Oxidation of Cytochromes *c* and c_2 by Bacterial Photosynthetic Reaction Centers in Phospholipid Vesicles. 1. Studies with Neutral Membranes†

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ABSTRACT: The oxidation of cytochrome c_2 by photosynthetic reaction centers isolated from *Rhodopseudomonas sphaeroides* and incorporated into unilamellar phosphatidylcholine vesicles was found to be kinetically similar to that observed earlier for reaction centers in low detergent solution [Overfield, R. E., Wraight, C. A., & DeVault, D. (1979) *FEBS Lett. 105*, 137–142]. At low ionic strength the kinetics were biphasic. The fast phase indicated the formation of a cytochrome–reaction center complex with an apparent binding constant, K_B, of about 10^5 M^{-1}. However, K_B decreased dramatically with increasing salt concentration, and no fast oxidation was detectable in 0.1 M NaCl. The slow cytochrome oxidation was first order in both cytochrome and reaction centers and, thus, second order overall. Deviations from theoretical second-order behavior were observed when the rate of the first-order back reaction of the primary photoproducts was significant compared to the cytochrome oxidation. This can cause serious overestimation of the second-order rate constant. The slow oxidation of cytochrome c_2 by reaction centers in phosphatidylcholine vesicles exhibited a 40% lower encounter frequency than with the solubilized reaction center. This was attributed to the much lower diffusion coefficient of the reaction center in the vesicle membrane than in solution. No effects of diminished dimensionality were detected with neutral vesicles. An activation energy of 8.0 ± 0.4 kcal·mol^{-1} was determined for the slow phase of cytochrome c_2 oxidation by reaction centers in solution and in vesicles of several different phosphatidylcholines, including dimyristoylphosphatidylcholine above and below its phase transition temperature. Thus, the physical state of the lipid did not appear to affect any rate-limiting steps leading to cytochrome oxidation. The ionic strength dependence of the slow kinetics of oxidation of cytochromes *c* and c_2 confirmed the electrostatic nature of the cytochrome–reaction center interaction, and the pH dependence indicated the titration of a group or groups, important to this interaction, at pH 9.5.

The oxidation of cytochrome c_2 by reaction centers in the photosynthetic bacterium, *Rhodopseudomonas sphaeroides*, is a good example of interaction between a peripheral and an integral membrane protein. The kinetics of oxidation of cytochrome c_2 in chromatophores and cells of *Rp. sphaeroides*, following flash excitation, have been resolved into two phases of roughly equal magnitudes with half-times of 3 and 200–400 μs (Ke et al., 1970; Kihara & Chance, 1969; Overfield et al., 1979; Dutton et al., 1975).

The oxidation of cytochrome c_2 by isolated, purified reaction centers from *Rp. sphaeroides* in solution has also been studied (Overfield et al., 1979; Prince et al., 1974). We have recently shown that biphasic oxidation kinetics in solution arise from different modes of interaction between the cytochrome and reaction center (Overfield et al., 1979). The fast phase, seen only at high concentrations of reactants, was first order with a half-time of a few microseconds, and was attributed to donation from a bound cytochrome c_2. Fast, first-order kinetics were also reported by Ke et al. (1970) for the oxidation of mammalian cytochrome *c* by reaction centers. The slow phase, which dominated the kinetics at low concentrations, was second order, as first described by Prince et al. (1974). At high concentrations, however, the slow phase reached a pseudo-first-order limit with a half-time of 200–400 μs (Overfield et al., 1979). Biphasic kinetics of cytochrome c_2 oxidation have also been reported using reaction centers incorporated into crude soybean lipid vesicles (Dutton et al., 1976). Although biphasic kinetics are observed under these widely varying conditions, we have found that their appearance in solution is dependent on ionic strength, due to the ionic nature of the binding process (Overfield et al., 1979). We have also reported that biphasic kinetics are not seen at high ionic strength with reaction centers incorporated into neutral vesicles of phosphatidylcholine (Overfield & Wraight, 1978). Using well-defined, unilamellar bilayer vesicles, we have, therefore, studied the influence that the lipid environment of the reaction center and the ionic composition of the solution have on the oxidation of cytochrome c_2. In this paper we describe the reaction for phosphatidylcholine vesicles (zero net surface charge).

Materials and Methods

Reaction centers were prepared from *Rp. sphaeroides*, strain R26, as described previously (Overfield et al., 1979; Wraight, 1979). Cytochrome c_2 was isolated and purified by the method of Bartsch (1971).

