This effect, which will be dealt with more fully later (see Fig. 1), appears to correspond to the course of oxidation of cytochrome c_2, or the Rieske Fe-S protein, neither of these two redox components has an E_m value that is significantly pH dependent in this pH range, so the effect is not unexpected. Having established that the E_h should be maintained low enough to reduce cytochrome c_2, we repeated the experiment using a fixed E_h of 220 mV, as shown in Fig. 7B. This yielded a simple Henderson-Hasselbalch curve and although the scatter is fairly wide, the extent of proton binding still

falls off with an apparent pK at about 7.5. Under these conditions the same value was obtained both in the presence (■) and absence (●) of valinomycin.

The effect of valinomycin on the pK of the agent responsible for H^+_{II} binding. As was shown in Fig. 1, valinomycin is able to increase the maximum extent of H^+_{II} binding from up to 0.8 H^+_{II}/e^- to 1.0 H^+_{II}/e^-. In addition, when cytochrome c_2 was essentially oxidized and $(BChl)_2$ was 90% reduced (E_h = +380 mV) before flash activation, H^+_{II} uptake could be stimulated from near 0.0 to 0.8 H^+_{II}/electron by the additon of valinomycin. This is demonstrated further in Fig. 8A and B, where the effect of increasing valinomycin concentrations is monitored at redox potentials such that cytochrome c_2 is reduced (Fig. 8A) or oxidized (Fig. 8B) prior to xenon flash activation. In the chromatophores used for the experiments depicted in Fig. 8, the reaction center concentration was 0.2 μM, and it can be seen in Fig. 8B that at E_h = 380 mV (cytochrome c_2 oxidized before activation) about 0.02 μM valinomycin is required to induce the maximal extent of proton binding.

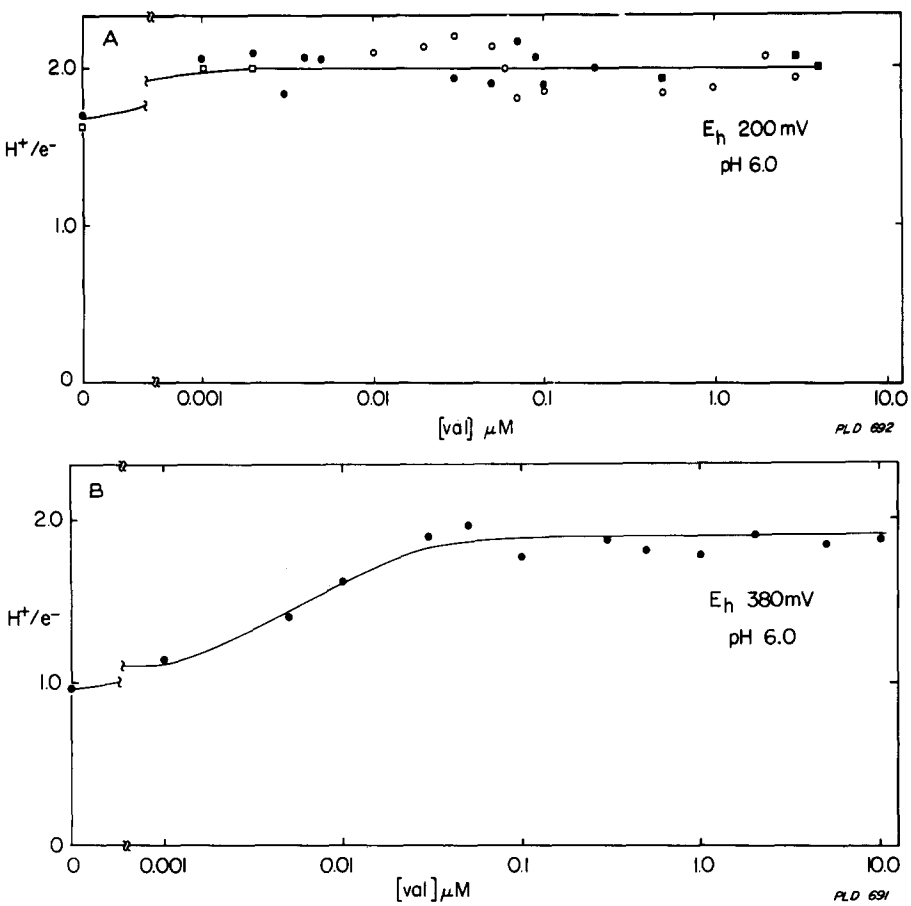

Fig. 8. Titrations of the extent of proton binding (H^+_I and H^+_{II}) in the presence of different concentrations of valinomycin, with cytochrome c_2 chemically reduced (A) or oxidized (B) prior to activation. The different symbols in (A) represent different preparations. Otherwise, the conditions are as in the legend to Fig. 1 without antimycin.

In contrast, at redox potentials (e.g., E_h = 200 mV) such that cytochrome c_2 is reduced prior to flash activation (Fig. 8A), addition of less than 0.002 μM valinomycin is sufficient to increase the amount of H^+_{II} bound in this experiment from 0.8 H^+_{II}/e^- to 1.0 H^+_{II}/e^-.

We have investigated the possibility that the lack of H^+_{II} binding, when cytochrome c_2 was oxidized prior to flash activation, is caused by a pK shift from 7.5 (with cytochrome c_2 reduced) down to well below the pH of measurement (pH 6.0). In such a case, the effect of valinomycin could be to bring the pK back up to higher values. Evidence in support of this suggestion is provided in pH titrations of the extent of H^+_{II} binding at different valinomycin concentrations with chromatophores poised at a redox potential of 380 mV (Fig. 9A).

