Biochimica et Biophysica Acta, 681 (1982) 512–518
Elsevier Biomedical Press

BBA 41144

EXOGENOUS CYTOCHROME *c*-DEPENDENT LIGHT-INDUCED MEMBRANE POTENTIAL GENERATION IN *RHODOSPIRILLUM RUBRUM* CHROMATOPHORES

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(Received January 19th, 1982)
(Revised manuscript received May 7th, 1982)

Key words: Cytochrome c; Membrane potential; Chromatophore; Bacterial photosynthesis; Charge transfer; (R. rubrum)

Rhodospirillum rubrum **chromatophores associated with a planar phospholipid macromembrane by bivalent cations in the presence of quinone, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate generate a transmembrane electrical potential difference in the light. Photoelectrical activity is also observed if chromatophores are preincubated with cytochrome *c*; the maximum values of responses are reached upon subsequent addition of ascorbate and menadion in the absence of bivalent cations and TMPD. The cytochrome *c*-dependent responses of the illuminated chromatophores are inhibited by Ca^{2+} and prevented by quinones. The possibility of cytochrome *c* (c_2) translocation across the chromatophore membrane and the mechanism of charge transfer across the planar phospholipid membrane are discussed.**

Introduction

The electrogenic cyclic chain in chromatophores of the nonsulfur purple bacteria may function at two regimes: (1) as a chain involving a complete set of redox components and (2) as a shortened chain involving only the P-870 bacteriochlorophyll reaction center complex, ubiquinone and cytochrome c_2 [1,2]. The data on electrochromic changes in the carotenoid absorption spectrum [3–5], on the dependence of millisecond (see Ref. 6) and nanosecond [7] bacteriochlorophyll afterglow on the membrane potential indicate that the light-induced transmembrane electron transfer is carried out by the 'cytochrome c_2-P-870 bacteriochlorophyll dimer – the intermediate short-lived electron acceptor I – the primary (tightly bound) quinone' system. Cytochrome c_2 is located on the inner side of the chromatophore membrane [8,9], and the primary quinone is arranged on its outer side [3]. The secondary (loosely bound) quinone is considered to be a transmembrane H^+ carrier [10–12].

Electrical activity of chromatophores isolated from purple and green bacteria was demonstrated [11,12] by means of the direct electrometric method [13]. When associated with a planar phospholipid macromembrane separating two compartments with electrolyte solutions, illuminated chromatophores generate an electrical potential difference across the macromembrane.

The photoelectrical responses observed in the chromatophore-planar membrane system increase significantly after addition of quinones (1,4-naphthoquinone, menadion, ubiquinone) with TMPD or phenazine methosulfate [11]. Quinones are necessary because decane, a planar membrane component, extracts loosely bound quinones from the chromatophores. With respect to TMPD and its

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Abbreviation: TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.

functional analogues, these compounds penetrating through membranes probably shunt the cytochrome segment of the photosynthetic redox chain [11,14].

In this paper, we report that exogenous cytochrome *c* produces a stimulatory effect which is the same as that of TMPD in the chromatophore-planar membrane system. The data are interpreted as indication of cytochrome *c* translocation across the chromatophore membrane. The mechanism of charge transfer across the planar phospholipid membrane is also discussed.

Methods

Cells of *Rhodospirillum rubrum* (wild-type strain No. 1 MGU) were grown and the chromatophores were isolated as described previously [15].