Egg phosphatidylcholine (egg PC)[1] was purified from egg yolks by the method of Singleton et al. (1965) and stored under acetone and nitrogen at −20 °C until used. Dimyristoylphosphatidylcholine (DMPC) was obtained from Sigma (Sigma Chemical Co., St. Louis, MO). Dioleoylphosphatidylcholine (DOPC) was obtained from Sigma and from Supelco, Inc. (Bellafonte, PA).

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[1] Abbreviations used: [RC_0], concentration of reaction centers accessible to rereduction by external cytochrome; LDAO, lauryldimethylamine *N*-oxide (Ammonyx LO); PC, phosphatidylcholine (diacyl-*sn*-glycero-3-phosphocholine); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine (diacyl-*sn*-glycero-3-phosphoserine); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Caps, cyclohexylaminepropanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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Unilamellar bilayer vesicles were made by the method of Brunner et al. (1976), which was extended to include incorporation of reaction centers. The lipid was dried with anhydrous diethyl ether under nitrogen and vacuum in a "Rotovap" (Büchi) and weighed. Sodium cholate (Sigma) was stored in powder form at −20 °C and freshly prepared as a 0.4 M solution. The lipid was solubilized in a flask with a buffer solution containing 0.1 M NaCl, 10 mM Tris, pH 8.0, and 40 mM cholate. After a clear mixture was obtained by rotating the flask with a few glass beads, it was cooled on ice. Reaction centers were added to a maximum of 10% by volume. The protein to lipid ratio typically used was about 0.08 by weight. This mixture was passed on a Sephadex G-75 column (2.5 × 35 cm) that was preequilibrated with 0.1 M NaCl and 10 mM Tris, pH 8.0 at 3 °C. The vesicles, which ran in the void volume, were eluted in twice the applied volume and were ready for use, or could be dialyzed to remove the salt. For incorporation into DMPC vesicles, which undergo a phase transition at about 24 °C, the column was run at 37 °C. The first run produced a broad reaction center band, which was concentrated by ultrafiltration to the original volume. Half the original amount of cholate was added, and the clear mixture was run on Sephadex G-75 a second time.

Detergent removal during vesicle preparation was determined with ^{14}C-labeled LDAO, prepared according to Applebury et al. (1974). Vesicle samples were counted in a Nuclear-Chicago Model II liquid scintillation system using 10 mL of a toluene–Triton X-100 cocktail (15:8) and 1 mL of aqueous vesicle suspension. Reaction centers at OD_{800} = 1 quenched 10% of the counts.

Differential scanning calorimetry was performed on a Perkin-Elmer Model II calorimeter. Samples were run under helium gas. The temperature was raised at 5 °C/min and the sensitivity set at 0.2 mcal/s.

The kinetics of the electron-transfer reactions were measured with a dual beam, unchopped spectrophotometer. The probe light of two wavelengths crossed horizontally in a 1 × 1 cm cuvette and was measured with S-20 photomultipliers. A dye laser (Phase-R DL-1100), operating at 590 nm with rhodamine 6G dye, provided a short, single turnover flash (400-ns pulse width) that was near saturating (>95%) for reaction center activation. The laser flash was delivered through the bottom of the cuvette, and the photomultipliers were protected with Corning 4-96 or interference filters. Temperature control was achieved by holding the cuvette in a massive brass block channeled for circulation. The temperature in the cuvette was measured with a copper–constantan thermocouple referenced to ice–water and, unless stated otherwise, was 25 °C. The sample was kept anaerobic by flushing continuously with high-purity argon. The pH and the ambient redox potential (E_h) in the cuvette were monitored with glass and platinum electrodes referenced to a calomel electrode, and were maintained.

Typical experimental protocol involved deoxygenating 5 mL of buffer solution for 20 min. Reaction centers were introduced at about 0.5 μM. 1,4-Naphthoquinone was then added to 20 μM in 10 μL of ethanol and equilibrated for 10 min. Minute additions of dithionite were used to adjust the ambient redox potential to about +120 mV. The naphthoquinone maintained the redox potential and provided a slow cyclic pathway ($t_{1/2} \simeq 1$ s) for electron flow from the reaction center acceptor complex to the cytochrome. Four minutes dark time between flashes was allowed for the system to return to equilibrium.

The back reaction was studied in single beam mode at 540 nm, which is isosbestic for cytochrome oxidation. The most commonly used wavelength pair for cytochrome changes was 550–540 nm, where a small P^+ signal persists and must be subtracted. The pair 520–480 allowed virtually complete P^+ cancellation and was also used, especially for studying the changes with very small cytochrome concentrations.