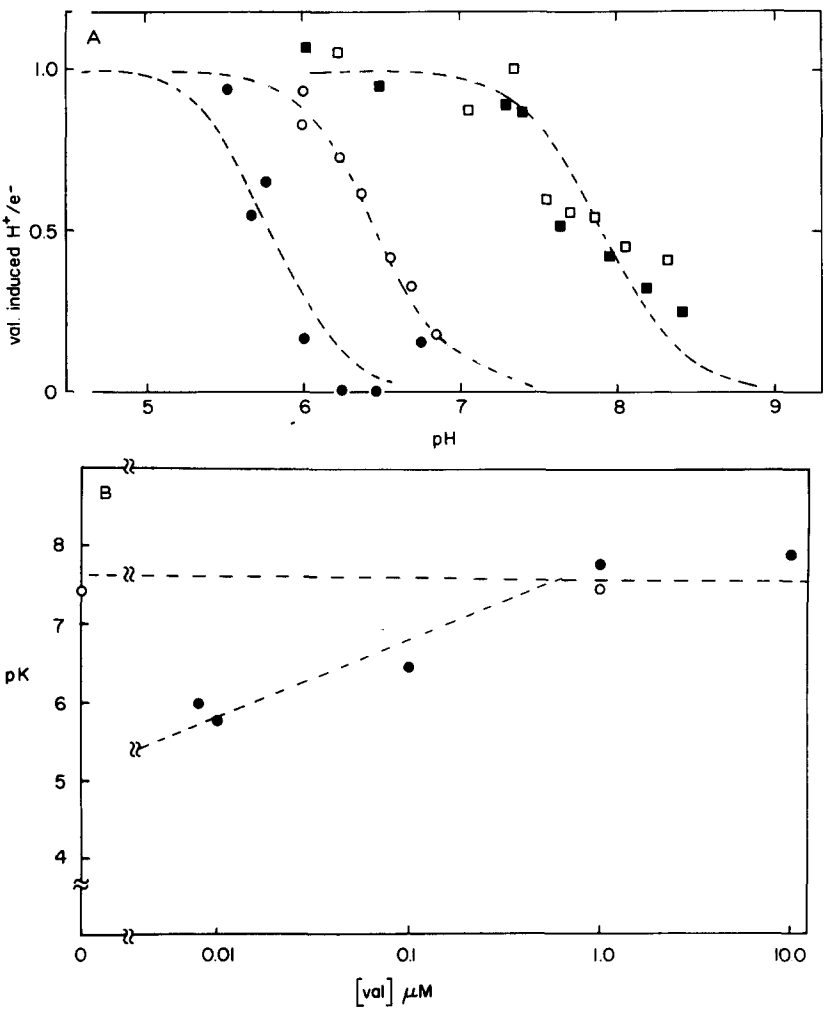

Fig. 9. pH titrations of the extent of H^+_{II} binding. (A). The titrations were done at E_h = 380 mV without antimycin. As in Fig. 7, the contribution of H^+_I has been subtracted. The titrations were done in the presence of 0.01 μM (●) 0.1 μM (■) and 10 μM valinomycin (□). (B). The apparent pK of the H^+_{II} binding agent at each valinomycin concentration at E_h = 380 mV (●) and E_h = 200 mV (○).

We have been unable to find a pH indicator dye suitable for studying pH changes below pH 5.5, but at this pH and at a redox potential of 380 mV in the absence of valinomycin, H^+_{II} binding is not seen; thus the apparent pK on the agent binding H^+_{II} is below 5.0 under these conditions. Addition of 0.001 μM valinomycin brings the measured pK on the H^+_{II} binding agent from this unmeasurable value to a value of 5.8. Higher concentrations of the ionophore shifts the pK still further, and at 0.02 μM valinomycin it reaches a value of 7.5 coinciding with the value obtained with cytochrome c_2 reduced. Higher valinomycin concentrations have little further effect on the measured pK. In addition to the E_h = 380 mV titrations (closed circles), the open circles demonstrate the constancy of the pK of the H^+_{II} binding agent in the presence of valinomycin when cytochrome c_2 is reduced before activation (E_h < 200 mV), as was shown in Fig. 7.

Thus, in summary, the highest pK apparent for H^+_{II} binding is 7.5. This value is lowered to below pH 5.0 if cytochrome c_2 is oxidized before activation, but valinomycin can overcome this effect, returning the pK to 7.5.

The effect of the redox state of cytochrome c_2 before activation on the pK of ferrocytochrome b_{50}

