Biochimica et Biophysica Acta, 591 (1980) 346—355
© Elsevier/North-Holland Biomedical Press

BBA 47866

EFFECTS OF SURFACE POTENTIAL AND MEMBRANE POTENTIAL ON THE MIDPOINT POTENTIAL OF CYTOCHROME *c*-555 BOUND TO THE CHROMATOPHORE MEMBRANE OF *CHROMATIUM VINOSUM*

SHIGERU ITOH

Department of Biology, Faculty of Science, Kyushu University 33, Fukuoka 812 (Japan)

(Received September 7th, 1979)

Key words: Membrane surface potential; Redox potential; Cytochrome c-555, Chromatophore membrane; Bacterial photosynthesis (Chromatium vinosum)

Summary

The values of midpoint potential (E_m) of cytochrome *c*-555 bound to the chromatophore membranes of a photosynthetic bacterium *Chromatium vinosum* was determined under various pH and salt conditions.

After a long incubation at high ionic concentrations in the presence of carbonylcyanide *m*-chlorophenylhydrazone, which was added to abolish electrical potential difference between the inner and outer bulk phases of chromatophore, the E_m value was almost constant at pH values between 4.0 and 8.4. With the decrease of salt concentration, the pH dependence of the E_m value became more marked. Under low ionic conditions, E_m became more positive with the decrease of pH. Addition of salt made the value more positive or negative at pH values higher or lower than 4.5, respectively. Divalent cation salts were more effective than monovalent cation salts in producing the positive shift of E_m at pH 7.8.

The E_m value became more positive when the electrical potential of the inner side of the chromatophore was made more positive by the diffusion potential induced by the K^+ concentration gradient in the presence of valinomycin.

These results were explained by a change of redox potential at the inner surface of the chromatophore membrane, at which the cytochrome is assumed to be situated, due to the electrical potential difference with respect to the outer solution induced by the surface potential or membrane potential change.

The values for the surface potential and the net surface charge density of the inner surface of the chromatophore membrane were estimated using the Gouy-Chapman diffuse double layer theory.

Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

Introduction

The midpoint redox potential (E_m) of the membrane-bound cytochrome *c*-555 in chromatophores of *Chromatium vinosum* is reported to change depending on ionic conditions of the medium by Case and Parson [1]. From the analysis using the Debye-Hückel theory, these authors concluded that the different ionic dependences of the activity coefficients of oxidized and reduced forms of the cytochrome result in such E_m change. However, quantitative explanation for the observed shift of E_m was not fully given by the analysis. Recent studies on the reactions and redox equilibria of membrane-bound electron transfer components [2—5] indicated that the Gouy-Chapman diffuse double layer theory gives a better information for analyzing the phenomena on the membrane surface than the Debye-Hückel theory which was developed to explain the behavior of small ions in solution. In the present study the salt and pH effects on E_m of the membrane-bound cytochrome *c*-555 were analyzed according to the former theory.

Theory

As discussed by Hinkel and Mitchell [6] or more recently by Walz [5], when an electrostatic potential difference exists between the bulk solution and a site in the membrane, E_m of the redox couple on the membrane takes a different value from the standard redox potential, E°, since a change in the chemical potential of electrons (redox potential) should compensate the difference in the electrical potential for the equilibration of electrochemical potential of electrons. The following relationship should exist between the values of E_m and E°.

$$E_m = E^\circ + \Delta\varphi \tag{1}$$

where $\Delta\varphi$ denotes the electrostatic potential difference at the membrane with respect to the outer solution where the redox potential is measured by electrodes. Such relation is observed in the cases of the membrane-potential-induced shifts of E_m of cytochrome *a* in mitochondria [6] and of cytochrome c_2 in chromatophore of *Rhodopseudomonas sphaeroides* [7].

