KINETICS AND STOICHIOMETRY OF PROTON BINDING IN *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

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1. Introduction

A currently popular preoccupation in bioenergetics is the stoichiometry of proton (H^+) binding or release during electron transfer in mitochondria [1,2], chloroplasts [3–5] and bacterial membranes (see [6] for references). Chromatophores from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* possess a unique combination of advantages [7]. They permit the determination of the time-resolved binding of protons following flash-activation as well as the number of H^+ bound for an electron moving through their ubiquinone-cytochromes b/c_2 (Q-b/c_2) oxidoreductase. Because the system is cyclic and there is no input of external oxidising or reducing equivalents, the redox state of the Q-b/c_2 system can be adjusted prior to activation and the flash-induced reactions can be referred to this starting state.

Microsecond H^+ binding accompanying electron transport was first revealed in photosynthetic bacteria by Chance et al. [8]. Chemiosmotic models [9] predicted the existence of a second, slower, antimycin sensitive phase of proton binding. This was later detected by Cogdell et al. [10] but strangely, only in the presence of valinomycin and K^+ ions. In a previous paper [11] we further characterized the rapid ($t_{1/2} \sim 120\ \mu s$, pH 7.0), antimycin insensitive proton binding (designated H_I^+) and established that 1.0 ± 0.1 H_I^+ is bound per electron delivered from the reaction centre to the Q at the outer side of the chromatophore membrane. In this report we show that under appropriate conditions the antimycin sensitive proton (designated H_{II}^+) is bound (up to 0.9 H_{II}^+/e) in addition to H_I^+ but without the need for valinomycin. We also show that its rate of uptake is influenced by the redox state of ubiquinone cytochrome b/c_2 (Q-b/c_2) oxidoreductase carrier designated Z [10,12–14], which requires $2e^-$ and $2H^+$ for reduction at equilibrium. It has been established [14] that this component Z must be in the reduced state if there is to be rapid electron transport through the Q-b/c_2 oxidoreductase.

2. Materials and methods

Chromatophores free from externally added buffer were prepared from *Rps. sphaeroides* strain Ga as described [11,15]. $(BChl)_2^+$ generated following a flash was estimated using the extinction coefficient of 29.8 mM^{-1} cm^{-1} for the $\Delta A_{605-540\,nm}$ [15]. Redox potentiometry in combination with spectrometry and flash-activation was carried out as described [15–17], as were the determination of the extent of proton uptake [8,10,11]. The dye used to monitor changes in the pH of the external medium (chlorphenol red, pK 6.0; obtained from British Drug Houses, Poole, England) does not bind significantly to the chromatophore membrane [11] (i.e., < 5% of the dye added (50 μM) bound to 25 times the chromatophore concentration used in proton binding experiments). Valinomycin and antimycin were obtained from Sigma, St Louis, USA. Because several components of the Q-b/c_2 oxidoreductase have pK values (e.g., see [7,11,24]), all the experiments reported here were performed at pH 6.0.

3. Results and discussion

3.1. *Multiple single turnover activation*

Figure 1 shows how proton uptake behaves during a train of near-saturating single-turnover flashes in the presence of valinomycin (K^+). Valinomycin is present to remove the constraint of $\Delta\psi$ which in the absence of the ionophore builds up to ~400 mV [18] (positive inside the chromatophore) in a matter of 3 or 4 turnovers [12,19] and greatly slows down electron transfer. In the presence of valinomycin ΔpH very slowly builds up to replace $\Delta\psi$ but many turnovers