Photoelectrical activity of isolated chromatophores was monitored using a planar phospholipid membrane [11,16]. A Teflon cuvette consisting of two compartments separated by a membrane (Teflon) filter (9 mm in diameter, 200 μm thickness and pore diameter less than 50 μm) was used (Fig. 1A). The membrane filter was impregnated with a 10% solution of soybean phospholipids (asolectin) in decane. Each compartment contained 50 mM Tris-HCl buffer (pH 7.6). Chromatophores were added to one compartment at a concentration corresponding to a bacteriochlorophyll absorbance of about 2 units at 880 nm at an optical path length of 1 cm. Then, the solutions in both compartments were supplemented by 20–40 mM $CaCl_2$ or $MgSO_4$. Bivalent cations neutralizing the negative surface charge of phospholipids give rise to association of chromatophores with the planar membrane. Subsequent illumination resulted in generation of an electrical potential difference across the planar membrane. It was registered using an Ag/AgCl electrodes. Actinic light of saturating intensity ($\lambda > 660$ nm) was used. Solutions in the experimental cuvette were mixed by means of a magnetic stirrer.

Cytochrome *c* (type VI) from horse heart was provided by Sigma Chemical Co.

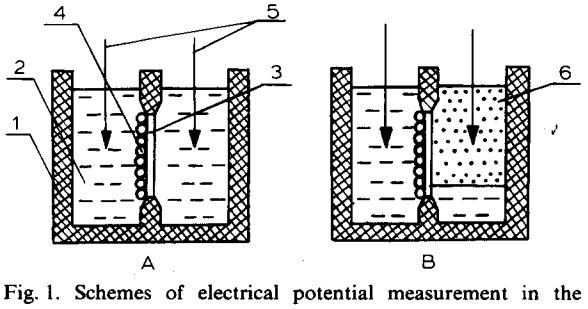

Fig. 1. Schemes of electrical potential measurement in the chromatophore-planar membrane system. (1) Teflon cuvette, (2) incubation mixture, (3). Teflon filter impregnated with asolectin solution in decane, (4) immobilized chromatophores, (5) measuring electrodes, (6) asolectin solution in decane.

Results

As reported previously [11], quinone in combination with TMPD and ascorbate is required for producing maximum photoelectrical responses in

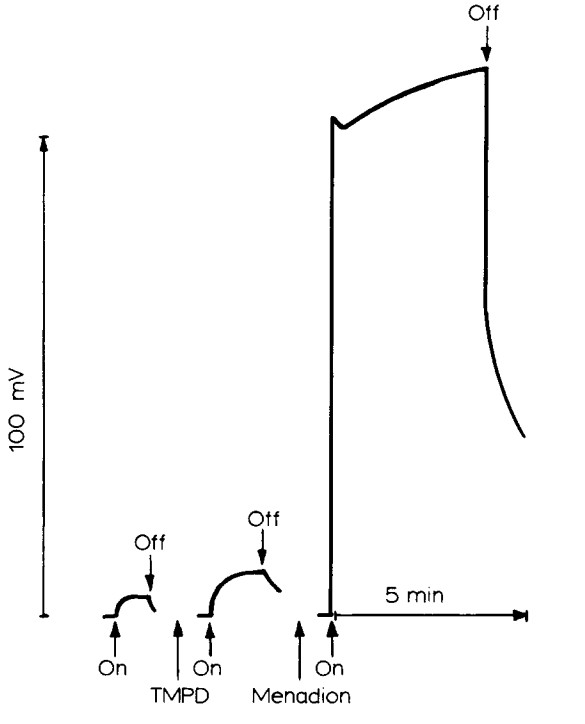

Fig. 2. TMPD and menadion effect on the light-induced generation of an electrical potential difference by *R. rubrum* chromatophores associated with a planar phospholipid membrane. The incubation mixture contained 5 mM Tris-ascorbate. The chromatophores were preincubated with 40 mM $CaCl_2$ for 45 min. Additions (here and below, to both compartments of the experimental cuvette): 0.2 mM TMPD, 0.1 mM menadion. On and Off, switching on and off the light. Positive charging of the chromatophore-free compartment is shown as an electrical potential increase.