In the absence of the externally applied field, too, an electrostatic potential difference on the membrane surface with respect to the bulk outer solution exists due to the presence of immobilized charges on the membrane surfaces [2—5] (Fig. 1A). The relation of the electrical potential difference, ψ_o (surface potential), to the net surface charge density, q, and the bulk concentration of a salt with $z:z$ ion composition, C_b, can be expressed as follows according to the theory,

$$\psi_o = \frac{2RT}{zF} \sinh^{-1}\left(\sqrt{\frac{\pi}{2RT\epsilon C_b}} \cdot q\right) \tag{2}$$

where ϵ is the dielectric constant of water and other symbols have their usual meanings. The difference of the electrical potential on the membrane surface changes the chemical potential of ions at the surface. Activity of ion at the

surface (a_s) and in the outer solution (a_b) is then expressed as follows

$$a_s = a_b \exp\left(-\frac{zF\psi_0}{RT}\right) \tag{3}$$

The higher valence cation and anion salts decrease the negative and positive surface potential, respectively, more effectively than the salts of monovalent ions.

The value of E_m of a non-protonating redox component on the negatively charged surface at the equilibrium can be given, from Eqns. 1 and 2 [5], as

$$E_m = E^0 + \psi_0 = E^0 + \frac{2RT}{zF} \sinh^{-1}\left(\sqrt{\frac{\pi}{2RT\epsilon C_b}} \cdot q\right) \tag{4}$$

Thus the salt- or pH-induced change of the surface potential (i.e., change in C_b or q in Eqn. 4) is expected to affect E_m of the nonprotonating membrane component.

On the other hand, for a component which binds proton(s) upon reduction, the relation below will hold when it is in equilibrium with protons in the solution [5].

$$E_m = E^0 + \psi_0 - \frac{2.3RT}{F} pH_s$$

$$= E^0 - \frac{2.3RT}{F} pH_b \qquad \left(pH_s = pH_b + \frac{F}{2.3RT}\psi_0\right) \tag{5}$$

where pH_s and pH_b denote the values of pH at the surface and in the bulk solution. The relationship in the bracket comes from Eqn. 3. Eqn. 5 indicates

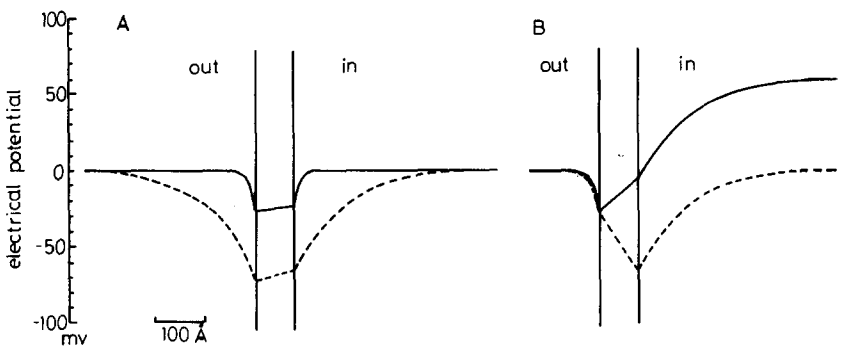

Fig. 1. Schematic representation of electrostatic potential across the chromatophore membrane. A, at the equilibrium after a long incubation in 1 mM (- - - - - -) or 100 mM (———) KCl in the presence of CCCP, which abolishes electrical and protic potential differences between the inner and outer bulk phases (membrane potential is expected to be zero). B, before (- - - - - -) and after (———) addition of 10 mM KCl in the presence of valinomycin and 0.1 M choline chloride in the outer solution (membrane potential is changed). Cation concentration in the inner chromatophore space was assumed to be 1 mM in Fig. 1B. Net charge density of the outer surface of the chromatophore (unknown in this organism) was chosen to be 10% larger than that of the inner surface (—1.2 $\mu C/cm^2$ estimated from the results in Figs. 3 and 4 at pH 7.8). The experiments in Figs. 2 and 3 correspond to the situation in Fig. 1A and that in Fig. 4 to the situation in Fig. 1B.

that E_m will not be affected by the surface potential change at the equilibrium.