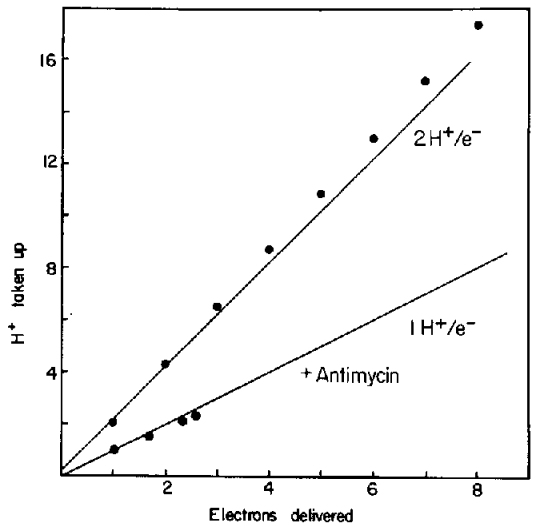

Fig.1. Multiple single turnover flash H^+ binding in *Rps. sphaeroides* chromatophores. Chromatophores (reaction centre concentration 0.2 μM) were suspended in the anaerobic cuvette in 100 mM KCl, pH 6.0. Diaminodurene (5 μM), phenazine methosulphate (5 μM) and phenazine ethosulphate (5 μM) were present together with chlorphenol red (50 μM) and the redox potential (E_h) was adjusted to +210 mV. The upper trace reflects ΔA_{586} nm of chlorphenol red in the presence of 0.5 μM valinomycin and the lower trace, changes in the presence of 2 μM antimycin. The flash-induced pH changes are calibrated by the addition of 2.5 μM HCl to provide a value for H^+ bound per flash. Measurement of the extent of the flash induced $(BChl)_2^+$ formed under the same conditions provides the number of electrons delivered to the Q-b/c_2 oxidoreductase. Chromatophores were excited by a train of 8 near-saturating single turnover xenon flashes spaced 25 ms apart. Each point represents the average of 16 such flash trains spaced 40 s apart in the presence of antimycin and at least a minute apart in the presence of valinomycin.

are required before any energy feedback from ΔpH is apparent. Under these conditions each single-turnover flash elicits the uptake of two protons (i.e., $2H^+$ per e^- entering the Q-b/c_2 oxidoreductase). The 25 ms flash interval chosen in the experiment shown in fig.1 is not critical to the H^+/e^- ratio; it is suitable to allow 99% of the carriers of the reaction centre and Q-b/c_2 oxidoreductase to return to their equilibrium redox state before the next flash. Each flash elicits the same response for at least 32 turnovers.

Addition of antimycin eliminates one of the protons bound (H^+_{II}) on each turnover, leaving only the antimycin insensitive H^+_I. Under the conditions of fig.1, H^+_I binding is limited to a maximum of three turnovers because in the presence of antimycin, flash-oxidized cytochrome c_2 is reduced very slowly with a $t_{1/2}$ of ~ 300 ms compared with the $t_{1/2}$ of ~ 1.5 ms found in its absence. This means that after three turnovers spaced 25 ms apart, the cytochromes c_2 and the $(BChl)_2$ accumulate in the oxidized state and this stops further light-induced electron transfer activity.

3.2. *Some current schemes for Q-b/c₂ oxido-reduction*

Figures 2A and B show two current views of how the system might be considered to work. Starting under optimum conditions for the operation of the cycle with Z, cytochrome c_2 and $(BChl)_2$ reduced before activation we will first discuss the model of fig.2A:

(i) $(BChl)_2$ goes oxidized in < 10 ps and after some intermediary reactions (see [20]) the Q outside the reaction center is reduced in $t_{1/2}$ ~ 120 μs [11].

(ii) This Q^- binds H^+_I in $t_{1/2}$ ~ 120 μs to form Q·H [11].

(iii) Meanwhile on the inside, ferrocytochrome c_2 is oxidized and reduces $(BChl)_2^+$ in 30 μs (or 300 μs depending on its position, see [15].

(iv) ZH_2 reduces ferricytochrome c_2 producing what is likely to be a metastable Z·H [14] in $t_{1/2}$ ~ 1.5 ms;

(v) Z·H then reduces ferricytochrome b_{50} (the subscript refers to the E_m value at pH 7.2) directly or indirectly, in $t_{1/2}$ ~ 1.5 ms (see [14,21].