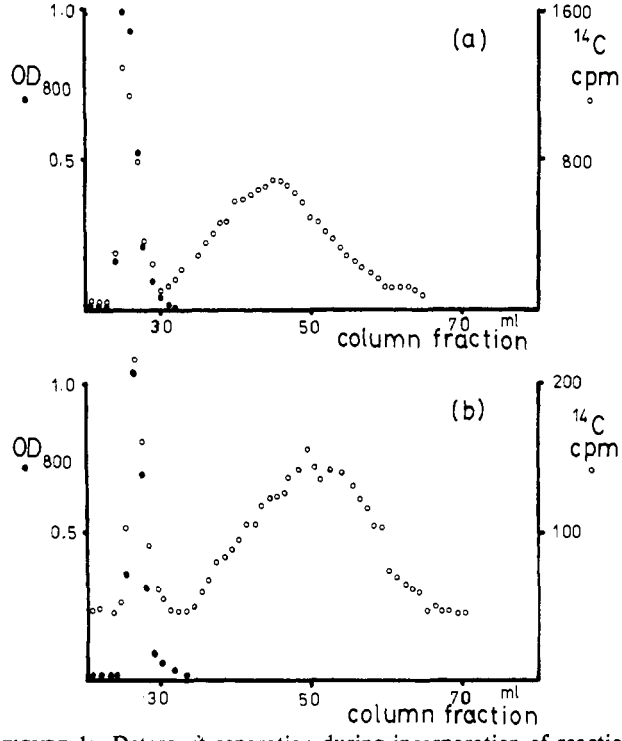

FIGURE 1: Detergent separation during incorporation of reaction centers into egg phosphatidylcholine vesicles. Fractions (1 mL) from a G-75 Sephadex column were analyzed for reaction centers by absorbance at 800 nm (●) and for LDAO by ^{14}C scintillation counting (○). Column temperature, 3 °C.

Results

Detergent Removal and Vesicle Characterization. Effective removal of the cholate in the cholate–Sephadex procedure for making unilamellar bilayer lipid vesicles was established by Brunner et al. (1976). In order to test the effectiveness of the procedure at removing the detergent (lauryldimethylamine *N*-oxide (LDAO)) used in the reaction center isolation procedure, reaction centers were purified with LDAO labeled in the α position with ^{14}C. Column fractions from the Sephadex G-75 column were assayed for reaction center and ^{14}C content. As Figure 1a shows, 80% of the LDAO was removed from the reaction center–vesicle fractions in a single pass. The same percentage was removed even after the initial detergent level in the reaction center was lowered fivefold by ammonium sulfate precipitation followed by exhaustive dialysis (Figure 1b). This suggests that a phase separation of micellar forms occurs on the Sephadex column and that detergent removal is achieved by partitioning of these smaller aggregates into the beads, as suggested for cholate alone (Brunner et al., 1976). Typically, the LDAO content of the vesicles was estimated to be 3–5 molecules per reaction center. Freeze-fracture electron microscopy of the reaction center containing vesicles showed them to be unilamellar with a diameter of 550 ± 180 Å. An effective molecular weight of 9 × 10^6 was used for estimating the number of reaction centers per vesicle.

Following a saturating flash, reaction centers incorporated into neutral phosphatidylcholine vesicles were only 50–60% rereduced on a rapid time scale by exogenously added cytochrome. This was measured directly by resolution of the P^+ signal at 540 nm, an isosbestic wavelength for cytochrome c_2

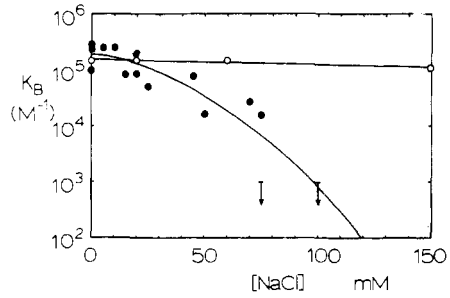

FIGURE 2: Salt dependence of apparent cytochrome c_2–reaction center binding. Reaction centers in egg PC vesicles (●): $[RC_0] = 0.39\ \mu M$, [cytochrome c_2] = 0.96 μM. Reaction centers in egg PS vesicles (○): $[RC_0] = 0.41\ \mu M$, [cytochrome c_2] = 1.1 μM. Buffer: 10 mM Tris, pH 8.0, and 20 μM naphthoquinone. K_B was calculated from the amplitude of the fast phase of cytochrome c_2 oxidation. The arrows at 80 and 100 mM indicate that values were below the limit of detection of the fast phase, equivalent to $K_B \simeq 10^3\ M^{-1}$.

oxidation. When preformed reaction center-containing egg PC vesicles were sonicated in the presence of a high (50 μM) cytochrome c_2 concentration and exogenous cytochrome was removed by passage on a Bio-Gel P-300 column, half the reaction centers were again rapidly rereduced. Addition of excess exogenous cytochrome to these samples resulted in rapid rereduction of all the reaction centers. Thus, it appears that the reaction centers were incorporated randomly in the neutral vesicles with half of the cytochrome reaction sites facing inward. In all subsequent experiments, cytochromes c and c_2 were only added externally. The fraction of reaction centers facing inward, which was kinetically invisible in the dual beam mode, was discounted in the rate determinations.