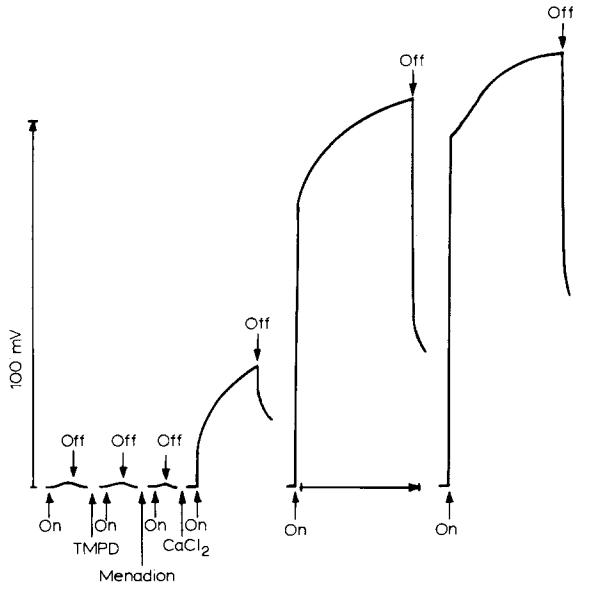

Fig. 3. The dependence of photoelectrical activity on bivalent cations in the *R. rubrum* chromatophores-planar membrane system. The incubation mixture contained 5 mM Tris-ascorbate. Additions: 0.2 mM TMPD; 0.1 mM menadion; 40 mM $CaCl_2$. The light was switched on 10, 50 and 60 min after $CaCl_2$ addition.

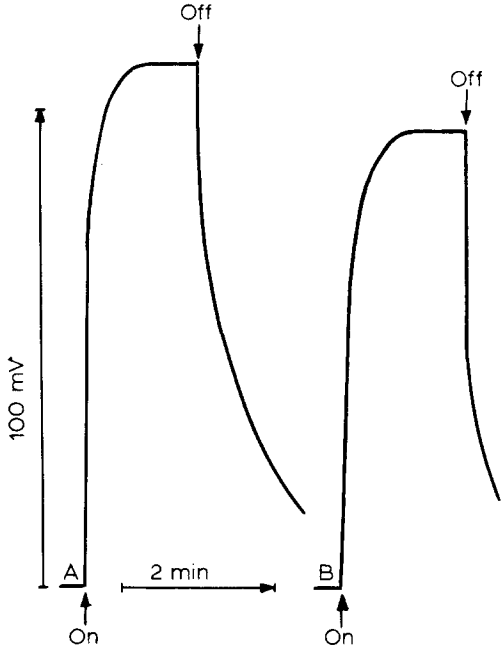

Fig. 4. Effect of substitution of an aqueous solution in the chromatophore-free compartment of the experimental cuvette for the 10% asolectin solution in decane on photoelectrical responses of immobilized *R. rubrum* chromatophores (see Fig. 1). (A) After 1.5 h incubation with 20 mM $MgSo_4$, the solution in the chromatophore-containing compartment was replaced by the initial chromatophore-free solution with 20 mM $MgSO_4$. 0.2 mM TMPD, 0.1 mM menadion and 5 mM Tris-ascorbate were added to both compartments. (B) Aqueous solution in the chromatophore-free compartment was substituted for the 10% asolectin solution in decane. The electrical resistance of the measuring phospholipid membrane was equal to $2.5 \cdot 10^9$ and $1.9 \cdot 10^9$ Ω in A and B, respectively.

the chromatophore-planar membrane system. Similar data are presented in this work. Fig. 2 shows that the light-dependent electrical potential difference across the planar membrane is equal to about 4 mV in the presence of ascorbate and increases up to 120 mV on addition of TMPD and menadion.

Photoelectrical activity is not exhibited in the absence of bivalent cations associating chromatophores with planar membrane (Fig. 3, see also Ref. 11). Photoelectrical responses emerge on addition of Ca^{2+} (or Mg^{2+}), increase while the process of chromatophore association with planar membrane proceeds and remain constant thereafter.