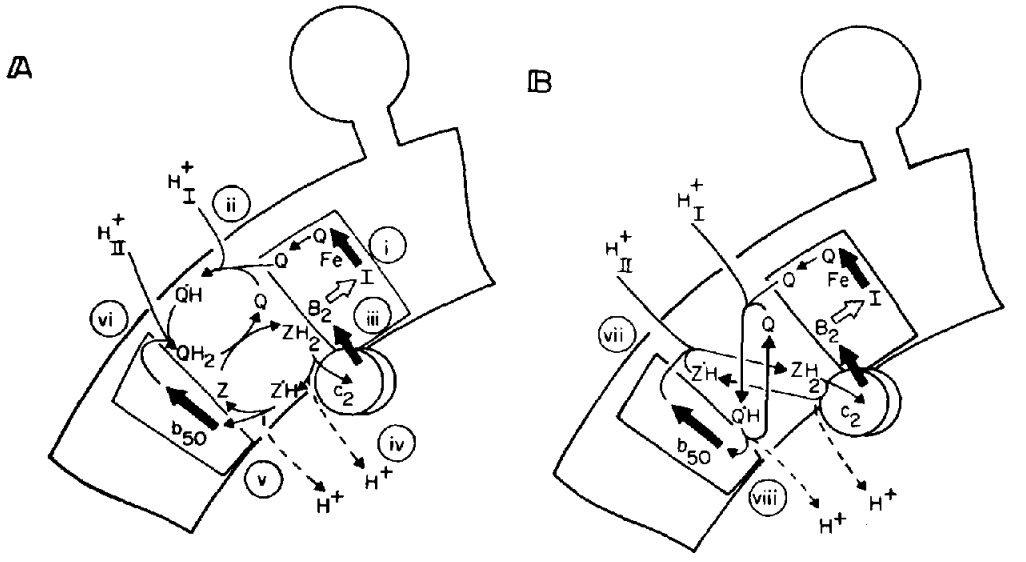

Fig.2. Two schemes of electron and proton transfer in *Rps. sphaeroides*. The open arrow in the reaction centre is the light reaction. The heavy arrows are considered electrogenic and are registered by carotenoid bandshifts [12,14] phase I ($B^+_2 \rightarrow I \rightarrow QFe$) and phase II cytochrome $c_2^+ \rightarrow B_2$, association with the reaction centre protein, and the millisecond antimycin sensitive, phase III in the Q-b/c_2 oxidoreductase. The dashed lines for the H^+ release on the inside represents the uncertainty regarding the fate of these protons under fully coupled conditions. The location of cytochrome b_{50} is uncertain but it does not appear to be in functional contact with the external aqueous phase [24]; some evidence [24] places it on the inside, but other evidence [14,24] is inconsistent with this location. The knob on the outside represents the ATPase. See text for further details.

(vi) Ferrocytochrome b_{50} then reacts directly or indirectly with Q·H from step (ii) to produce QH_2 (H^+_{II} binding) which in turn reacts with Z to produce ZH_2 and complete the cycle.

The addition of antimycin stops ferricytochrome c_2 reduction on the first turnover [15,21]; in model 2A this could be rationalised as an inhibition at step (iv) or (v) since either point of action would stop step (vi) and thereby explain the antimycin sensitivity of H^+_{II}. This scheme is effectively a Mitchell-type Q-cycle [22].

Figure 2B shows an alternative scheme in which reactions (v) and (vi) of fig.2A are replaced by (vii) and (viii). The principle difference between the schemes lies in whether Z'H leads to the reduction of cytochrome b_{50} and Q'H leads to the oxidation of cytochrome b_{50} (2A) or vice versa (2B). The model 2A requires that Q and Z be constrained on separate sides of the membrane whereas 2B requires both Q and Z to be able to move almost completely through the membrane. Both models can explain the antimycin sensitivity of H^+_{II} and suggest that H^+_{II} binding will be governed in rate by the millisecond oxidation–reduction reactions of ZH_2/Z.

3.3. *The second proton bound without the aid of valinomycin*

Chromatophores poised so that cytochrome c_2 is oxidized before activation, bind H^+_I (1.0 H^+_I/e^-) but not H^+_{II}. In contrast, when cytochrome c_2 is reduced before activation, H^+_{II} is bound in addition to H^+_I. The amount of H^+_{II} varies with preparation from 0.5–0.9 H^+_{II}/e^-. The variance may reflect the reaction centres that are devoid of cytochrome c_2 (damaged incurred during preparation [15]) which produces a situation equivalent to having some of the cytochrome c_2 oxidized before activation. Valinomycin appears to be able to overcome the effects of oxidized cytochrome c_2 and brings the zero H^+_{II}/e^- value, observed when all cytochromes c_2 are oxidized before activation, up to 0.8 or 0.9. Indeed, valinomycin seems to be able to compensate for those reaction centres which lost their cytochromes during preparation, because the ionophore brings the 0.5–0.9 H^+_{II}/e^- ratio up to near 1.0

at potentials where cytochrome c_2 is reduced before activation. We believe this recovery is due to valinomycin-K^+ collapsing adverse charge-interaction between $(BChl)_2^+$ which remains oxidized for hundreds of milliseconds if not promptly reduced directly by cytochrome c_2 (see [11,23]) and the chemical group responsible for H_{II}^+ binding. In the presence of the $(BChl)_2^+$ charge the apparent pK of the H_{II}^+ binding group is shifted to a value below pH 5 (this is further discussed [7]). This latter role of valinomycin may be distinct from its principle role in fig.1 which was to collapse transmembrane $\Delta\psi$ build-up.