In the present study relation between the surface potential and the E_m value of cytochrome *c*-555, which seems to be a nonprotonating component within the pH range tested and is expected to obey Eqn. 4, was studied. Redox titrations were carried out after preincubating chromatophores in the media of various pH and ionic conditions in the presence of CCCP to equilibrate the electrical potential and pH in the solutions inside chromatophores with those in the outer solution [10] as shown in Fig. 1A since the cytochrome is probably situated on the inner surface of the chromatophore vesicles [11]. Then the effect of salt or pH on the surface potential of the inner surface of the chromatophore as shown schematically in Fig. 1A is expected to be measured by the change of E_m. The effect of diffusion potential on the redox state of the cytochrome was also studied (Fig. 1B).

Materials and Methods

C. vinosum was grown photoautotrophically in the medium of Bose. The cells harvested were once washed with a medium containing 2 mM $MgCl_2$, 50 mM KCl and 50 mM Tris-HCl buffer, pH 7.8, and resuspended in the same medium. Chromatophores were prepared by passing the cell suspension through a French Pressure Cell (Ohtake) at 1000 kg/cm^2. The cell debris were precipitated by a centrifugation at 10 000 $\times g$ for 30 min. The supernatant was further centrifuged for 1 h at 100 000 $\times g$ and the resultant pellet was washed in the same medium. The precipitate obtained after washing was then suspended in distilled water and the suspension was centrifuged at 10 000 $\times g$ for 30 min to remove large fragments. An aliquot of the chromatophore suspension was diluted with the reaction medium (final bacteriochlorophyll concentration, 10—15 μM) and used for measurements.

Bacteriochlorophyll concentrations of chromatophores were determined in acetone/methanol (7 : 2, v/v) extracts using the absorption coefficient of 75 $mM^{-1} \cdot cm^{-1}$ at 772 nm [12].

Redox titration of cytochrome *c*-555 was done by measuring absorption change at 422 nm upon excitation with a single-turnover Xe flash of 20 μs duration at saturating intensity (Sugawara, s-3A) with a single beam spectrophotometer (Union Giken) at varied redox potentials which were determined by a Pt-Ag/AgCl electrode (Toa, PS115C). Before reductive titration the chromatophores were preincubated in a mixture of 100 μM ferricyanide, 10 μM phenazine methosulfate, 10 μM *N,N'*-tetramethyl-*p*-phenylenediamine, 0.4 μM CCCP, 1 mM phosphate buffer and varied concentrations of salt for more than 10 h at 4°C unless otherwise mentioned. Reductive titration then were carried out by adding small aliquots of 100 mM ascorbate or dithiothreitol at room temperature. Back titration was carried out sometimes with air or ferricyanide as oxidant. In the cases of titrations at pH 4 and 5, the chromatophores were preincubated in a medium comprising above mixture of redox reagents and phosphate buffer at pH 5.4 for more than 10 h, and then an aliquot of succinic acid solution was added to adjust pH and incubated for another 30 min before the titration.

Results

Effect of surface potential

Fig. 2 shows the pH dependence of the E_m of cytochrome *c*-555 measured after long incubations in low- and high-ionic media containing CCCP. The existence of two cytochromes for a reaction center changes a little the shape of the titration curve of this cytochrome measured by the flash-induced absorption change from the Henderson-Hasselbach relation and shifts the apparent E_m value approx. 20 mV more positive than the true E_m as reported by Case and Parson [1,9]. The pH dependence of the E_m value measured depended on ionic conditions of the medium as expected from the result of these authors. In the presence of 0.3 M KCl, E_m was only slightly pH dependent within the pH range tested. On the other hand, at a low ionic concentration a significant pH dependence of E_m was observed. E_m became more negative as the medium pH increased. A large change of the value of E_m was observed at pH values between 4 and 6 but the change was less in the higher pH region. Addition of KCl at pH values less than 4.5 made the E_m negative, while that at higher pH values made it more positive.