Being immobilized, the chromatophores remain associated with the planar membrane and retain photoelectrical activity after replacement of the solution in the chromatophore compartment of the cuvette by a chromatophore-free solution containing bivalent cations (Fig. 4A). The thickness of the measuring phospholipid membrane does not considerably influence the values of electrical responses. Fig. 4B shows that the light-dependent response of immobilized chromatophores decreases insignificantly when the aqueous solution in the chromatophore-free compartment is substituted for the asolectin solution in the decane and the measuring electrode is plunged into this solution (see the scheme of the experiment in Fig. 1B). The experiment simulates an increase in the planar membrane thickness from 200 μm (Fig. 4A) to 4–5 mm (Fig. 4B). The electrical resistance of the phospholipid membrane was unlikely to decrease under these conditions.

In further experiments, the cytochrome *c* effect on electrical activity of chromatophores was investigated. Illumination of chromatophores preincubated with cytochrome *c* in the absence of biva-

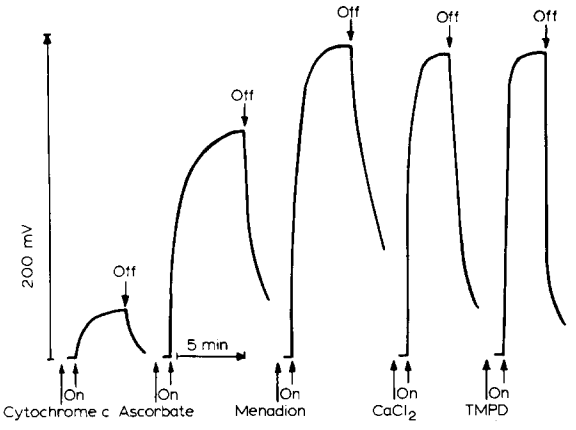

Fig. 5. Cytochrome *c* effect on the light-dependent generation of electrical potential difference by *R. rubrum* chromatophores in the system with the planar phospholipid membrane. Additions: 50 μM horse heart cytochrome *c*, 5 mM Tris-ascorbate, 0.1 mM menadion, 40 mM $CaCl_2$, 0.2 mM TMPD. The chromatophores were preincubated with cytochrome *c* for 90 min. The light was switched on 30 min after addition of $CaCl_2$.

lent cations generated an electrical potential difference across the planar membrane (Fig. 5). The photoelectrical response of about 30 mV was enhanced by ascorbate and menadion, and decreased insignificantly by Ca^{2+}. It did not increase on addition of TMPD. Notably, the cytochrome *c*-dependent photoelectrical responses in the chromatophore-planar membrane system are stimulated by quinone less effectively than those observed with TMPD (cf. Figs. 2 and 5). Thus, cytochrome *c* associated chromatophores with the

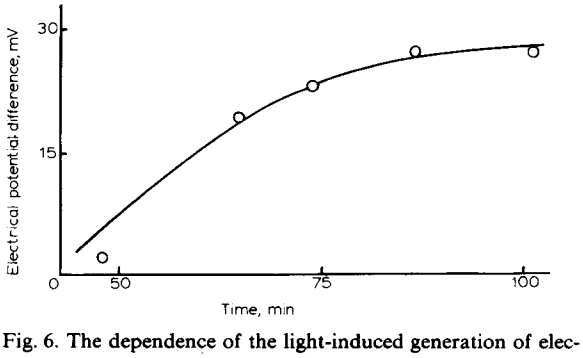

Fig. 6. The dependence of the light-induced generation of electrical potential difference by *R. rubrum* chromatophores in the system with the planar membrane on the incubation time with cytochrome *c* (50 μM). The incubation mixture did not contain Ca^{2+}, ascorbate, menadion and TMPD.

