Oxidation of Cytochromes *c* and c_2 by Bacterial Photosynthetic Reaction Centers in Phospholipid Vesicles. 2. Studies with Negative Membranes†

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ABSTRACT: The oxidation of cytochromes *c* and c_2 by reaction centers from *Rhodopseudomonas sphaeroides*, incorporated into unilamellar phospholipid vesicles, was studied as a function of the negative surface charge of the membrane and ionic conditions of the medium. The overall kinetics of oxidation of cytochrome c_2 were biphasic and qualitatively similar to those for neutral vesicles in low salt. However, the fast phase, indicative of a binding equilibrium between cytochrome and reaction centers prior to flash activation, was independent of salt concentration. For phosphatidylserine vesicles the apparent binding constant, K_B, was 0.7–2 × 10^5 M^{-1}. The binding enthalpy, ΔH_B, was estimated at −7 kcal·mol^{-1}. In 0.1 M NaCl, or lower, the concentration dependences of the rate of the slow phase of cytochrome c_2 oxidation indicated that the cytochrome was restricted, on the time scale of the reaction, to the surface of a single phosphatidylserine (PS) vesicle and encountered the reaction centers by two-dimensional diffusion. In low salt medium, the association with the membrane was sufficiently tight that lateral diffusion of the cytochrome was retarded. Increasing the NaCl concentration up to 0.1 M enhanced the rate of reaction, in marked contrast to the behavior in free solution or with neutral vesicles. In the absence of NaCl, the lateral diffusion coefficients for cytochromes *c* and c_2 were estimated at 0.6–2 × 10^{-10} and 0.3–1 × 10^{-9} cm^2·s^{-1}, respectively. In 0.1 M NaCl, the values were 0.3–1 × 10^{-9} and 0.3–1 × 10^{-8} cm^2·s^{-1}, respectively. Thus, under nonoptimal conditions, the diffusion coefficient approached values more often associated with integral proteins. The effective mobility of cytochrome c_2, measured in subsaturating flashes, was governed both by the diffusive process and the rate of dissociation from an unactivated reaction center. With PS vesicles, in 0.1 M NaCl, the apparent activation energy for oxidation was 8 kcal·mol^{-1}, and the dissociation rate was slow, such that the cytochrome was effectively immobile. In low salt, at low temperature, similar behavior was observed, but at high temperature the rate of oxidation exhibited negligible activation energy, and the dissociation rate was sufficient to allow mobility of the cytochrome, despite a smaller diffusion coefficient on the membrane in low salt. The transition from effective to ineffective cytochrome mobility was abrupt, and the temperature at which it occurred was a sensitive function of salt concentration. It is concluded that oxidation of cytochrome c_2 by reaction centers in PS vesicles can be characterized by two-dimensional diffusion, which, under appropriate conditions, can give rise to either an acceleration or a retardation of the reaction.

Consideration of the effects of dimensionality on reaction rates in membrane systems has been largely theoretical in nature (Adam & Delbrück, 1968; Richter & Eigen, 1974; Berg & Purcell, 1977). Nevertheless, two-dimensional diffusion has long been suggested to be possibly significant in mitochondrial electron transport (Bücher, 1953) and has been indicated to occur during the oxidation of cytochrome *c* by cytochrome oxidase–lipid aggregates (Roberts & Hess, 1977). In the preceding paper (Overfield & Wraight, 1980) we have shown that the oxidation of cytochrome *c* and c_2 by reaction centers, isolated from *Rhodopseudomonas sphaeroides* and incorporated into neutral (zwitterionic) phospholipid vesicles, is very similar to that by reaction centers in solution, with no indication of restricted diffusion of the cytochrome. However, we show here that when reaction centers are incorporated into vesicles with a negative surface charge, the reaction is profoundly affected and is rate limited by the two-dimensional diffusion of the cytochrome on the vesicle surface. Acceleration of the reaction rate can result from the restricted dimensionality, as the theoretical studies have suggested, but is limited by a counterbalancing reduction in the diffusion coefficient of the cytochrome.