Overall Kinetics of Cytochrome c_2 Oxidation. In the presence of 0.1 M NaCl, the oxidation of cytochrome c_2 by reaction centers incorporated into phosphatidylcholine vesicles was monophasic even at the highest concentrations of reactants used. We have previously shown that, under conditions eliciting biphasic kinetics, the fast phase ($t_{1/2} \simeq 1$–4 μs) is indicative of a binding equilibrium between cytochrome c_2 and reaction centers (Overfield et al., 1979). Binding constants estimated from the proportion of fast phase agreed well with direct estimates by equilibrium chromatography (Overfield et al., 1979) and equilibrium dialysis (Rosen et al., 1979). The lack of any detectable fast phase using phosphatidylcholine vesicles at high ionic strength, therefore, suggests no significant binding, and we could detect none using equilibrium chromatography.

In the absence of salt, biphasic cytochrome oxidation kinetics were observed, and a fast phase ($t_{1/2} \simeq 2\ \mu s$) was seen with an apparent equilibrium binding constant, K_B, of $0.8–3 \times 10^5\ M^{-1}$. In low salt, therefore, the kinetics with phosphatidylcholine vesicles were very similar to those observed for free reaction centers (Overfield et al., 1979). However, K_B for reaction centers in PC vesicles was very sensitive to ionic strength (Figure 2). Above 80 mM NaCl, K_B had decreased by at least 100-fold, and the fast phase was no longer detectable. By contrast, K_B for reaction centers in phosphatidylserine vesicles ($0.7–2 \times 10^5\ M^{-1}$ in low salt) was insensitive to salt concentrations up to 150 mM (Figure 2). For reaction centers in free solution, K_B was only slightly sensitive to the ionic strength, decreasing by a factor of 2 at 100 mM NaCl (not shown).

The rest of the work reported here addresses only the slow phase of cytochrome c_2 oxidation, which, in most cases, was the only detectable component. However, under conditions giving rise to biphasic kinetics (low salt, high concentrations) the slow phase was easily separable from the fast component.

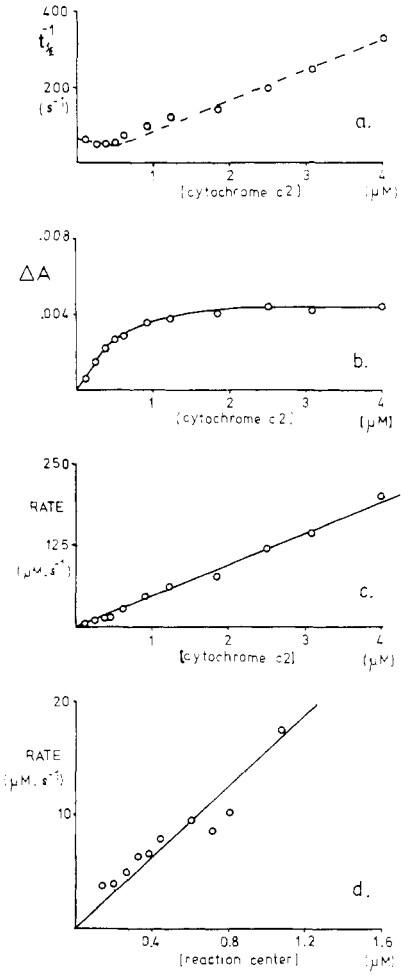

FIGURE 3: Kinetic characteristics of the slow phase of cytochrome c_2 oxidation by reaction centers in egg PC vesicles: buffer, 0.1 M NaCl, 10 mM Tris, pH 8.0, and 20 μM naphthoquinone; (a, b, and c) reaction centers in egg PC vesicles ($[RC_0] = 0.66\ \mu M$) titrated with cytochrome c_2; (d) cytochrome c_2 (0.4 μM) titrated with reaction centers in egg PC vesicles; (a) inverse half-time of cytochrome oxidation; (b) extent of cytochrome oxidation; (c) product of inverse half-time (from a) and extent (from b), i.e., rate of cytochrome oxidation; (d) rate of cytochrome oxidation, as in c.