The results can be explained by assuming that the change of surface potential of the chromatophore membrane depends on the pH and salt concentration of the medium according to Eqn. 4 (see Fig. 1A). At pH values less than 4.5, the membrane surface of the chromatophore is assumed to be positively charged, with a positive surface potential; then the addition of KCl will decrease the positive surface potential by screening the charges. On the other hand, at pH values higher than 4.5, where the surface is negative, addition of KCl will decrease the negative surface potential (Eqn. 2). These changes of the

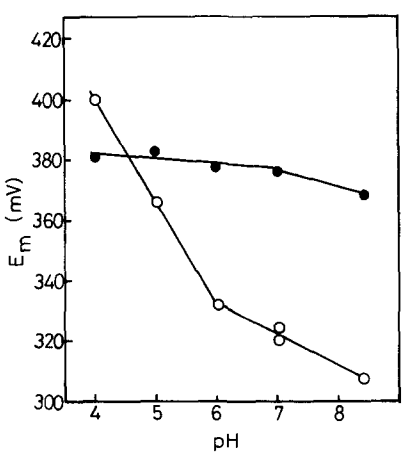

Fig. 2. pH dependence of E_m of cytochrome *c*-555 in low and high ionic media. ○, no KCl. ●, in the presence of 0.3 M KCl. The values of E_m were measured after incubating chromatophores in the reaction medium containing 0.4 μM CCCP as described in Materials and Methods. The E_m values are approx. 20 mV more positive than the true E_m value of the cytochrome due to the existence of two cytochrome molecules per one reaction center [9]. E_m values determined from the absorption changes upon the second flash excitations (given 20 ms after the first flash), which were approx. 40 mV lower than those measured by the first flash [9], also showed similar pH and ionic dependences (not shown).

surface potential by KCl addition will change the value of E_m according to Eqn. 4, showing the negative (below pH 4.5) and positive (above pH 4.5) shifts of E_m.

Dependence of the salt effect at pH 7.8 on the ionic species is shown in Fig. 3. Salt of monovalent cations (KCl, K_2SO_4 and NaCl) were effective in increasing E_m in similar cation concentration ranges. Divalent cation salts ($MgCl_2$, $MgSO_4$ and $CaCl_2$) were more effective in lower concentration ranges than the monovalent cation salts. When the chromatophores were preincubated for shorter times (e.g., 30 min) these salt effects did not fully develop (especially in the case of divalent cations) suggesting that the penetration of salt into the internal space of chromatophores is necessary for a change of E_m.

These results with salts of mono- and divalent cations indicate that the effectiveness of the salt depends on the valence of cations, and not on the ionic strength as originally proposed by Case and Parson [1].

The solid lines in Fig. 3 represent theoretical curves for the dependences of the value of E_m on the concentrations of 1 : 1 and 2 : 2 symmetrical salts. The lines were calculated according to Eqn. 4 with q values of −1.2 and −1.7 $\mu C/cm^2$ by assuming $\psi_o = 0$ at $E_m = 383$ mV, at which potential the crossover of the salt effect occurred (Fig. 2). On the right-hand ordinate of Fig. 3 a scale for the ψ_o value thus calculated is shown. The values of E_m experimentally determined somewhat deviated from the theoretical curves. However, the difference observed between the effectiveness of the mono- and divalent cation salts was almost as expected from the theory. The q value of -1.2 ± 0.4 $\mu C/cm^2$ was found to be most appropriate to explain the experimental results. Within this range of q value, larger absolute values of q were estimated from the