Materials and Methods

Reaction centers were prepared from *Rp. sphaeroides*, strain R26, as previously described (Overfield et al., 1979), and cytochrome c_2 was isolated by the method of Bartsch (1971). Cytochrome *c* (Type III, horse heart) was obtained from Sigma Chemical Company, St. Louis, MO. Phosphatidylserine (PS)[1] was made from egg phosphatidylcholine (PC) by an enzymatic transesterification of the head group (Confurius and Zwaal, 1977) using cabbage phospholipase D (type I, Sigma). The purified lipid chromatographed to a single spot on silica gel thin-layer plates in acid, neutral, and basic solvent systems (Neilsen, 1975). Egg phosphatidylcholine for the transesterification was purified by the method of Singleton et al. (1965). A crude phosphatidylserine preparation, from Avanti Biochemicals, Inc. (Birmingham, AL), was also used in some experiments.

Phosphatidylserine vesicles containing reaction centers were formed in a manner similar to that employed for egg PC vesicles (Overfield & Wraight, 1980). However, it was necessary to sonicate the lipid–cholate mixture (prior to adding reaction centers) to obtain a clear solution. Phosphatidylserine was dried, weighed, and dispersed with 40 mM sodium cholate,

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[1] Abbreviations used: PS, phosphatidylserine (diacyl-*sn*-glycero-3-phosphoserine); PC, phosphatidylcholine (diacyl-*sn*-glycero-3-phosphocholine); Tns, 2-*p*-toluidinylnaphthalene-6-sulfonate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Caps, cyclohexylaminepropanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

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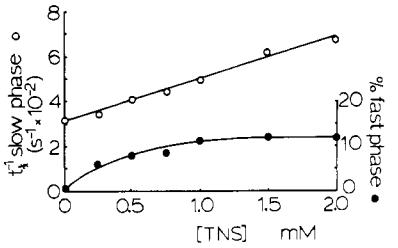

FIGURE 1: The effect of Tns on the oxidation of cytochrome c_2 by reaction centers in egg PC vesicles. Reaction centers (2.2 μM) and cytochrome c_2 (7.2 μM) in 0.1 M NaCl, 10 mM Tris, pH 8.0, and 20 μM 1,4-naphthoquinone.

10 mM NaCl, and 10 mM Tris, pH 8.0. The mixture was sonicated in a water-jacketed vessel, under nitrogen, for 10 min. The temperature was monitored and was not allowed to exceed 30 °C. The clear dispersion was cooled on ice, and reaction centers were added and incubated for 10 min. The detergent–lipid phase separation was carried out on a Sephadex G-75 column preincubated with 10 mM NaCl and 10 mM Tris, pH 8.0, at 3 °C. Electron micrographs of the reaction center-containing vesicles showed them to be 400 ± 100 Å in diameter.

Laser and xenon flash activated kinetics of cytochrome oxidation were measured under pH and redox potential controlled conditions as described in the preceding paper (Overfield & Wraight, 1980). Unless stated otherwise, all measurements were done at 25 °C.

Results

Effect of Adsorption of Negative Charge onto Neutral Phosphatidylcholine Vesicles. The kinetics of cytochrome c_2 oxidation by reaction centers incorporated into egg PC vesicles were monitored at increasing concentrations of toluidinylnaphthalene-6-sulfonate (Tns). This amphiphile has been shown to adsorb to phospholipid vesicles and to impart to them a negative surface charge (McLaughlin & Harary, 1976). In the presence of 0.1 M NaCl, cytochrome c_2 oxidation by reaction centers in PC vesicles exhibits monophasic second-order kinetics (Overfield & Wraight, 1978, 1980). Addition of Tns accelerated this second-order process and induced a faster phase of oxidation (Figure 1). Fast oxidation kinetics for reaction centers in solution have previously been attributed to the formation of a cytochrome–reaction center complex (Overfield et al., 1979). At saturating levels of Tns, the apparent binding constant (K_B) for the formation of this complex was estimated at 2×10^4 M^{-1}, in 0.1 M NaCl.