The Rate Law for the Slow Phase of Cytochrome c_2 Oxidation. The rate law for the slow cytochrome c_2 oxidation kinetics in 0.1 M NaCl was investigated by titrating a fixed concentration of reaction center-containing egg PC vesicles with cytochrome c_2 (Figure 3a–c). The dependence of the inverse half-time on cytochrome concentration was as expected for a second-order reaction (Bashford et al., 1979), exhibiting a minimum at a 1:1 ratio of reactants (Figure 3a). For a simple reaction time course, the product of the inverse half-time (Figure 3a) and the extent of oxidation (Figure 3b) is equal to the initial rate, and Figure 3c shows this to be a linear function of the cytochrome c_2 concentration. Thus, the reaction is first order in cytochrome. A similar plot obtained as a function of reaction center concentration (Figure 3d) shows this dependence also to be first order, confirming the overall second-order character of the reaction.

In some cases, the inverse half-time did not increase as the concentration of cytochrome was decreased below that of reaction centers, but stayed at a constant value. This apparent concentration independence is shown in Figure 4 for reaction centers in DMPC vesicles. It arises from the effect of the back reaction, as an independent pathway for the rereduction of P^+, competing with cytochrome oxidation. The effect is particularly marked near the 1:1 concentration ratio, since the sec-

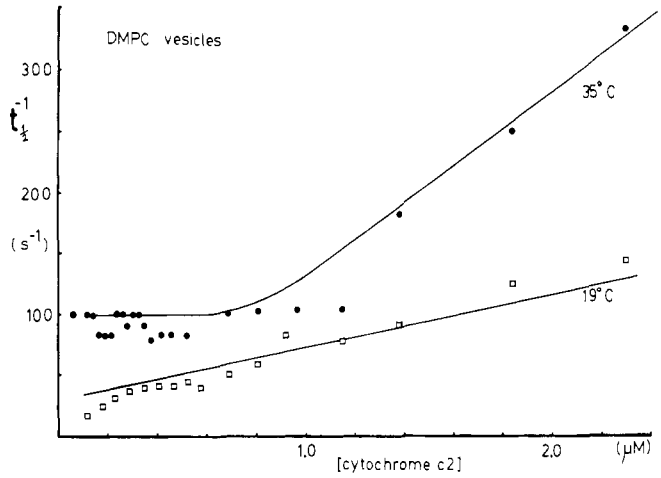

FIGURE 4: Oxidation of cytochrome c_2 by reaction centers in DMPC vesicles. Buffer as in Figure 3. Reaction centers in DMPC vesicles ($[RC_0]$ = 0.8 μM) were titrated with cytochrome c_2. Data points obtained at 35 °C (●) and at 19 °C (□). Lines are fitted curves with bimolecular rate constants of 9×10^7 $M^{-1}\cdot s^{-1}$ (35 °C) and 3.8×10^7 $M^{-1}\cdot s^{-1}$ (19 °C) and a back reaction rate constant of 10 s^{-1} for both.

ond-order kinetics of the cytochrome oxidation are highly nonexponential and the process can be severely truncated. Numerical simulations of competing first- and second-order reaction mechanisms were performed with an IBM continuous system modelling program (CSMP III). The back-reaction half-time varies from 0.1 to 1 s depending on the state of quinone acceptor complex of the reaction center (Wraight, 1979) and on the lipid or detergent environment (Overfield, 1979). Appropriate values were obtained for each preparation and in most cases were in the range of 0.12–0.18 s. For the preparation in Figure 4, the back-reaction half-time was 0.1 s. The fitted curves are shown. It is particularly noteworthy that when the second-order process is not significantly faster than the first-order one, the depenence of the half-time on reactant concentration can appear essentially linear, as for the DMPC vesicles at 19 °C. The general effect is to cause an overestimation of the second-order rate constant when determined from the 1:1 concentration point.

Ionic Strength and pH Dependence. Increasing the ionic strength of the solution with sodium chloride retarded the slow phase of oxidation of cytochrome c_2 by reaction centers, both in egg phosphatidylcholine vesicles (Figure 5a) and in solution at low concentrations (<0.1%) of LDAO (Figure 5b). Since no quantitative interpretation of the ionic strength dependence will be attempted, the data have not been corrected for the effect of the competing back reaction. For cytochrome c_2 the correction is generally small and does not affect the overall shape of the curve. The limiting values for the observed rate constant for cytochrome c_2 oxidation at low ionic strength were 4×10^8 $M^{-1}\cdot s^{-1}$ for egg phosphatidylcholine vesicles, and 7×10^8 $M^{-1}\cdot s^{-1}$ in solution.