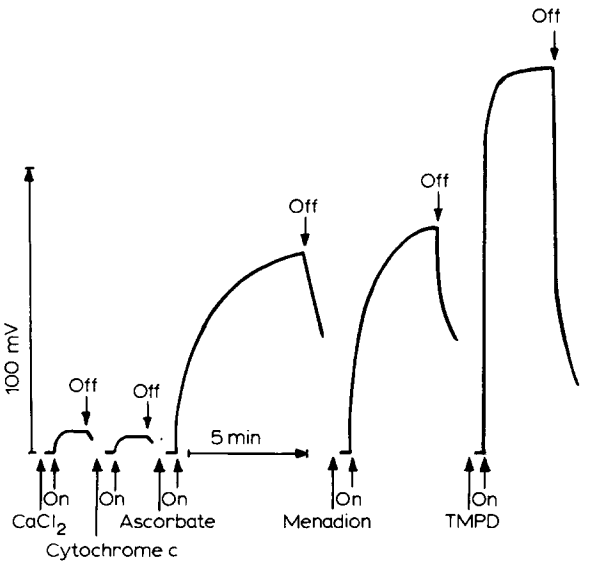

Fig. 7. Effect of preincubation with Ca^{2+} on cytochrome *c*-dependent photoelectrical responses of *R. rubrum* chromatophores in the system with the planar membrane. Additions: 40 mM $CaCl_2$, 50 μM cytochrome *c*, 5 mM Tris-ascorbate, 0.1 mM menadion, 0.2 mM TMPD. The chromatophores were incubated with $CaCl_2$ for 40 min. The light was switched on 80 min after addition of cytochrome *c*.

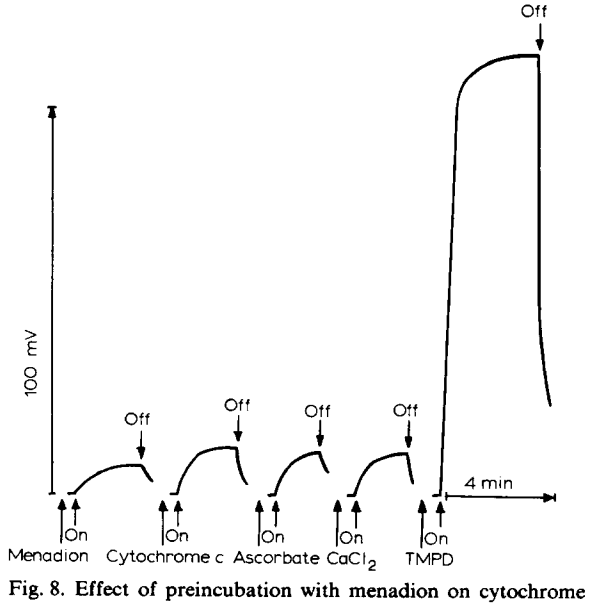

Fig. 8. Effect of preincubation with menadion on cytochrome *c*-dependent photoelectrical responses of *R. rubrum* chromatophores in the system with the planar membrane. Additions as indicated in Fig. 7. The light was switched on 20, 75 and 40 min after addition of menadion, cytochrome *c* and $CaCl_2$, respectively.

planar membrane and so TMPD was not required.

Fig. 6 shows the dependence of electrical potential difference generation in illuminated chromatophores associated with a planar membrane on the incubation time. Upon cytochrome *c* addition, a slow increase in photoelectrical responses occurs, reaching the maximum value in about 80 min.

Preincubation of chromatophores with Ca^{2+} decreased the values of photoelectrical responses observed on subsequent addition of cytochrome *c*, ascorbate and menadion (Fig. 7, compare with Fig. 5). TMPD enhanced the response.

Cytochrome *c*-dependent photoelectrical responses of chromatophores were prevented by menadion. As Fig. 8 shows, cytochrome *c* does not influence the light-induced generation of an electrical potential difference across the planar membrane if the chromatophores have been preincubated with menadion. The light responses do not increase after Ca^{2+} is added, but are markedly stimulated by TMPD. The prevention of cytochrome *c*-dependent electrical responses of chromatophores was demonstrated using ubiquinone-6 introduced into the planar membrane and not merely with menadion (data not shown).