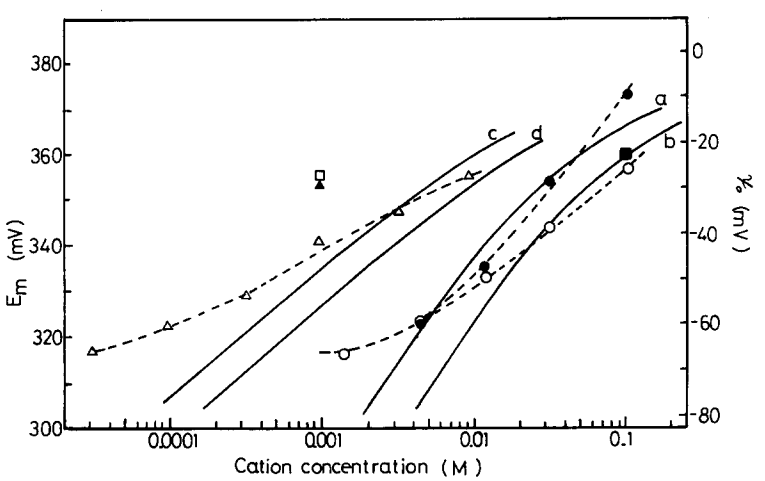

Fig. 3. Dependence of E_m of cytochrome *c*-555 on cation concentrations at pH 7.8. ○, KCl. ●, K_2SO_4. ■, NaCl. △, $MgCl_2$. ▲, $MgSO_4$. □, $CaCl_2$. The values of E_m were measured after incubating chromatophores in the reaction media containing 0.4 μM CCCP and varied concentrations of salts as described in Materials and Methods. 1 mM Tricine buffer, pH 7.8, was used in place of phosphate buffer. Solid lines show the theoretical curves calculated by assuming $\psi_o = 0$ at $E_m = 383$ mV with q value of −1.2 (curves a and c) or −1.7 $\mu C/cm^2$ (curves b and d). Curves a and b for 1 : 1 symmetrical salts. Curves c and d for 2 : 2 symmetrical salts. The values of experimentally determined E_m are approx. 20 mV more positive than the true E_m of the cytochrome [9].

values of E_m at higher salt concentrations. This observation seems to be reasonable, since at neutral pH the difference between pH values at the surface and in the bulk solution will decrease, i.e., surface pH will increase with the increase of salt concentration, and this will make the surface more negative due to the deprotonation of surface groups.

Effect of diffusion potential

If the cytochrome is situated on the inner side of the chromatophore membrane, changes in the membrane potential (potential difference between the outer and inner solutions) should also affect the E_m value as shown in Fig. 1B. The chromatophores were preincubated in 0.1 M choline chloride and the redox mediators at an E_h of approx. 350 mV for 30 min and then valinomycin and varied amounts of KCl solution were added (in this type of experiment CCCP was not present). Redox state of the cytochrome was monitored by the flash induced absorption change before and after the KCl addition. When the full titration was done in the presence of 0.1 M choline chloride at pH 7.8, an E_m value of 340 mV was obtained. This value was more negative than that observed with 0.1 M KCl or 0.05 M K_2SO_4 (Fig. 3) and seemed to reflect the situation that penetration of salt into the internal space of the chromatophore is necessary to decrease the surface potential in the vicinity of the cytochrome. When KCl was added in the presence of valinomycin, change in the redox level of the cytochrome was induced. The change took approx. 1 min to attain the new steady level with a half-time of 15 s. From the E_h value and the redox level of the cytochrome determined by the flash 1 min after adding KCl, the value of E_m was calculated (Fig. 4). A rise of E_m of approx. 20 mV was