The same ionic strength dependence was observed for the oxidation of horse heart cytochrome *c* by reaction centers (also Figure 5), which exhibited a similar second-order rate law to that of cytochrome c_2. The positive curvature at salt concentrations greater than 50 mM is partly due to the effect of the back reaction on the cytochrome oxidation kinetics. The limiting values for the observed rate constants at low ionic strength were 1×10^9 $M^{-1}\cdot s^{-1}$ for reaction centers in egg phosphatidylcholine vesicles, and 6×10^9 $M^{-1}\cdot s^{-1}$ in solution.

The oxidation of cytochrome c_2 by reaction centers in egg phosphatidylcholine vesicles was pH dependent and was reversibly retarded above pH 9 (Figure 6).

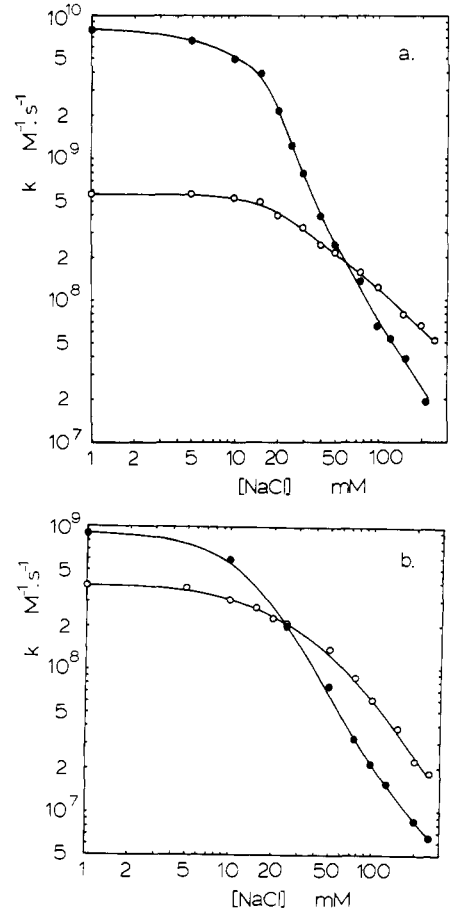

FIGURE 5: Salt dependence of cytochrome *c* and c_2 oxidation by reaction centers. Buffer as in Figure 2. (a) Reaction centers (0.5 μM) in solution (<0.1% LDAO): (●) cytochrome *c* (0.5 μM); (○) cytochrome c_2 (0.5 μM). (b) Reaction centers in egg PC vesicles ($[RC_0]$ = 0.65 μM): (●) cytochrome *c* (1.3 μM); (○) cytochrome c_2 (1.3 μM).

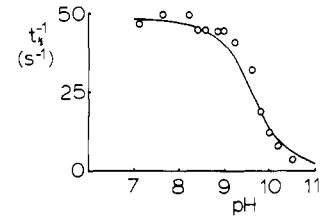

FIGURE 6: pH dependence of cytochrome c_2 oxidation by reaction centers in egg PC vesicles. Buffer: 0.1 M NaCl, 20 μM naphthoquinone, and 3 mM each of Mes, Mops, Caps, Tris, and Tricine, $[RC_0]$ = 0.7 μM, [cytochrome c_2] = 1.1 μM. Reaction centers were reconstituted with excess ubiquinone-10 to give a back reaction half-time of 0.7 s at pH 7.0. The curve is drawn for a single titratable group with p*K* = 9.6.

Temperature Dependence. The temperature dependence of cytochrome c_2 oxidation was measured for reaction centers in phosphatidylcholine vesicles of various types and compared with that for reaction centers in low LDAO solution (Figure 7).

The data were determined with excess cytochrome and are not corrected for distortion by the back reaction which was not marked (<20%). A single activation energy of 8.0 ± 0.4 $kac\cdot mol^{-1}$ clearly fits the data, over most of the temperature range, for reaction centers either in solution or incorporated into vesicles of various phosphatidylcholines. This observation is especially significant for the dimyristoylphosphatidylcholine vesicles, which underwent a phase transition at 24 °C. The heat of melting of this process, measured by differential