Discussion

Charge transfer across the planar membrane

The mechanism of energy-dependent generation of a transmembrane electrical potential difference across the planar membrane associated with ATPase-, cytochrome oxidase-, bacteriorhodopsin- and bacteriochlorophyll-containing liposomes and chromatophores was discussed earlier [11,13]. Switching on of the intrachromatophore electrical generator by illumination induces H^+ gradient generation across the chromatophore membrane (positive inside the membrane vesicle) and an electrical current across the planar membrane (positive in the chromatophore-free compartment) measured by macroelectrodes. Of some interest is the mechanism of charge translocation through the planar (thick) membrane.

In this paper, we report that photoelectrical responses of chromatophores are observed if the planar membrane thickness varies significantly. Substituting an aqueous solution for the asolectin solution in decane used for impregnation of the membrane filter does not cause any significant changes in the magnitude and shape of the chromatophore electrical responses (Fig. 4).

The electrical current across the planar membrane and the whole width of the phospholipid solution added may be due to formation of cylindrical micelles where the polar heads constitute the micelle main body and the hydrocarbon tails – the nonpolar envelope solvated with decane molecules. The inner hydrophilic phase of these micelles may function as proton and, perhaps, other ions conductor.

Cytochrome c effect on the chromatophore-planar membrane system

Cytochrome *c* isolated from mammalian mitochondria is similar to cytochrome c_2 from nonsulfur purple bacteria in a number of physicochemical properties [17,18]. There are several isoelectrically different forms of cytochrome c_2: eight forms have been isolated from *R. rubrum*, their isoelectric points ranging between 4.1 and 9.3 [19]. The isoelectric point of mitochondrial cytochrome *c* is 10.65 [20] and the charge of oxidized horse heart cytochrome *c* is +9.5 at pH 7.0 [21].

Both of the cytochromes are oxidized effectively by isolated P-870 reaction centers from *Rhodopseudomonas sphaeroides* [20] and *R. rubrum*[22]. The interaction between cytochrome *c* or c_2 and the reaction centers is electrostatic [20,23]. Data on the pH dependence of cytochrome oxidation by the reaction centers incorporated into phospholipid vesicles indicate that group(s) with a p*K* at pH 9.5 are essential for the cytochrome-reaction center interaction [23].

As a polycation, cytochrome *c* is known to bind to negatively charge phospholipids [24–28]. The electrostatic interaction apparently gives rise to association of chromatophores with the planar phospholipid membrane and thereby minimizes the need for Ca^{2+} or Mg^{2+} (Fig. 5).

Furthermore, cytochrome *c* produces another action: the maximum photoelectrical response unaffected by TMPD is generated by chromatophores in the presence of cytochrome *c*, ascorbate and menadion. Such an effect of cytochrome *c* as a TMPD functional substitute was observed before in experiments with proteoliposomes containing P-870 reaction center complexes from *R. rubrum*

[10]. The direction of the electrical field generated in the illuminated proteoliposomes (negative inside) was opposite to that generated in chromatophores (positive inside), thus pointing to opposite orientations of redox chain components in natural and artificial membranes [10,11]. Cytochrome *c* (c_2) interacts with reaction centers the outside of the proteoliposome membrane [10] and on the inside of the chromatophore membrane [8,9].

Cytochrome *c* is known to penetrate in part lipid bilayers [29,30] and monolayers [31,32], to induce phase separation of lipids [27] and nonbilayer structure formation in cardiolipin liposomes [33]. Interacting with phospholipids, cytochrome *c* forms a complex soluble in hydrocarbon solvents [34]. A model [33] for cytochrome *c* translocation across the hydrophobic membrane barrier by means of inverted lipid structures has been advanced. The main body of these structures is the cytochrome *c* surrounded by negatively charged phospholipid heads and its nonpolar envelope consists of phospholipid hydrocarbon tails.