As discussed above and in a previous paper [38], ferrocytochrome b_{50} has a functional pK at pH 7.4 (i.e., at pH <7.4 it requires at equilibrium 1 proton/single electron reduction, while at pH >7.4 a proton is not required). In addition to the E_m/pH relationship presented in Fig. 6, this pK was also revealed [38] by monitoring H^+_I reappearance from the chromatophore at different pH values in the presence of antimycin and uncoupler. Below the pK of cytochrome b_{50}, H^+_I was accepted by the cytochrome with an electron (the donor was considered to be Q · H), and so was released only slowly as the antimycin block was bypassed and the protonated ferrocytochrome was re-oxidized. Above the pK (but below the pK of H^+_I; i.e., between pH 7.5 and 8.5), H^+_I was found not to be required for reduction and so was free to promptly return in 1—2 ms to the outer aqueous phase aided by the uncoupler (nigericin and FCCP are equally effective in this capacity). The pH dependency of the transition from slow to prompt re-appearance of the H^+ provided the pK of cytochrome b_{50}. However, the measurements previously described [38] were carried out at a redox potential such that cytochrome c_2 was reduced prior to activation (e.g. E_h = 200 mV). We have repeated the experiment, but with the view to examine what happens when cytochrome c_2 is oxidized before activation (see Fig. 10) (e.g., E_h = 380 mV). Under these conditions it appears that the pK on cytochrome b_{50} has been shifted from 7.4 to a value below pH 5.0. Fig. 10 shows two redox titrations done at different pH values, of the extent of rapid re-appearance of H^+_I. At pH 7.4 (the pK) and E_h = 200 mV (cytochrome c_2 reduced) 50% of the protons were re-released rapidly with the other 50% released only on oxidation of the cytochrome b_{50}, in the seonds time domain; this confirms the pK at 7.4 observed when cytochrome c_2 is reduced [38]. However, as the redox potential is raised clearly fewer of the cytochromes b_{50} require a proton on reduction and a higher percentage of rapid H^+ release is observed. At an experimental pH of 5.8 and E_h = 200 mV, as expected nearly 100% of the cytochromes b_{50} (pK = 7.4) require a proton on reduction and

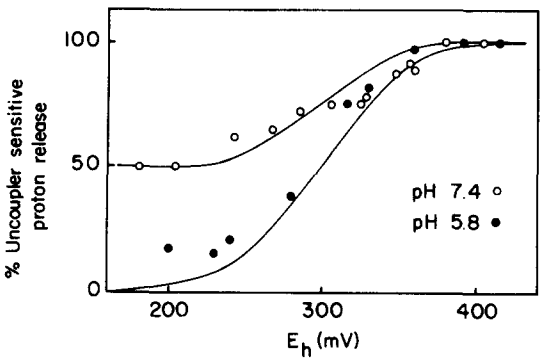

Fig. 10. Redox titrations of uncoupler-stimulated re-appearance of protons bound initially as H_I^+. The conditions were as described in the legend to Fig. 2; 2 μM antimycin and 5 μM *p*-trifluoromethyxophenylhydrazone (FCCP) were present. Uncoupler-stimulated H^+ re-appearance was complete within 25 ms of the flash, but uncoupler-insensitive protons were retained by the system for several seconds. Determinations made at pH 7.4 (○) (the p*K* of cytochrome b_{50}), and at pH 5.8 (●) (well below the p*K* of cytochrome b_{50}).

there is no prompt re-appearance of protons in the external aqueous phase; but as the E_h is raised so as to oxidize cytochrome c_2 before activation, the p*K* appears to shift to a value well below pH 5.8, since nearly all H_I^+ is rapidly released. We therefore conclude that the apparent p*K* on cytochrome b_{50} at high potentials must be at a pH below 5.0.

Discussion

The goal of this work and of our previous studies [1,2,8,16—18,20,29, 21,38] on the photosynthetic electron and proton transfer system is to determine the thermodynamic properties of the components involved, and the time course of the events. In describing the energy coupling processes we want to know for any instant in time the physical-chemical character of the agents carrying the energy, and to know how the energy is converted from one form to another. With different redox centers reduced (at equilibrium with known values of E_h and pH) before single-turnover excitation, we can see the altered patterns of behavior of electron transfer and of proton binding and release. Proton binding/release patterns as events coupled to flash-activated redox reactions provide unique ways of observing transient changes in equilibria that may be encountered during the conversion of redox and charge separative energy into chemical potential free energy. Integral to consideration of electron and proton transfer is the location of the individual redox components with respect to the chromatophore membrane. Of the components established to be primary to the reaction center Q-b/c_2 oxidoreductase, what is known of their membrane positions can be summarized as follows: (a). Cytochrome c_2 (two molecules/reaction center [2]) is on the inner membrane interface [15]. (b). Evidence suggests that the reaction center $(BChl)_2$ is near the center of the membrane dielectric [8,44]. (c). The reaction center primary quinone-iron complex (QFe) is at a position nearer the outer side of the chromatophore [8]. (d). At least one other secondary Q is associated with the reaction center and is

functionally close enough to the outer aqueous interface to permit proton binding concomitant with its reduction to occur with no detectable diffusional limitations ([20] and see ref. 12). (e). Z, as the reductant to ferricytochrome c_2 [16] must be functionally close at least to the cytochrome on the inner side of the membrane (see also [22]). (f). Cytochrome b_{50} is not in contact with the external aqueous phase [38] and there is evidence to suggest that it is in rapid contact with the inner aqueous phase [38] (see later) although some schemes have weighed this decision against other considerations and have it placed within the membrane [37] towards the outside.

Simple oxidation-reduction and H^+ binding-release

Proton binding that is coupled directly to an oxidation-reduction is usually presented as follows:

$$\begin{array}{ccccc} A & \rightleftharpoons & A^- & \rightleftharpoons & AH \\ & {\scriptstyle e^-} & & {\scriptstyle H^+} & \end{array} \qquad (1)$$

