THE INFLUENCE OF TRANSMEMBRANE POTENTIALS OF THE REDOX EQUILIBRIUM BETWEEN CYTOCHROME c_2 AND THE REACTION CENTER IN *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

Ken-ichiro TAKAMIYA and P. Leslie DUTTON

Johnson Research Foundation and Department of Biochemistry and Biophysics University of Pennsylvania Philadelphia, PA 19174, USA

Received 17 June 1977

1. Introduction

Current schemes and models of electron transfer in mitochondrial and photosynthetic energetics place considerable importance on both the membrane location of the components involved, and on whether the reduction of the individual electron carriers involves only an electron (and hence involves a net charge change) or both an electron and a proton (when the reduction is electrically neutral) (see refs. [1] and [2] for general articles). However, remarkably few direct experiments have been reported which take both these factors into account. In particular, it seems necessary to test whether redox components which are proposed to act across membranes actually do respond to the function ($\Delta\psi$ or ΔpH) which they are postulated to generate and maintain. The only report in this area is by Hinkle and Mitchell [3] who demonstrated alteration in the redox equilibrium poise between cytochrome *c* and *a* of mitochondria as a function of membrane potential differences ($\Delta\psi$) set up across the membrane by K^+ gradients in the presence of valinomycin.

In this paper we report related studies on chromatophores from the photosynthetic bacterium *Rhodopseudomonas sphaeroides.* In this organism the reaction center protein occupies the membrane with some portion of the protein in contact with the outer aqueous phase [4–6]. Two cytochrome c_2 molecules are functionally associated with the reaction center [7]; however, they are on the other side of the chromatophore membrane, occupying a position on the inner membrane-aqueous interface [8]. Following light excitation, the reaction center bacteriochlorophyll dimer $(BChl)_2$, forms $(BChl)_2^{+}$ and leads to the reduction of a ubiquinone-iron complex (QFe) forming $Q^{-}Fe$ (for more details, see ref. [9]). Since 1.0 H^+ is incorporated into the chromatophore per single turnover of each reaction center, this suggests that all reaction centers direct electrons (via QFe) to the ubiquinone complement functionally at the external surface of the chromatophore [10]. The overall reaction sequence, including the oxidation of one of the cytochrome c_2 molecules on the inside, therefore leads to ferricytochrome $c_2^{(+)}$ $(BChl)_2$ $Q^{-}Fe$ (with subsequent external H^+ binding) arranged across the profile of the membrane. It has been proposed that this sequence generates $\Delta\psi$ across the chromatophore membrane [7,10–13].

This report focuses on the influence of $\Delta\psi$ on the redox poise between cytochrome c_2 and $(BChl)_2$. The redox midpoint potential (E_m) values of these redox couples are 295 mV and 450 mV respectively and, consistent with their proposed role in generating $\Delta\psi$, it is known that electron transfer between them involves the necessary charge transfer over at least the pH 6–11 range [14,15].

There are several reasons why the method of Hinkle and Mitchell is not immediately applicable with chromatophores; for example, the E_m difference between $(BChl)_2$ and cytochrome c_2 is rather large to achieve good displays of redox equilibrium shifts

induced by diffusion potentials. This is especially the case if the cytochrome c_2 to $(BChl)_2$ electron transfer spans only part of the membrane, and if the maximum reliable diffusion potential generatable is less than 200 mV (see fig.1B). Instead we have studied the multiple, single-turnover flash activated redox reactions of $(BChl)_2$ and cytochrome c_2 in coupled chromatophores, where it is considered [11] that the first few turnovers generate $\Delta\psi$ values in the 400 mV region. In order to estimate the $\Delta\psi$ prevailing under the conditions of these experiments, we have measured the magnitude of the flash-induced carotenoid bandshift; the bandshift was calibrated as an indicator of $\Delta\psi$ via K^+ gradient-induced diffusion potentials in the presence of valinomycin.