It is possible that a similar mechanism of cytochrome *c* translocation proposed for model membranes functions in the chromatophore-planar membrane system used. The chromatophores associated with a planar membrane by bivalent cations may lose in addition to quinones [11] endogenous cytochrome c_2 due to extraction of cytochrome c_2-phospholipid complexes by decane, a component of the planar membrane. Cytochrome *c* added penetrates into the chromatophores but the effect produced is small because the bivalent cations neutralizing the negative charge of phospholipids, as shown for Ca^{2+} [33], compete with cytochrome *c*. Under these conditions, the photoelectrical activity of chromatophores is higher in the presence of TMPD (easily penetrating through membranes) than with cytochrome *c*. In the absence of bivalent cations, when the association of chromatophores with a planar membrane is due to cytochrome *c*, TMPD is ineffective (Fig. 5): the conditions are apparently favorable for cytochrome *c* translocation into the chromatophores; it is also possible that cytochrome *c* added prevents endogenous cytochrome c_2 extraction from the chromatophores.

Membrane potential generation in the illuminated chromatophores associated with the planar membrane and supplemented by cytochrome *c* seems to be due to the action of a shortened cyclic electron-transfer chain involving the P-870 reaction center, ubiquinone and cytochrome *c* (c_2). Such a suggestion is consistent with the data on the light-induced membrane potential generation in proteoliposomes containing the P-870 reaction center complexes and incubated with cytochrome *c* and ubiquinone (menadion) [10] as well as in the isolated [1] and intracellular [2] chromatophores treated with antimycin A . The planar membrane inactivates the cytochrome *b* region of the electron-transfer chain [11].

TMPD can act analogously to cytochrome *c* [10]. However, exogenous quinone (menadion) stimulates the photoelectrical activity of the chromatophores in the presence of TMPD to a greater degree than with cytochrome *c* (cf. Figs. 2 and 5). This can be explained by a dual effect of TMPD as an electron carrier. Along with a cytochrome *c*-like action (electron transfer from the residual loosely bound ubiquinone to the reaction center bacteriochlorophyll dimer on the inner side of the chromatophore membrane and H^+ release into the chromatophore interior), TMPD can be reduced competitively to the secondary quinone by the primary quinone and reoxidized by the bacteriochlorophyll dimer. The contribution of this nonelectrogenic transfer of electrons from the primary quinone to P-870 bacteriochlorophyll mediated by TMPD must be reinforced upon the extraction of the loosely bound quinone pool (by the planar membrane) and, vice versa, must be minimized upon the regeneration of this pool (by exogenous quinone).

Thus, the data obtained indicate the possibility of cytochrome *c* (c_2) translocation across the chromatophore membrane. Cytochrome c_2 synthesized in the cytoplasm is revealed in the periplasmic space of intact bacterial cells [8,19]; the mechanism considered may be involved in cytochrome c_2 translocation across the cytoplasmic membrane. In this connection, the observation on prevention of the cytochrome *c* effect on light-induced generation of membrane potential in chromatophores by added quinones (Fig. 8) seems to be important. It is possible that quinones as well as bivalent cations are involved in the regulation of cytochrome c_2 transsmembrane movement in bacterial cells.

Acknowledgement

We are grateful to Dr. A.V. Oleskin for help with preparation of the English version of the manuscript.