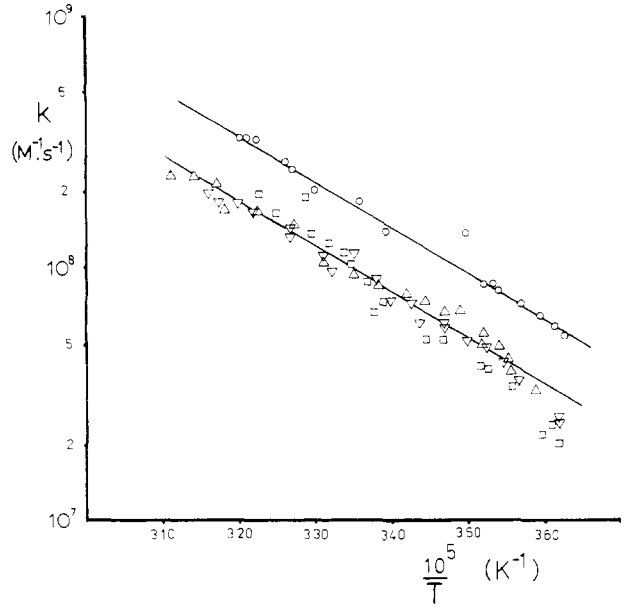

FIGURE 7: Temperature dependence of cytochrome c_2 oxidation by reaction centers. Buffer as in Figure 3, $[RC_0] = 0.5\ \mu M$, [cytochrome c_2] = 1.0 μM: (○) reaction centers in solution (0.1% LDAO); (△) reaction centers in egg PC vesicles; (□) reaction centers in DMPC vesicles; (▽) reaction centers in DOPC vesicles.

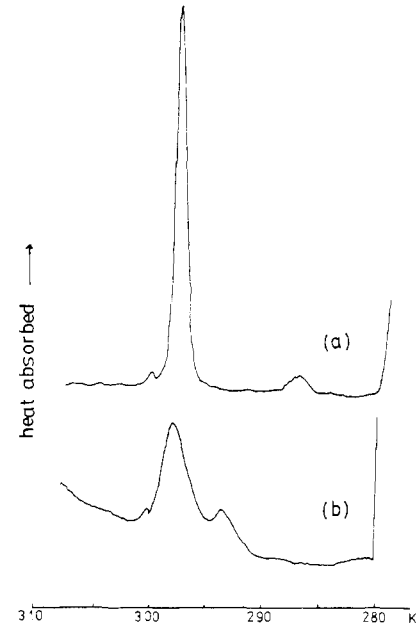

FIGURE 8: Thermotropic phase transitions of DMPC vesicles. Heat of melting was measured by differential scanning calorimetry. Buffer was 0.1 M NaCl and 10 mM Tris, pH 8.0; scan rate, 5 °C/min; vertical sensitivity, 0.2 mcal/s; (a) sonicated DMPC vesicles; (b) reaction center-containing DMPC vesicles from Sephadex G-75 column (5% protein w/w). Both samples were 20 mg of lipid/mL.

scanning calorimetry, is shown in Figure 8. After incorporation of reaction centers, the main transition was still readily observable at 24 °C, although somewhat broadened. However, the premelting transition was shifted considerably from 13 to 20 °C.

The major difference in the temperature dependence of the reaction on the neutral membrane compared with that in solution was in the Arrhenius preexponential factor, which was $10^{13}\ M^{-1}\cdot s^{-1}$ in solution and $6 \times 10^{12}\ M^{-1}\cdot s^{-1}$ for the vesicles.

Discussion

The oxidation of cytochromes *c* and c_2 by reaction centers in neutral vesicles is predominantly second order, and the kinetics are very similar to the slow phase of oxidation by reaction centers in solution (Overfield et al., 1979). At low ionic strength, the true second-order rate constants for the reaction in solution are $7 \times 10^8\ M^{-1}\cdot s^{-1}$ for cytochrome c_2 and $6 \times 10^9\ M^{-1}\cdot s^{-1}$ for cytochrome *c*. These values approach the diffusion limit given by the von Smoluchowski equation. However, the activation energy observed for cytochrome c_2, 8 kcal·mol^{-1}, is somewhat higher than expected for a diffusion-controlled reaction. Possibly this value reflects a barrier encountered in the close approach of the cytochrome and reaction center.

In an Arrhenius treatment of the rate constant for the cytochrome c_2 oxidation, the 40% reduction on going from solution to the neutral membrane resides in the preexponential factor. This parameter reflects the encounter frequency which is apparently reduced when the reaction is in a neutral membrane compared to when it is in free solution, probably for two reasons. Since the reaction center diffusion constant is greatly diminished in the membrane as compared to in solution, its contribution to the net diffusion constant is lost. The diffusion constant for cytochrome *c*, in solution, is about $10^{-6}\ cm^2\cdot s^{-1}$. If the diffusion constant for reaction centers were lowered from a likely value in solution of $0.5 \times 10^{-6}\ cm^2\cdot s^{-1}$ to a much lower value in the membrane, a 40% reduction in the association frequency would result. In addition, limitation of the approach to a hemisphere could also cause a small reduction in the association frequency, for steric reasons. It is interesting to note that the supposition that a reflecting membrane would cause increased association rates due to "quasi-two-dimensional diffusion" of the reactants (Berg & Purcell, 1977) was not borne out.