Thus with A oxidized before its flash-activated reduction, progressively less H^+ is bound per electron delivered as the pH of the aqueous phase, presumed in equilibrium with the redox couple, is raised describing the Henderson-Hasselbach curve through the pK of the AH/A^- pair. This may be illustrated by the pK = 8.5 apparent for the agent binding H_I^+ [20] and in Fig. 7 by the pK = 7.5 of H_{II}^+; analogously, cytochrome b_{50} appears to require a proton in addition to an electron only if the ambient pH is below the pK = 7.4 of the reduced citochrome b_{50} (see Fig. 10 and ref. 38). The second aspect of Eqn. 1 that we have used as a simple rationale for experiments in this paper is that prior reduction of A at equilibrium will prevent prompt H^+ binding after a single turnover activation. Thus the greater the state of reduction of the AH/A couple before activation, the less H^+ is bound after activation; this provides the Nernst curve through the E_m of the AH/A couple at a prescribed pH. Determination of E_m values of the redox couple at different pH values below the pK_{red} of the AH/A^- pair will show the characteristic decrease of 60 mV/increase in pH of one unit. The attenuations of H_I^+ and H_{II}^+ with lowering E_h titrations of Figs. 1 and 2 describe Nernst curves and display distinctive pH dependencies (Figs. 3 and 6) and prima facie are consistant with these expectations. However, there is room for doubt as to whether this is really a direct indication of the properties of the proton binding agents themselves. The arguments against us adopting the simple explanation are as follows:

(a) H_I^+. Fig. 6 showed that the E_m/pH dependency of H_I^+ results was explainable as a combination of effects from the E_m/pH dependencies of cytochrome b_{50} and the main 19 Q complement. The E_m values of Q and cytochrome b_{50} cross at pH 8 (Fig. 6); above this pH cytochrome b_{50} seems to prime importance in that no H_I^+ binding is seen following single-turnover activation when the cytochrome is reduced; at pH values below the cross-over point, the E_m is closer to the main 19 Q complement E_m although the n-value is not 2. On the second and subsequent turnovers the influence of cytochrome b_{50} is overruled at any pH value and the E_m/pH relationship is the same as the Q. (It is also interesting to note that the pK on the oxidized form of the Rieske Fe-S protein at 8.0 [34] is close to the break in the E_m/pH plot displayed by the H_I^+ agent(s)

on the first turnover (see Fig. 6); however, although this may ultimately prove to be relevant, a function has yet to be demonstrated for this almost ubiquitous redox center).

Although cytochrome b_{50} does seem to exert some control on the proton binding reaction following a single-turnover flash, it cannot itself be the direct H_I^+ binding agent because of the large kinetic discrepancy between H_I^+ binding and the slower cytochrome b_{50} reduction; it seems clear that H_I^+ binding occurs concomitantly with the reduction of the reaction secondary Q. In concert with this it has been shown that in *Rps. sphaeroides* H_I^+ binding does not occur following extraction of all quinones except the reaction center primary Q (Takamiya, K., unpublished result; see also refs. 11 and 12). In addition, the pK of H_I^+ binding extent observed at 8.5 is not simply that of ferrocytochrome b_{50} at 7.4 [20,38] and furthermore the pK shift displayed at high E_h by the H_I^+ agent (8.5 to 7.5; ref. 20) is markedly different from that of cytochrome b_{50} (7.4 to <5; Fig. 10). Thus is spite of the complicatons, it still seems probable that some form of Q is the agent (as flash generated Q^-) which actually binds H_I^+. If we consider that the main 19 Q complement as a whole was responsible for H_I^+ binding, the H_I^+ attenuation would not necessarily describe a simple Nernst curve characteristic of the E_m value of the entire complement as seen in ref. 12; it might tend toward the curves b and c plotted in the top right frame of Fig. 2 for the E_h range which describes (with lowering E_h) the 'last' Q to be reduced of the 19 Q complement. The half-reduction point for this is approx. 45 mV lower than the measured E_m of the 19 Q complement as a whole. As such, it would have half-reduction point at pH 6 at 105 mV not the observed 150 mV, and the course of oxidation reduction would not be a symmetrical Nernst curve (see Fig. 2). More elaborate arguments can be made using the quinones (see the legend of Fig. 2), but whether they are of value at this point is questionable since we do not yet known whether the 19 Q complement is directly kinetically involved in the reaction center Q-cytochrome b_{50} electron transfer sequence. It is unfortunate that we do not have any direct details on the reaction center-associated secondary Q which is perhaps the most likely candidate to be actually responsible for the H_I^+ binding. There is no reason why it would not have a similar E_m to the constituents of the 19 Q complement; current indirect indications are that the secondary Q can be a two electron redox center [40,41] but directly determined details of its n-value remain unknown.

(b) H_{II}^+. The binding of H_{II}^+ attenuates in the redox titration shown in Fig. 1 with the equilibrium reduction of the primary Q(Fe) of the reaction center and therefore with the chromatophore photochemistry, and it follows a similar E_m/pH dependency from pH 6—8 as displayed by Q(Fe) shown in Fig. 6. The actual equilibrium E_m value of the agent binding H_{II}^+, if indeed it is a redox agent, could be below this value. Alternatively, if the agent were formed during the flash activation processes in the Q-b/c_2 oxidoreductase, a dependency on Q(Fe) would also be encountered.

Electrochemical interactions affecting H^+ binding and release

In order to progress beyond descriptions of resting equilibrium it is necessary to tak e a more comprehensive view of Eqn. 1. This is shown in Eqn. 2 and is

presented graphically in Fig. 11:

$$\begin{array}{ccc} e^- + A^+H & \overset{E_{ma}}{\rightleftharpoons} & AH \\ {\scriptstyle pK_{ox}}\uparrow\downarrow & & \uparrow\downarrow{\scriptstyle pK_{red}} \\ e^- + H^+ + A & \overset{E_{mb}}{\rightleftharpoons} & A^- + H^+ \end{array} \qquad (2)$$

E_{ma} and E_{mb} represent the limiting E_m values of the redox/acid-base system either at acidic or basic pH values respectively, and pK_{ox} and pK_{red} are the pK values of the acid-base transitions of the oxidized and reduced forms of the redox couple. This fuller description is justified in Fig. 6 where pK_{ox} and E_{ma} are represented by the Rieske Fe-S protein (pK_{red} and E_{mb} are still unknown) and pK_{red} and E_{mb} are represented by cytochrome b_{50} and the reaction center primary quinone (pK_{ox} and E_{ma} unknown).