2. Materials and methods

Cells of *Rhodopseudomonas sphaeroides* Ga were grown anaerobically in the light with succinate as the carbon source, and chromatophores were prepared with a French pressure cell as described previously [7], using a sodium and potassium-free medium composed of 100 mM choline chloride buffered with 1 mM morpholinoethane sulfonate and adjusted to pH 7.0 with tetramethyl ammonium hydroxide. Flash-induced absorbance changes due to $(BChl)_2$, cytochrome c_2 and carotenoid bandshift were measured with the fast double beam spectrophotometer as previously described (see ref. [7]). Redox potentiometry was carried out as described before (see [7,10,

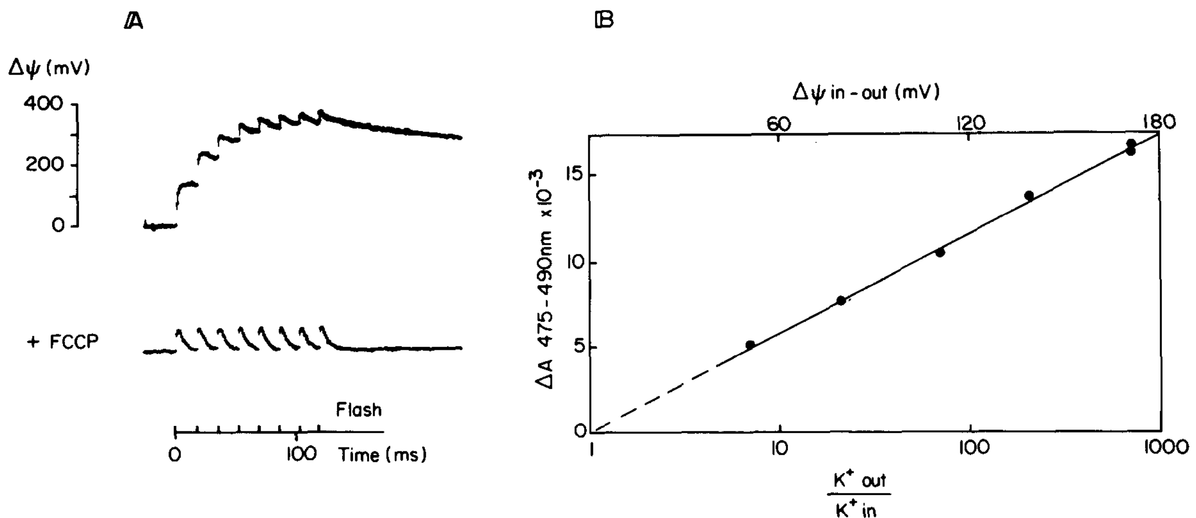

Fig.1. The multi-pulse kinetics of the carotenoid bandshift and membrane potential calibration estimated by potassium-pulsed carotenoid bandshift in *Rps. sphaeroides* Ga. Part A. Flash-induced carotenoid bandshift. Chromatophores (reaction center $[BChl]_2$ concentration, 0.09 μM) were suspended in 20 mM morpholinopropane sulphonate buffer (pH 7.0), containing 100 mM KCl and 1 mM $MgCl_2$ poised at a redox potential of 95 mV. Each flash (89% saturating, 6 μs full width at half height) was fired every 16 ms. The vertical scale is expressed in membrane potential (mV) calibrated as in B. The final concentration of FCCP, when added, was 20 μM. Part B. Calibration of transmembrane potential by potassium-pulse. Chromatophores (reaction center $[BChl]_2$ concentration, 0.16 μM) were suspended in the sodium and potassium-free 20 mM morpholinopropane sulfonate buffer (pH 7.0), containing 100 mM choline chloride. Experimental procedures were same as those in ref. [11]. Final concentration of valinomycin was 1 μM. The lower abscissa indicates the calculated ratio of the final injected potassium ion concentration to that which gave null change of carotenoid bandshift (in this case, the null change concentration was 52 μM) i.e., the final potassium concentration of each point can be obtained by multiplying 52 μM by the value of the abscissa. The upper abscissa indicates $\Delta\psi_{in\text{-}out}$ calculated from the equation:

$$\Delta\psi_{in-out} = \frac{RT}{F} \ln K^+_{out}/K^+_{in}.$$

13,16]). The redox mediation dyes used were 2 μM each of 2,3,5,6-tetramethylphenylene diamine, *N*-methyl phenazonium methosulfate, *N*-ethylphenazonium ethosulfate and approximately 100 μM of potassium ferrocyanide. These low dye concentrations did not have any significant effect on the reaction time courses in millisecond-range measurements. All measurements were made at a redox potential (E_h) value of 95 ± 5 mV where maximal multiflash-induced activity of the ubiquinone cytochrome b-c_2 oxidoreductase and carotenoid bandshifts are encountered (see ref. [15] for a review).