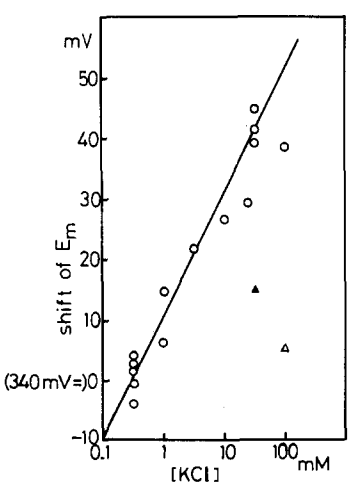

Fig. 4. Dependence of E_m on K^+ concentration added in the presence of valinomycin. Chromatophores were preincubated in 0.1 M choline chloride, 1 mM Tricine buffer, pH 7.8, 10 μM phenazine methosulfate, 10 μM *N,N'*-tetramethyl-*p*-phenylenediamine, 0.1 mM potassium ferricyanide and dithiothreitol at E_h = 350 ± 10 mV and then valinomycin (final concentration of 0.13 μM) was added followed by addition of varied concentration of KCl. Redox level of the cytochrome was measured by the flash-induced absorbance change at 422 nm at 1 min before and after the addition of KCl. Data on addition of NaCl in the place of KCl (△) and on addition of KCl in the absence of valinomycin (▲), respectively.

observed for every 10-fold increase of KCl concentration. When the valinomycin was added after KCl addition, the change of redox level was almost the same in extent. KCl addition without valinomycin or NaCl addition with valinomycin caused a smaller change in the redox level (Fig. 4) indicating that the change in the electrostatic potential was induced by the diffusion potential of K^+.

By the use of the Goldman equation, a +60 mV change of the electrostatic potential at the inner surface of the chromatophore is expected for the 10-fold increase of KCl concentration in the outer solution, while the observed shift of E_m was only +20 mV. Change of the surface potential on the outer surface is negligible in this case due to the pre-existence of choline chloride. The quantitative discrepancy is probably due to the decay of the diffusion potential after adding KCl, since the field-indicating carotenoid band shift [13,14] measured by the change of absorption difference between 483 and 500 nm decayed approx. 50% within the first minute after the KCl addition (Itoh, S., unpublished data).

This diffusion potential-induced reduction of the cytochrome was very slow and incomplete if *N,N'*-tetramethylphenylenediamine was omitted from the reaction medium, indicating that a membrane-permeant reductant is necessary.

These results confirm the view that cytochrome *c*-555 is situated in the membrane close to the inner surface of the chromatophore.

Discussion

Electrostatic potential difference, created either by the surface potential change of the inner surface of the chromatophore or by the membrane potential change induced by the diffusion potential of K^+, affected the E_m value of cytochrome *c*-555 as expected theoretically. The result in the present study indicated that a change in the surface potential of the inner surface of the chromatophore membrane induces pH- or salt-dependent change of the E_m value as expected from Eqn. 4. It is concluded that the pH-dependent change of E_m in low ionic medium represents the pH dependences of the surface potential and of the net surface charge density of the inner surface. The susceptibility of E_m to the diffusion potential supports this view and indicates that the change in the E_m value can be a probe of electrostatic potential difference at the inner surface.

The direction of the salt effect changed at approx. pH 4.5, suggesting that the net surface charge on the inner surface changes its sign at this pH. This pH was a little lower than the reported isoionic pH of the chromatophore membrane (pH 5.2 according to Case and Parson [1]), and may represent isoionic pH of the inner surface of the chromatophore which may be a little different from that of the outer surface.

The q value of -1.2 ± 0.4 $\mu C/cm^2$ (0.07 ± 0.02 elementary charge/100 $Å^2$) estimated at pH 7.8 corresponds to 0.2 ± 0.06 elementary charge/bacteriochlorophyll molecule provided that the surface area of one bacteriochlorophyll is 300 $Å^2$ [15]. This value is a little lower than that estimated for the outer surface of *R. sphaeroides* chromatophores by the measurement of the carotenoid band shift induced by the surface potential change (-2.8 $\mu C/cm^2$) [10]

and is similar to those estimated for chloroplast membranes [2—4]. Whether this difference between the values of q for the chromatophore membranes reflects the difference of the surfaces or comes from the difference of the methods used cannot be concluded yet.