Such schemes provide the framework for discussion of how oxidation-reduction and its coupled reactions measured under resting equilibrium conditions might behave under non-resting conditions emanating from an 'energized state' or from disequilibria that exist in the timescale of the functional events of the membrane system. An example of the latter case is provided by the reaction center primary quinone Q(Fe): although its equilibrium redox titration yields a −60 mV/pH unit variation ([31], see Fig. 6) up to pH 9.8 where there is a pK_{red}, the light-activated reaction center is energetically capable of putting an electron on Q(Fe) without an accompanying H^+; proton equilibration with Q^-(Fe) is slow relative to the residence time of the electron on the primary Q during its photoreduction (approx. 150 ps half-time) and subsequent re-oxidation by the secondary Q (approx. 100 μs half-time). Thus, as indicated

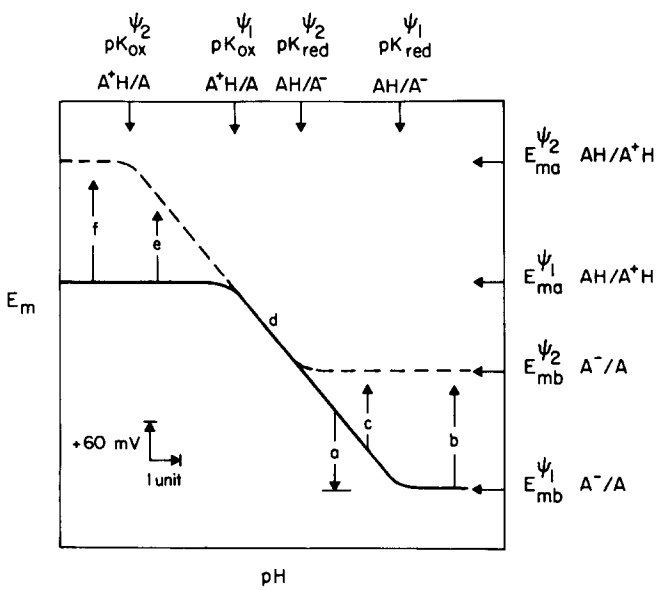

Fig. 11. The E_m/pH relationship of a redox couple and the effect induced by a positive change of electrical potential in its immediate environment from ψ_1 to ψ_2. See text for details.

by the point a in Fig. 11, the functional redox couple of the primary Q(Fe) is $Q^{\cdot-}/Q$, and the functional E_m is given by E_{mb} at −180 mV which is quite different from the Q·H/Q redox couple given at pH values in the physiological range (e.g. in Fig. 1 it is +45 mV at pH 6). For further discussion see refs. 31 and 39 and references cited therein.

In this paper we have extended the instances where, in contrast to the above example, an agent involved in H^+ binding and release appears to be in prompt contact with an aqueous phase and can be seen to move its pK to lower values during certain non-resting activities. The observations all seem to be dependent on the chemical oxidation of cytochrome c_2 prior to flash activation; they are (i) a decrease in pK, from 8.5 to 7.5 of the agent binding H_I^+ [20]; (ii) a decrease in pK from 7.5 to <5.0 of the agent binding H_{II}^+; (iii) a decrease in pK from 7.5 to <5.0 on ferrocytochrome b_{50} which governs internal H^+ release in the presence of antimycin. We are unable to account for these observations in toto, but to speculate, we consider that a likely cause may be the increased lifetime of the light-generated reaction center $(BChl)_2^+$ under these conditions, rather than a direct effect of the oxidized cytochrome c_2 or the Rieske Fe-S protein. The presence of long-lived $(BChl)_2^+$ in the center of the membrane dielectric may, through charge interaction, affect other redox carriers in the vicinity. The failure to observe a maximum $2H^+$/electron (depending on the preparation, values vary between 1.4—1.8) is roughly consistent with the fact that our preparations possess only 55—80% of the full complement of functionally intact cytochrome c_2. In reaction centers which lack cytochrome c_2, the flash-generated $(BChl)_2^+$ will have a long lifetime (in the hundreds of milliseconds range).

The pK shift to lower values of a simple, non-redox linked acid-base group which comes under the influence of a change of electrical potential from say ψ_1 to a more positive value, ψ_2, caused by the presence of $(BChl)_2^+$ can readily be understood by the stabilization of the base because of its net negative character when compared to the protonated form. Fig. 11 shows the behavior of the redox system of Eqn. 2 to a similar change in electrical potential increasing from ψ_1, the resting value, to ψ_2, a value more electropositive. As indicated for the simple acid-base example, the pK_{ox} and pK_{red} both more to lower values, the former because the protonated form A^+H is destabilized with respect to A, the latter because A^- is stabilized with respect to AH. There are, however, concomitant changes in the E_{ma} and E_{mb} values for the same reasons; at pH values much higher than pK_{red}, where the system is described essentially by the A/A^- couple, the reduced form is stabilized making the redox component become a better oxidizing agent and so its E_{mb} goes up. Similarly at pH values well below the pK_{ox}, where the redox system is described by the A^+H/AH couple, the oxidized form is destabilized and the E_{ma} also goes up. Such influence, affecting both pK and E_m values, moves the E_m/pH relationship en bloc diagonally along the −60 mV/pH unit slope and so pK values change according to $(\psi_2 - \psi_1)/60$ and E_{ma} and E_{mb} change according to $(\psi_2 - \psi_1)$.