Calibration of the carotenoid bandshift as an indicator of $\Delta\psi$ was done by the K^+ diffusion potential method of Jackson and Crofts [11]. Figure 1A shows the carotenoid bandshift induced by eight successive flashes. The bandshift's calibration is shown in fig.1B. At $\Delta\psi$ values greater than 180 mV (where reliable diffusion potentials required in the calibration are difficult to achieve), it is necessary to assume that the calibration maintains linearity. Although we cannot be certain, this assumption is not unreasonable; recent studies [17] extended to below the abscissa of fig.1B have shown that the linearity holds over at least a total of 300 mV. The calibration indicates that the first flash elicits a $\Delta\psi$ change (inside minus outside) of 140 mV. After the fourth or fifth flash a pulsed steady state is established. There is small amount of rapid decay occurring but this approaches completion in the dark period between the flashes. The peak $\Delta\psi$ value indicated is as high as ∿400 mV, in good agreement with previous findings during continuous illumination [11,18]. The course of the collapse of the state, consistent with its electrical nature, is accelerated by uncouplers (see figure) or valinomycin which, although not shown, is the same (see refs. [11–13,15,19]).

3. Results and discussion

Figure 2 shows measurements on $(BChl)_2$ and cytochrome c_2 to determine their flash-induced oxidation and subsequent dark reduction patterns. The first four or five turnovers build up to a relatively stable (and uncoupler or valinomycin sensitive) level of oxidation with a rapid reductive phase which is complete within approximately 10 ms of each flash. The actual extent of the relatively stable level of oxidation is obtained by addition of antimycin which, in drastically slowing cytochrome c_2 re-reduction, provides the absorbance changes for 0–100% oxidation of $(BChl)_2$ and cytochrome c_2. The data from figs 1 and 2 are analyzed in detail in fig.3. Part A shows simply the fractions of $(BChl)_2$ and cytochrome c_2 stably oxidized after each flash, obtained from the top and bottom traces in fig.2. In Part B, the extents of oxidation of cytochrome c_2 and $(BChl)_2$ are presented in terms of their individual E_h values calculated from the data in Part A and the resting E_m values of cytochrome c_2 (295 mV) and $(BChl)_2$ (450 mV). Viewed in this way, the individual E_h values of the two redox couples are not the same during the flash sequence, and by this criterion appear not to be in equilibrium. However, if cytochrome c_2 is located on the inside of the chromatophore vesicle [8] and $(BChl)_2$ is contained at some point X within the membrane dielectric (X is O on the outside and 1.0 if on the inside of the chromatophore) and if these components are at equilibrium with the $\Delta\psi$, application of the simple Nernst expressions to each component is inadequate. Instead, an expression (see fig.3C) is required that takes account of both the magnitude of $\Delta\psi$ after each turnover, and the relative positions of the components within the membrane. A modified presentation (after refs. [3,20]) is shown in fig.3 Part C. Here the Nernst expression is corrected for the $\Delta\psi$ given by the carotenoid bandshift in fig.1A and a best fit for the relative positioning of $(BChl)_2$ and cytochrome c_2 in the membrane profile. E'_h is defined as the 'local' E_h, relative to an external electrode.

With the exception of the first turnover [which consistently yields a high 'local' E'_h value for $(BChl)_2$ relative to that of cytochrome c_2, (see legend of fig.3 for a comment)] there is a good agreement (< 15 mV) in the E'_h values of the two components after each flash if X values of 0.6 and 1.0 are given to $(BChl)_2$ and cytochrome c_2 respectively. These results indicate that the redox poise between $(BChl)_2$ and cytochrome c_2 is in equilibrium with the prevailing $\Delta\psi$. As such, the results are consistent with the idea that the 150 mV ΔE_m between $(BChl)_2$ and cytochrome c_2 harnessed to the generation of charge separation across the inner 40% of the low dielectric part of the membrane profile.