The slowing of the oxidation of both cytochrome *c* and c_2 with increasing ionic strength was previously noted in high detergent solution (Prince et al., 1974) but not well understood. The isoelectric points (p*I*) of the species involved are 6.1 for the reaction center (Prince et al., 1974), 5.5 for cytochrome c_2 (Meyer, 1970), and 10.65 for cytochrome *c* (Ke et al., 1970). The ionic strength dependence indicates that although the two cytochromes have opposite net charges at pH 8.0, both act as positive species in their interaction with the negative reaction center. The effective charge at the interaction site of reaction centers from *Rhodospirillum rubrum* has been estimated at about −4.6 (Rickle & Cusanovich, 1979). A positive charge distribution at the heme cleft face, arising from about 11 lysine residues, is common to both cytochromes (Takono et al., 1973; Salemme et al., 1973). Since it is now widely believed that the mechanism of oxidation (and reduction) of cytochrome *c* involves electron transfer from the heme at the heme cleft, it appears that the *local* charge distribution controls the ionic interaction of both cytochromes with the reaction center. The effect of pH on the oxidation of cytochrome c_2 is also consistent with this view. Cytochrome *c* is known to undergo a gross alteration in properties and structure with a p*K* at pH 9.3 (Davis et al., 1974), but the similar transition in cytochrome c_2 occurs at pH 7.9 (Pettigrew et al., 1975). Thus, the decline in oxidation rate above pH 9 is not associated with this change. Although we cannot ascribe the observed pH dependence to effects on the cytochrome rather than the reaction center, the lysine charges of the heme cleft face are attractive candidates for titration in the range of pH 9–10. Furthermore, cytochrome c_2 from *R. rubrum* exhibits a similar pH dependence, with an apparent p*K* of 9.6, for oxidation by either reaction centers or ferricyanide (Rickle & Cusanovich, 1979).

For reasons which are not clear, Prince et al. (1974) did not observe an effect of pH on the oxidation of cytochrome c_2 by

reaction centers solubilized in high detergent, although a decrease in rate at high pH was seen for cytochrome *c*. It is possible that the high concentration of LDAO (2%) which they used affected this property of the reaction. At concentrations as low as 0.2%, LDAO has been shown to drastically alter the redox midpoint potential of cytochrome c_2 (Rickle & Cusanovich, 1979), and therefore, it probably alters its structure. We have found that the rate of cytochrome oxidation is slowed by LDAO above 0.2%. At low LDAO concentration (<0.1%), we have observed a pH dependence very similar to that described here for reaction centers in neutral vesicles (unpublished observations).

The temperature dependence of the oxidation of cytochrome c_2 by reaction centers incorporated into phosphatidylcholine vesicles was indistinguishable for all phosphatidylcholines investigated. This indicates that the membrane viscosity was not a factor in the reaction rate. This is especially borne out in the case of dimyristoylphosphatidylcholine vesicles, which were shown to undergo a thermotropic phase transition at 24 °C. The viscosity is much higher in the gel state than in the fluid state (Cogan et al., 1973), but no break in the rate was seen.

It should be noted that the marked differences in the concentration dependence of the kinetics at temperatures above and below the phase transition for DMPC, shown in Figure 4, are attributable to the effect of the back reaction and do not represent a qualitative change in response to the lipid phase transition. Nevertheless, we have not attempted an extensive analysis with corrections for competition from the back reaction and cannot rule out some small influence of the physical state of the lipid.

Thus, it appears that the encounter processes leading to electron donation from the cytochrome to the reaction center are not significantly affected by the hydrophobic milieu of the lipid bilayer. The absence, at high ionic strength, of a fast phase of cytochrome c_2 oxidation by reaction centers in phosphatidylcholine vesicles reflects a weaker binding of cytochrome to reaction center, compared to the solutional case. As we show in the accompanying paper (Overfield & Wraight, 1980), the appearance of fast kinetics is particularly sensitive to the surface charge of the vesicle and, hence, to the nature of the phospholipid head group. However, in the neutral (zwitterionic) membrane, we may conclude that electron transfer from cytochrome to reaction center is dominated by ionic interactions between the proteins themselves.

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