According to the results by Case and Parson [1], not only the E_m value of cytochrome *c*-555 but also that of *P*-870 in *Chromatium* chromatophore depended on the ionic strength of the medium in similar extents. The result can be explained in a way similar to that for cytochrome *c*-555 using the q value estimated in the present study. The value of E_m of another component, cytochrome *c*-552, which also seems to be a nonprotonating component, has been reported to show less dependence on the ionic strength [1]. This may reflect a difference in the local charge distribution in the vicinity of this cytochrome. It remains to be studied. E_m of other electron carriers, X and Y (primary and secondary electron acceptors) were reported to be independent of ionic strength. The results are as expected, since these components were shown to protonate upon reduction, and therefore their E_m should be independent of ionic conditions of the medium according to Eqn. 6 as indicated by Walz [5].

Effect of electrostatic potential difference at the membrane surface due to the surface potential should affect E_m of many membrane-bound electron carriers. In the case of cytochrome c_2 of *R. sphaeroides*, E_m becomes more positive when the cytochrome is detached from the negative membrane surface [16]. A study to find out how the redox states of such dissociable components are affected by the surface potential will be published elsewhere [17].

The inner surface of the chromatophore membrane is regarded to be homologous to the outer surface of the cell membrane in photosynthetic bacteria [18]. The surface of the cell membrane will experience the changes in pH or ionic conditions of the culture medium through the cell wall, which is permeable for small ions. It is important to know how the surface-potential-induced changes in the redox states and in the reactivities [2] of the membrane-bound electron carriers situated on or near the surface affect the energy transduction performed in the cell.

Acknowledgements

The author thanks Dr. M. Nishimura for his valuable suggestions and criticism during the work. This work was supported by a financial aid from the Ministry of Education.

References

1 Case, G.D. and Parson, W.W. (1973) Biochim. Biophys. Acta 292, 677—684
2 Itoh, S. (1978) Biochim. Biophys. Acta 504, 324—340
3 Itoh, S. (1979) Biochim. Biophys. Acta 548, 579—595
4 Nakatani, H.Y., Barber, J. and Forrester, J.A. (1978) Biochim. Biophys. Acta 504, 215—225
5 Walz, D. (1979) Biochim. Biophys. Acta 505, 279—353
6 Hinkel, P. and Mitchell, P. (1970) Bioenergetics 1, 45—60
7 Takamiya, K. and Dutton, P.L. (1977) FEBS Lett. 80, 279—284
8 McLaughlin, S. (1977) in Current Topics in Membrane and Transport, Vol. 9 (Bronner, F. and Kleinzeller, A., eds.), pp. 71—144, Academic Press, New York
9 Case, G.D. and Parson, W.W. (1971) Biochim. Biophys. Acta 253, 187—202

10 Matsuura, K., Masamoto, K., Itoh, S. and Nishimura, M. (1979) Biochim. Biophys. Acta 547, 91—102
11 Tieda, D.M., Leigh, J.S. and Dutton, P.L. (1978) Biochim. Biophys. Acta 503, 524—544
12 Clayton, R.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A., Vernon, L.P., eds.), pp. 495—500, Antioch Press, Yellow Springs, Ohio
13 Case, G.D. and Parson, W.W. (1973) Biochim. Biophys. Acta 325, 441—453
14 Knaff, D.B. and Carr, J.W. (1979) Arch. Biochem. Biophys. 193, 379—384
15 Packham, N.K., Berriman, J.A. and Jackson, J.B. (1978) FEBS Lett. 89, 205—210
16 Dutton, P.L., Petty, K.M., Bonner, H.S. and Morese, S.D. (1975) Biochim. Biophys. Acta 387, 536—556
17 Matsuura, K., Takamiya, K., Itoh, S. and Nishimura, M. (1980) J. Biochem. 87, 1431—1437
18 Oelze, J. and Drews, G. (1972) Biochim. Biophys. Acta 265, 209—239