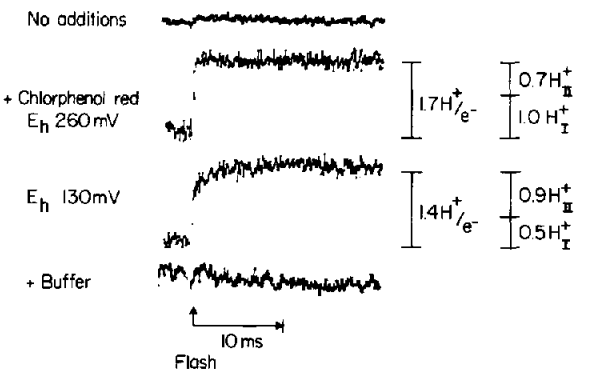

Fig.3. Single turnover flash H^+ binding in *Rps. sphaeroides* chromatophores. Conditions as in fig.1 except for the absence of valinomycin and antimycin. Each trace represents the average of 64 single turnover flashes spaced 40 s apart. The top and bottom traces are baselines, obtained as indicated, for the adjacent experimental traces measured under similar conditions. The scales on the experimental traces represent the extent of H^+ binding per single turnover flash ($1e^-$) obtained by calibrating the ΔH^+ by standardized HCl and relating this to the known reaction centre concentration. On the right is a breakdown of the contribution to the total H^+/e^- by H_I^+ and H_{II}^+. At E_h 130 mV and 260 mV at pH 6 H_I^+ is 0.5 and 1.0, respectively (see [7]).

3.4. *Details of the extent and kinetics of H_{II}^+* binding

The models of fig.2 predict that H_{II}^+ should be bound with a $t_{1/2}$ close to 1.5 ms but only if Z is reduced before activation. The extent of millisecond H_{II}^+ binding should be as dependent on the state of Z reduction as is the re-reduction of flash oxidized cytochrome c_2 and also the carotenoid bandshift phase III [14]. Carotenoid bandshift phase III (see fig.2 legend) is thought to be a response to an electrogenic movement of an electron in the Q-b/c_2 oxidoreductase (drawn as a thick arrow in fig.2) from a component near the inside to a component near or on the outside of the membrane. In terms of the models of fig.2, H_{II}^+ should be bound after or simultaneous with the ZH_2 reduction of cytochrome c_2 by ZH_2 and the Z'H mediated electrogenic reaction registered by carotenoid bandshift phase III. Consistent with this is the finding that cytochrome c_2 reduction, carotenoid bandshift phase III and H_{II}^+ are inhibited by one antimycin per electron transfer system [23]. Furthermore, fig.3 shows that when Z is reduced prior to activation (e.g., E_h 130 mV at pH 6) H_{II}^+ binding is very close to $t_{1/2}$ 1.5 ms.

At this point it would seem that many of the overall kinetic features of reaction centre driven electron and proton transfer in the Q-b/c_2 oxidoreduction are explained and that the main concepts behind the simple models of fig.2 are correct. However a remarkable anomaly emerges when further attention is given to the extent and kinetics of H_{II}^+ binding following single turnover activation.

If the models of fig.2 are correct, then with Z oxidized prior to activation, H_{II}^+ binding would not be expected to occur in the millisecond time range since neither cytochrome c_2 is re-reduced nor is there any significant formation of carotenoid bandshift phase III. Strangely however, under these conditions (fig.3, second trace) the rate of H_{II}^+ binding is much faster than was encountered with Z reduced before activation; at an E_h of 260 mV at pH 6 (Z oxidised, cytochrome c_2 reduced) the $t_{1/2}$ is estimated to be about 0.2 ms. This halftime is much faster than any known reactions involving the central, antimycin sensitive part of the Q-b/c_2 oxidoreductase under any conditions. Nevertheless the proton binding still has the character of H_{II}^+ in that it is still antimycin sensitive. Two protons can therefore be bound per electron delivered into the Q-b/c_2 oxidoreductase before any electron has moved through what classically would be regarded as the energy conservation site or through the requisite steps of a chemiosmotic 'loop' or Q-cycle sequence.

In conclusion, the chromatophore reaction centre-ubiquinone-cytochromes b/c_2 oxidoreductase system operates to bind $2H^+$ for every electron delivered into the system. If the system is examined under optimum energy conserving conditions (i.e., Z in the reduced

form before activation) the $t_{1/2}$ of H_{II}^{+} fits in well with expectations derived from other reactions known in the Q-b/c_2 oxidoreductase and supports current electron and proton transfer schemes. However the anomolous behaviour of H_{II}^{+} under other conditions indicates that the schemes may ultimately prove to be inadequate when it comes to describing electron transfer events at the mechanistic level.

Acknowledgements

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