The observed shift on the pK_{red} value of cytochrome b_{50} serves to illustrate the behavior described in Fig. 11. We have previously shown [38] that with cytochrome c_2 reduced before activation (i.e., no long-lived $(BChl)_2^+$ after activation) the response of the cytochrome b_{50} to reduction, by what is pro-

posed to be Q·H was to release or retain the proton according to the ambient pH; this yielded the same pK_{red} value that was measured from its E_m/pH relationship determined under resting conditions at equilibrium. It was concluded that there were no activated effects on the pK_{red} and that the cytochrome was in rapid contact with an aqueous phase. This work also indicated, from the uncoupler sensitivity of the rate of re-appearance of a released H^+ in the external water of the chromatophore, that the point of release was inside the chromatophore. The work presented here confirms this and demonstrates that in the presence of a long-lived $(BChl)_2^+$ cytochrome b_{50} fails to retain the H^+ offered with an electron by Q·H, even at an ambient pH of 5.8. This implies that the pK_{red} moved to well below this value. According to Fig. 11, during the lifetime of $(BChl)_2^+$, the E_{mb} of cytochrome b_{50} will move to a value well above the equilibrium E_m value at pH 5 (i.e. $E_{mb} > 170$ mV). The similar pK shift of the H_I^+ binding agent ($Q{\cdot}H/Q^-$) from pH 8.5 to 7.5 will cause a 60 mV increase in the E_{mb} couple (Q^-/Q). Also, if the H_{II}^+ binding agent is a redox couple its apparent pK shift from 7.5 to <5.0 will also induce an E_{mb} increase of over 150 mV.

Thus in general, the pulsed reduction of a redox center associated with the membrane but in rapid contact with an aqueous phase, if exposed to a local change in electrical potential from within the membrane dielectric, will display patterns of H^+ binding and functional E_m values which may be dramatically altered when compared to the resting state. Fig. 11 can serve to illustrate several instances starting at rest in different pH ranges indicated by the arrows: At point b and f there will be no change in H^+ binding capability following activation since H^+ exchange is expected neither at ψ_1 nor ψ_2; however, the E_{ma} or E_{mb} values will rise with the increased positive potential ($\psi_2 - \psi_1$). At point c there will be a change in the relationship of the reduced form with the proton; after activation and creation of ψ_2, the reduced redox agent will not require a proton. The approximate extent of the E_m change encountered at point c will be $(\psi_2 - \psi_1) - 60$ ($pK_{red}^{\psi 2} -$ pH at c). At point d no change in behavior during the experiment will be detected, and the reduced form will always require a proton. At point e there will be a change in teh relationship of the oxidized form (A^+H) with the proton. If the potential change to ψ_2 is felt before A^+H is reduced to AH, a proton will first be released and later bound again as A is reduced. If ψ_2 is established after reduction no change in behavior will be detected. The approximate E_m change encountered at point e will be $(\psi_2 - \psi_1) - 60$ (pH at e $- pK_{ox}^{\psi 2}$).

The above cases provided by cytochrome b_{50}, H_I^+ and H_{II}^+, and the case of the reaction center primary Q illustrate two different aspects of alteration in the physical-chemical properties of redox centers during electron flow. Another type of demonstrated change in resting equilibrium properties was provided by the shifts in the redox poise between $(BChl)_2$ and cytochrome c_2 [44], and earlier between cytochrome a and cytochrome c in mitochondria [49], which were proposed to emanate from a delocalized transmembrane potential change (see also refs. 50, 51) and required a functionally coupled membrane. These examples, although fragmentary, provide some ground rules to consider other aspects of Fig. 11 and to reveals other cases. Progress in this area seems important if we are to understand better the nature of the reactions coupled to electron transfer.

The effect of valinomycin on the pK shifts

Valinomycin seems to be able to overcome the effect of the long-lived $(BChl)_2^+$ in restoring the pK of the agent binding H_{II}^+ back up to 7.5 (Fig. 7B). Note that high concentrations of valinomycin are required to achieve this. The turnover rate of valinomycin is 2000 s^{-1} (Mueller, P., personal communication) so that concentrations of valinomycin roughly stoichiometric with reaction center concentrations are necessary to modify the reaction within 200 μs. Lower concentrations of valinomycin are required to reconstitute $2H^+$/electron at potential where cytochrome c_2 is reduced before the flash. In this case the ionophore is only required to collapse charge interaction due to $(BChl)_2^+$ in those reaction centers deficient in cytochrome c_2.

Valinomycin is unable to restore the other parameters which are altered by the prsence of $(BChl)_2^+$, it fails to accelerate electron transport through the Q-b/c_2 oxidoreductase significantly or to raise the pK either on the agent binding H_I^+ or cytochrome b_{50}. In the latter case the experiments on cytochrome b_{50}-mediated internal H^+ release, which provided the pK on the cytochrome, were carried out in the presence of very high concentrations of FCCP which should dissipate charge interactions in a manner similar to valinomycin. Perhaps this suggests that the Q and b-cytochrome are positioned with respect to the reaction center such tha the interactions are not prone to interference by ionophores, possibly because of inaccessibility. In contrast, perhaps the agent binding H_{II}^+ responds to a more delocalized interaction with the $(BChl)_2^+$ More experiments will be necessary before the real nature of these charge interactions are understood, but it is becoming increasingly clear that local electric fields and other types of interaction may control the chromatophore electron and proton transport processes.