References

1 Remennikov, V.G. and Samuilov, V.D. (1979) Biochim. Biophys. Acta 548, 216–223
2 Remennikov, V.G. and Samuilov, V.D. (1980) Arch. Microbiol. 125, 271–275
3 Jackson, J.B. and Dutton, P.L. (1973) Biochim. Biophys. Acta 325, 102–113
4 Takamiya, K. and Dutton, P.L. (1977) FEBS Lett. 80, 279–284
5 Packham, N.K., Greenrod, J.A. and Jackson, J.B. (1980) Biochim. Biophys. Acta 592, 130–142
6 Fleischman, D.E. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 513–523, Plenum Press, New York
7 Borisov, A.Yu., Godik, V.I., Kotova, E.A. and Samuilov, V.D. (1980) FEBS Lett. 119, 121–124
8 Prince, R.C., Baccarini-Melandri, A., Hauska, G.A., Melandri, B.A. and Crofts, A.R. (1975) Biochim. Biophys. Acta 387, 212–227
9 Hochman, A. and Carmeli, C. (1977) Arch. Biochem. Biophys. 179, 349–359
10 Drachev, L.A., Kondrashin, A.A., Samuilov, V.D. and Skulachev, V.P. (1975) FEBS Lett. 50, 219–222
11 Drachev, L.A., Frolov, V.N., Kaulen, A.D., Kondrashin, A.A., Samuilov, V.D., Semenov, A.Yu. and Shulachev, V.P. (1976) Biochim. Biophys. Acta 440, 637–660
12 Krasinskaya, N.P. and Samuilov, V.D. (1977) J. Bioenerg. Biomembranes 9, 171–180
13 Drachev, L.A., Jasaitis, A.A., Kaulen, A.D., Kondrashin, A.A., Liberman, E.A., Nemecek, I.B., Ostroumov, S.A., Semenov, A.Yu. and Skulachev, V.P. (1974) Nature 249, 321–324
14 Remennikov, V.G. and Samuilov, V.D. (1979) Biochim. Biophys. Acta 546, 220–235
15 Isaev, P.I., Liberman, E.A., Samuilov, V.D., Skulachev, V.P. and Tsofina, L.M. (1970) Biochim. Biophys. Acta 216, 22–29
16 Drachev, L.A., Kaulen, A.D., Samuilov, V.D., Severina, I.I., Semenov, A.Yu., Skulachev, V.P. and Chekulaeva, L.N. (1979) Biofizika 24, 1035–1042
17 Salemme, F.R., Kraut, J. and Kamen, M.D. (1973) J. Biol. Chem. 248, 7701–7716
18 Ambler, R.P., Meyer, T.E. and Kamen, M.D. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 472–475
19 Bartsch, R.G. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 249–279, Plenum Press, New York
20 Ke, B., Chaney, T.H. and Reed, D.W. (1970) Biochim. Biophys. Acta 216, 373–383
21 Barlow, G.H. and Margoliash, E. (1966) J. Biol. Chem. 241, 1473–1477
22 Van der Rest, M. and Gingras, G. (1974) J. Biol. Chem. 249, 6446–6453
23 Overfield, R.E. and Wraight, C.A. (1980) Biochemistry 19, 3322–3327
24 Dutton, P.L., Wilson, D.F. and Lee, C.P. (1970) Biochemistry 9, 5077–5080
25 Kimelberg, H.K., Lee, C.P., Claude, A. and Mrena, E. (1970) J. Membrane Biol. 2, 235–251
26 Vanderkooi, J., Erecinska, M. and Chance, B. (1973) Arch. Biochem. Biophys. 154, 219–227
27 Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261–310
28 Brussel. G.B. and Griffith, O.H. (1976) Biochemistry 15, 2925–2929
29 Gulik-Krzywichi, T., Shechter, E., Luzzati, V. and Foure, M. (1969) Nature 223, 1116–1117
30 Papahadjopoulos, D., Mascarello, M., Eylar, E.H. and Isac, T. (1975) Biochim. Biophys. Acta 401, 317–335
31 Morse, P.D. and Deamer, D.W. (1973) Biochim. Biophys. Acta 298, 769–782
32 Kimelberg, H.K. and Papahadjopoulos, D. (1971) J. Biol. Chem. 246, 1142–1148
33 De Kruijff, B. and Cullis, P.R. (1980) Biochim. Biophys. Acta 602, 477–490
34 Das, M.L. and Grane, F.L. (1964) Biochemistry 3, 696–704