The accuracy of the 40% value, taken from the

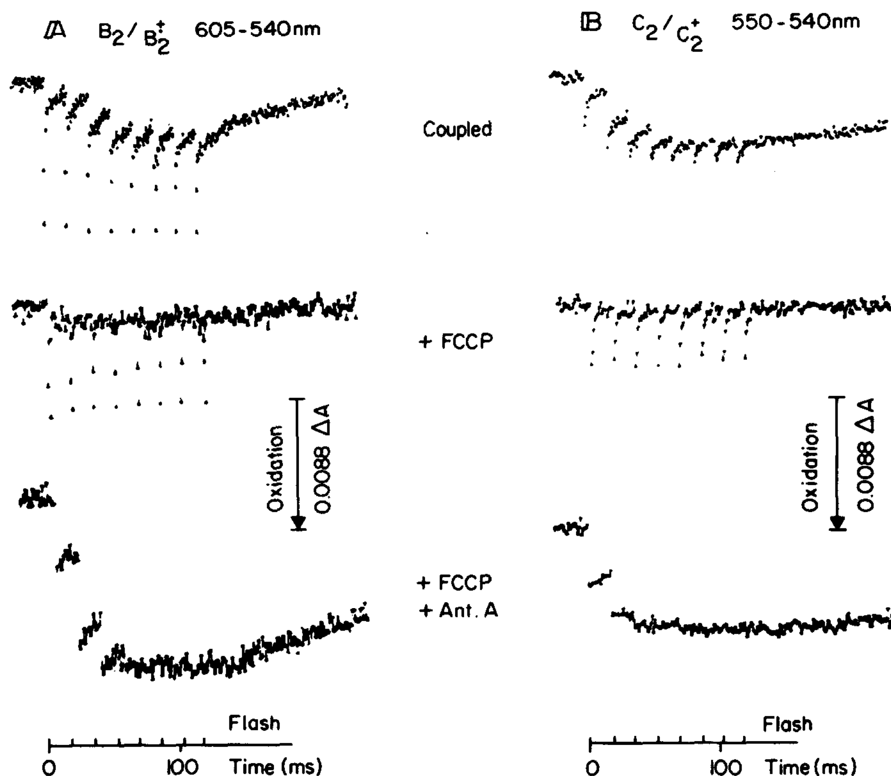

Fig.2. The multi-pulse flash-induced kinetics of reaction center bacteriochlorophyll dimer $(BChl)_2$, and of cytochrome c_2 in *Rps. sphaeroides* Ga. All conditions were the same as that in fig.1A, except that the measuring wavelengths were 605–540 nm for $(BChl)_2$ and 550–540 nm for cytochrome c_2. In this figure and fig.3 $(BChl)_2$ and cytochrome c_2 are abbreviated B_2 and c_2. Final concentration of antimycin A, when added, was 4 μM.