Some further considerations and problems

H_{II}^+ binding and the redox state of Z. We have discussed before that based on our current working models [18], H_{II}^+ should be bound in the ms time range and be bound only if Z is reduced before activation. With Z reduced before activation, H_{II}^+ has a half-time of about 1.8 ms consistent with the models; however, with Z oxidized before activation not only is H_{II}^+ still evident (Fig. 1), it is bound with a shorter half-time of 0.2 ms. The E_h dependency of the half-time shown in Fig. 12 describes a curve in the region of the Nernst curve of the ZH_2/Z couple, and possible sources of this effect have been discussed elsewhere [46,47].

The kinetics of H_{II}^+ binding and cytochrome b_{50} reduction. It can be demonstrated that a high potential, where there is only one electron [on $(BChl)_2$] in each system, both H_I^+ and H_{II}^+ are bound before cytochrome b_{50} is reduced as shown in Fig. 13. The half-time of b_{50} reduction at pH 6 and 380 mV is approx. 30 ms, (a time much slower than the approx. 2 ms half-time when cytochrome c_2 is reduced before activation, see ref. 38). Fig. 13B shows that the 30 ms half-time is not affected by the presence of valinomycin (which as we have already discussed is necessary in order to see the H_{II}^+ binding at 380 mV). It is possible that not all the electrons remain on cytochrome b_{50} at high potential since addition of antimycin doubles the extent of b_{50} reduction (see Fig. 13C) although the reduction half-time is not affected. The inset of

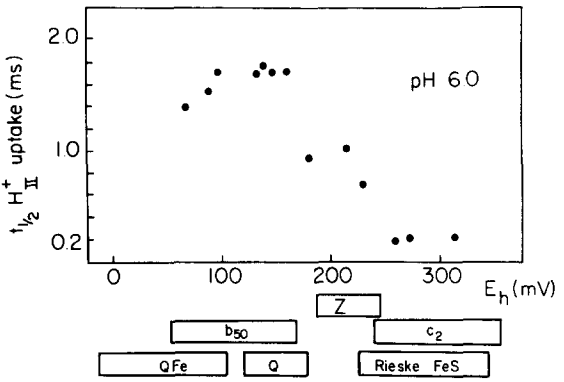

Fig. 12. The half-time of H^+_{II} binding as a function of E_h. The conditions were as described in the legend to Fig. 1, with neither valinomycin nor antimycin. Contributions from the faster binding H^+_I were subtracted from the total change after construction of semilogarithmic plots; thus it is tentatively assumed that H^+_{II} binding follows simple first order kinetics.

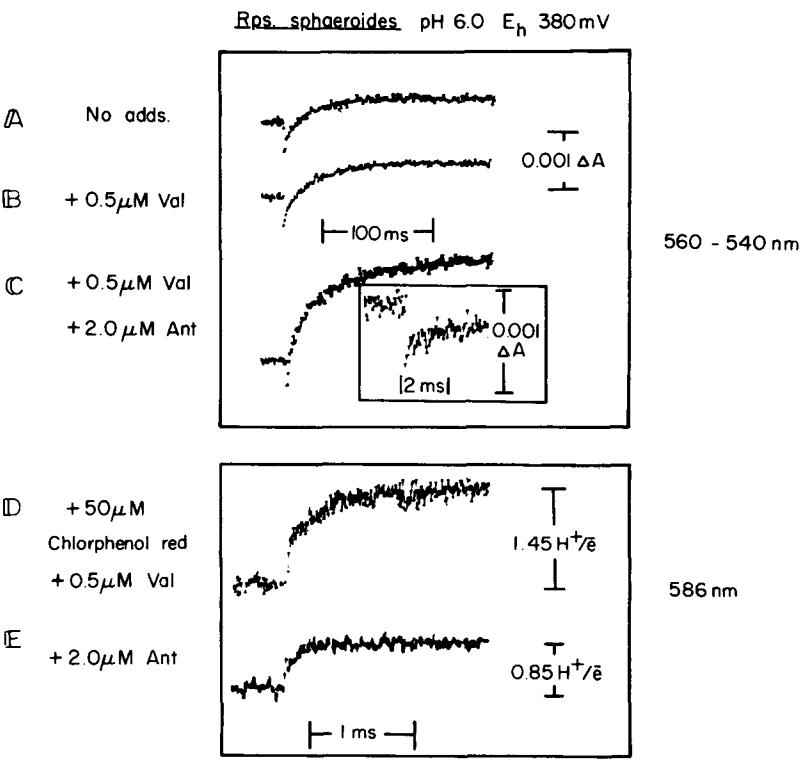

Fig. 13. Comparison of cytochrome b_{50} reduction kinetics with H^+ binding at E_h = 380 mV. Traces A, B, and C represent absorption changes in cytochrome b_{50} (measured at 560 to 540 nm). A, with no additions; B, plus 0.5 μM valinomycin; C, plus 0.5 μM valinomycin and 2.0 μM antimycin. The inset in C shows the initial change displayed on a faster time scale. The spike represents oxidation of the reaction center (see ref. 39). Traces D and E show proton binding under identical conditions to B and C but on a much faster time scale.

Fig. 13C confirms that complex transient kinetics that may be coincident with H^+_{II} binding do not occur during the 'spike' seen in traces 13A, B, and C. The very first downward movement is a contribution at 500 to 540 nm from $(BChl)^+_2$ which, because cytochrome c_2 is not available promptly to re-reduced it, simply goes oxidized and remains so during the time course of the experiment. The recovery of the absorbance decrease and formation of a spike results from the absorbance increase due to cytochrome b_{50} reduction (see [47]). Traces D and E in Fig. 13 show that both H^+_I and H^+_{II} can be bound at high potentials, in a time that is well before cytochrome b_{50} is seen to go reduced. This result complements the 0.2 ms H^+_{II} binding half-time with cytochrome c_2 reduced but Z oxidized (Fig. 12; ref. 18) in suggesting that the system has the capability to bind both protons with one electron without the electron fully moving through the Q-b/c_2 oxidoreductase. Thus from the results presented here it seems that the sequencing of electron and H^+ translocation is not following current, perhaps over-simplified, chemiosmotic expectations.

The rathe low pK values apparent on H^+_I and H^+_{II}. Essentially no protons are bound by the Q-cytochrome b/c_2 oxidoreductase above pH 9.5. If these data are correct, a consequence with regard to a simple chemiosmotic model of electron and H^+ transfer in the reaction center Q-b/c_2 oxidoreductase is that the flash-induced carotenoid bandshift (commonly used as a measure of membrane potential alteration) should be dramatically modified at high pH. This is because any reaction which is normally (at low pH values) regarded as an 'electroneutral' transmembrane hydrogen carrying step will become electrogenic and thereby counter the work done in the previous electrogenic reactions. Fig. 14 shows that at high pH all phases of the carotenoid bandshift are seen, indicating the usual multipulse patterns of membrane potential buildup, despite the absence of detectable H^+ binding (lower right hand trace). This 'paradox' deserves further study.

Other protons? Although this work has been done under the premise that there are only two protons involved, one antimycin sensitive and the other one not, we cannot rule out the possibility that there may be others. For example, is H^+_{II} measured with Z oxidized bound by the same agent as that measured with Z reduced before activation? More accurate kinetic measurements may help resolve this problem. A similar question can be asked regarding the H^+ bound in the presence of valinomycin (Fig. 1) at E_h values about 100 mV at pH 6.0; the depression seen in the extent could mean a switch from one binding reaction to another, dependent on the state of reduction of say cytochrome b_{50}.

Proton to electron ratios. Under conditions that would be described as 'optimal' we have shown that a maximum of close to $2H^+$ may be bound on every turnover (see also [18]) of the reaction center Q-b/c_2 oxidoreductase. In this paper we have shown how a number of factors can operate to reduce the number of H^+ bound on each turnover. These include redox components being chemically reduced or chemically oxidized before activation. Equivalent to the latter case may be the fact that not all reaction centers have any functionally intact cytochrome c_2. Another source of variation may arise from differing concentrations of reaction center and Q-b/c_2 oxidoreductase in the membrane. It has been shown from antimycin inhibition titrations [48] and from estimates

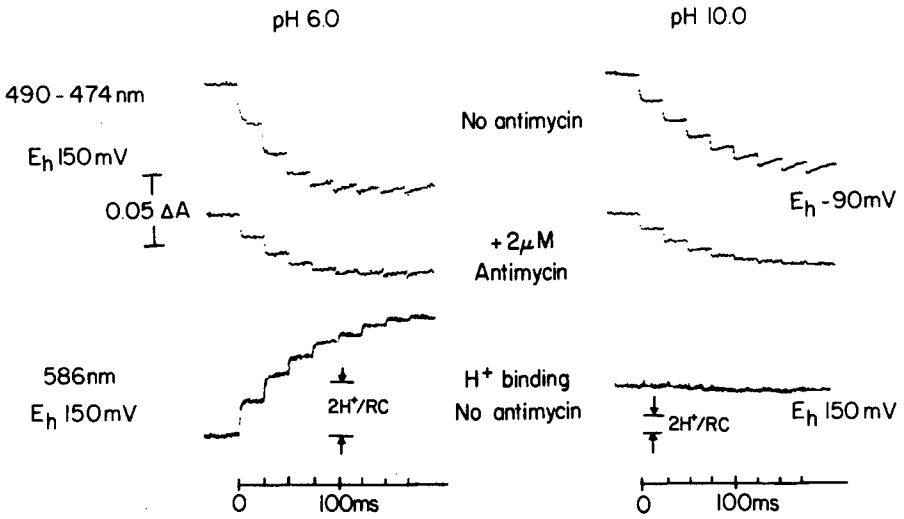

Fig. 14. The carotenoid bandshift and proton binding at high (pH 10) and low (pH 6) pH. Carotenoids were measured at 490 to 474 nm following a train of 8 flashes 25 ms apart. Although the extent of the change is smaller at pH 10, in the absence of antimycin, all three phases of the carotenoid bandshift are still evident. (Phases I and II are associated with the rapid reaction center and cytochrome c_2 reactions; phase III occurs in the ms time range, is antimycin sensitive and requires that Z be reduced before flash activation, see [16]). In both cases addition of 2 μM antimycin abolishes phase III. The redox potential at pH 10 is set 250 mV lower than at pH 6 so that the pH dependent ZH_2/Z was reduced before activation. Conditions for the H^+ binding experiments were as in the legend to Fig. 1, using 50 μM chlorophenol red at pH 6 and 50 μM phenol violet at pH 10. The E_h was 150 mV for both pH value.

of the amount of Z/reaction center [22] that there is often less Q-b/c_2 oxidoreductase present in the chromatophore membrane than reaction center protein; it appears that the number of Z molecules [22], or antimycin molecules bound to the chromatophore/reaction center [48] falls into the 0.6—0.9 range.

It seems likely that in the experimental measurements of H^+/e^- ratios in bacteria, chloroplasts, and mitochondria, whole numbers will be the exception rather than the rule.

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