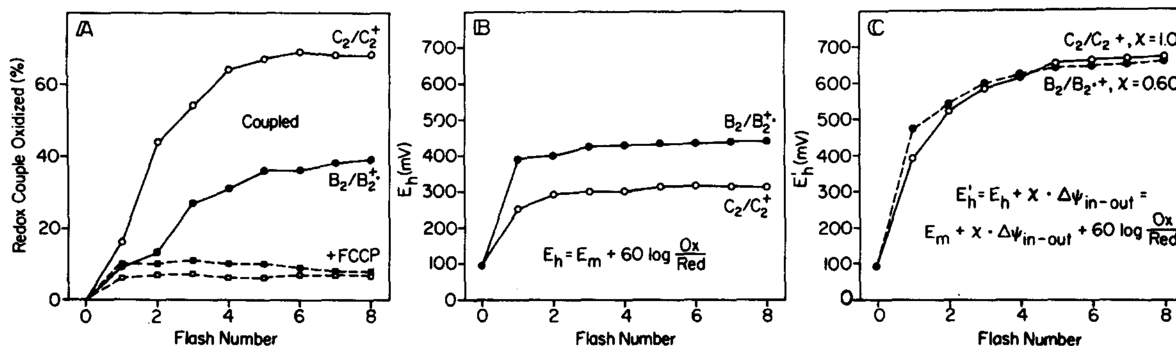

Fig.3. Oxidation-reduction levels and redox potentials of $(BChl)_2$ and c_2 10 ms after flash. Part A. Percentages of oxidized form of $(BChl)_2$ (●,■) and cytochrome c_2 (○,□) 10 ms after each flash were calculated from the data in fig.2. Part B, E_h values calculated from Part A using the equation shown. Part C. 'Local' E_h values (E'_h) calculated are shown using data from Part B of this figure and fig.1 and X-values of 0.6 and 1.0 for $(BChl)_2$ and cytochrome c_2. X is relatively insensitive to measurement errors; for example an error of E'_h of 23 mV after the second or 37 mV after the sixth flashes would yield 0.1 difference in X. The discrepancy after the first flash may arise from the high sensitivity of the E_h value of $(BChl)_2$ at low oxidation levels. It could be accounted for by the small amount of absorbance decrease after the first flash which is evident even in the presence of uncoupler; this area deserves a more detailed examination.

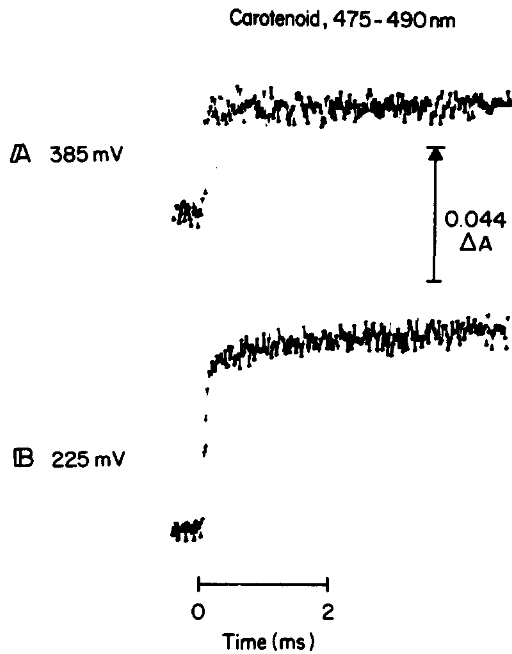

Fig.4. The extents of the carotenoid bandshifts phases I and II in *Rps. sphaeroides.* Chromatophores (reaction center $[BChl]_2$ concentration, 0.18 μM) taken from the same stock suspension as that in fig.1,2, and 3 were suspended in the same buffer as that in fig.1A. Antimycin A (4 μM) was present to eliminate carotenoid bandshift phase III (see ref. [7,13,15]). In Part A with chromatophores poised at an E_h value of 385 mV where cytochrome c_2 is >95% chemically oxidized and $(BChl)_2$ is 90% reduced, the flash elicits only the $(BChl)_2^{+} \rightarrow Q^{-}Fe$ reaction which generates a 90% yield of carotenoid bandshift phase I. In Part B, at an E_h value of 200 mV, where cytochrome c_2 is also reduced before activation, the flash elicits both phases I and II resulting from the formation of cyt c_2 (+) → $(BChl)_2$ → Q^{-} Fe.

X-value of $(BChl)_2$, is of course, contingent on the accuracy of the $\Delta\psi$-value obtained from the carotenoid bandshift. Although currently we do not have an independent way of checking the $\Delta\psi$-values in these experiments, there is an alternative way of estimating the $(BChl)_2$ position from the carotenoid bandshift. This is shown in fig.4, and the experimental details are described in the legend. The measurement is based on the studies done by Dutton and co-workers [7,13, 15,16] who showed that the light-induced electrical potential differences generated in the membrane dielectric, as seen by the carotenoids, occurred in two successive steps. In *Rps. sphaeroides,* the first step carotenoid bandshift phase I [7,13,15] is associated with the photo-oxidation of $(BChl)_2$ and the $< 10^{-9}$ s halftime reduction of QFe. The second step (carotenoid bandshift phase II) is associated with the $10^{-5}-10^{-4}$ s electron transfer from ferrocytochrome c_2 to the light generated $(BChl)_2^{+}$. The two steps identified are consistent with the above findings that $(BChl)_2$ is somewhere within the membrane dielectric, since electric field alterations are registered by the carotenoid for both steps. As such, the ratio of the extent of phase I shown in fig.4A to that of the sum of phases I and II shown in fig.4B provides a value for X; in this case the value is 0.54, which is reasonably close to the value obtained from the above measurements of $(BChl)_2$ and cytochrome c_2 and estimated $\Delta\psi$. It is also in close agreement with data taken from several previous studies in which carotenoid bandshift phases I and II were resolved [7,13, 15]; these also yield an X-value of 0.54 with a standard error of 0.01 from six measurements done on different preparations under different conditions.

From the above lines of evidence and the assumption that the low dielectric part of the membrane profile is homogeneous and is 40 Å thick, we can calculate a distance between cytochrome c_2 and $(BChl)_2$ in the profile of the membrane of 16–18 Å. However, although there is general self-consistency between the parameters we have measured, a detailed quantitative examination will rest on the aquisition of information on the local or average membrane dielectric constant, and on the local concentrations or the vectorial organization of the carotenoids.

Acknowledgements

The authors are indebted to Drs R. C. Prince, D. DeVault, J. S. Leigh, Jr. and C. L. Bashford for valuable discussions and to Mrs. Heather Bonner for preparing the chromatophores. This work was supported by United States Public Health Service Grant GM 12202 and National Science Foundation Grant PCM 76-14209. P.L.D. acknowledges his PHS RCDA No. 1-K4-GM 70771.

References

[1] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[2] Dutton, P. L. and Wilson, D. F. (1974) Biochim. Biophys. Acta 346, 165–212.
[3] Hinkle, P. and Mitchell, P. (1970) Bioenergetics 1, 45–60.
[4] Reed, D. W. and Raveed, D. (1972) Biochim. Biophys. Acta 283, 79–91.
[5] Steiner, L. A., Lopes, A. D., Okamura, M. Y., Ackerson, L. C. and Feher, G. (1974) Fed. Proc. 33, 1461, Abstr. 1345.
[6] Feher, G. and Okamura, M. Y. (1976) in: Brookhaven Symposia in Biology: No. 28 (Olson, J. M. and Hind, G. eds), Brookhaven National Laboratory, Upton.
[7] Dutton, P. L., Petty, K. M., Bonner, H. S. and Morse, S. D. (1975) Biochim. Biophys. Acta 387, 536–556.
[8] Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandria, B. A. and Crofts, A. R. (1975) 387, 212–227.
[9] Dutton, P. L., Prince, R. C., Tiede, D. M., Petty, K. M., Kaufmann, K. J., Netzel, T. L. and Rentzepis, P. M. (1976) in: Brookhaven Symposia in Biology: No. 28 (Olson, J. M. and Hind, G., eds), Brookhaven National Laboratory, Upton.
[10] Petty, K. M. and Dutton, P. L. (1976) Arch. Biochem. Biophys. 172, 335–345.
[11] Jackson, J. B. and Crofts, A. R. (1969) FEBS Lett. 4, 185–189.
[12] Jackson, J. B. and Crofts, A. R. (1971) Eur. J. Biochem. 18, 120–130.
[13] Jackson, J. B. and Dutton, P. L. (1973) Biochim. Biophys. Acta 325, 102–113.
[14] Prince, R. C. and Dutton, P. L. (1977) Biochim. Biophys. Acta 459, 573–577.
[15] Dutton, P. L. and Prince, R. C. (1977) in: The Bacteria (Ornston, L. N. and Sokatch, J. R., eds), Vol. VI, Academic Press, Inc., New York, in press.
[16] Dutton, P. L. (1971) Biochim. Biophys. Acta 226, 63–80.
[17] Matsuura, K. and Nishimura, M. (1977) Biochim. Biophys. Acta 459, 483–491.
[18] Casadio, R., Baccarini-Melandri, A. and Melandri, B. A. (1974) Eur. J. Biochem. 47, 121-128.
[19] Nishimura, M. (1970) Biochim. Biophys. Acta 197, 69–77.
[20] Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction. Bodmin: Glynn Research.