Kinetics of Cytochrome c_2 Oxidation by Reaction Centers in Phosphatidylserine Vesicles. Tns has a rather low adsorption equilibrium for phospholipid vesicles in aqueous dispersions (McLaughlin & Harary, 1976) and also induced light scattering in our samples. Further experiments on the influence of a negative surface charge on the cytochrome oxidation were, therefore, performed with phosphatidylserine vesicles. Approximately 90% of the reaction centers incorporated into egg PS vesicles were rapidly rereduced by excess, exogenous cytochrome c_2. This may be compared to the 50% obtained for reaction centers in egg PC vesicles (Overfield & Wraight, 1980) and suggests a high degree of orientation in PS vesicles. Electron micrographs showed the vesicles to be apparently intact. A strong orienting effect of negative surface charge has previously been reported, for cytochrome oxidase, by Eytan et al. (1976).

As expected from the effect of Tns on the reaction with PC vesicles, the extent of the fast phase of cytochrome c_2 oxidation was promoted in PS vesicles. With the nanosecond kinetic spectrophotometer of D. DeVault, the fast phase was resolved to a single exponential, with a half-time of 2 μs, which was not noticeably affected by NaCl concentrations up to 0.15 M. This value for the half-time is very similar to that observed in vivo and for reaction centers in solution, although in the latter case some dependence on salt concentration was observed (Overfield et al., 1979). The apparent equilibrium constant for binding of cytochrome c_2 to reaction centers in egg PS vesicles, calculated from the extent of the fast oxidation phase, was $0.7–2 \times 10^5$ M^{-1} in the absence of NaCl and was insensitive to the salt concentration (see Figure 2 of the preceding paper: Overfield & Wraight, 1980).

Direct binding determinations by the method of gel filtration or equilibrium chromatography (Ferguson-Miller et al., 1978) revealed the binding of cytochrome c_2 to the PS vesicles themselves ($K_B \simeq 10^7$ M^{-1} in the absence of NaCl), which was more extensive than that expected for binding to the reaction centers ($K_B = 10^5$ M^{-1}). Binding of the cytochrome c_2 to reaction centers in solution has been demonstrated directly, by equilibrium dialysis (Rosen et al., 1979).

Rate Law for the Slow Phase of Cytochrome c_2 Oxidation. Titration of reaction center-containing egg PS vesicles in 0.1 M NaCl with cytochrome c_2 showed the reaction to be first order in cytochrome (Figure 2). The dependence of the half-time on total cytochrome concentration (Figure 2a) conformed to expectation for an overall second-order reaction (Bashford et al., 1979). In Figure 2a–c, the amount of cytochrome bound to reaction centers was less than 5% of the total throughout the concentration range. However, up to 16% of the reaction centers were complexed before the flash at the highest cytochrome concentration. This can be determined directly from the fraction of fast phase in the cytochrome oxidation kinetics (Figure 2b). The lines drawn in Figures 2a and 2c are, therefore, corrected for the fractions of cytochrome and reaction center complexed before the flash, using $K_B = 10^5$ M^{-1} determined from Figure 2b. The corrected second-order rate constant was $5 \pm 1 \times 10^8$ $M^{-1} \cdot s^{-1}$ at 25 °C in 0.1 M NaCl.

At a fixed cytochrome concentration, the kinetic behavior as a function of the concentration of reaction centers in PS vesicles was unexpected (Figure 2d). At high ratios of cytochrome to reaction centers, the rate increased linearly with concentration, and the reaction was clearly second order overall. However, at low ratios the rate of reaction became independent of reaction center concentration. The line drawn in Figure 2d is corrected for the dark equilibrium binding of cytochrome and reaction center, which accounts for up to 18% of the cytochrome at the highest reaction center concentrations. The onset of concentration independence is clearly not due to complexation of cytochrome with reaction centers.

Ionic Strength and pH Dependences. The ionic strength dependence of cytochrome oxidation by reaction centers in PS vesicles is shown in Figure 3. Compared to the reaction in solution or on PC vesicles (Overfield & Wraight, 1980), the oxidation of both cytochromes c and c_2 at low ionic strength was retarded. As the salt concentration was increased, the rate of reaction accelerated, in marked contrast to the reaction in solution or on PC vesicles. At the maximum, in 0.05–0.1 M NaCl, the rate for cytochrome c_2 oxidation on PS vesicles was actually faster than the corresponding rate on PC vesicles. At still higher salt concentrations, the rates declined in much the same way as observed for the other systems.

At low ionic strength, the extent of oxidation of both cytochromes on egg PS vesicles was also diminished. In the absence of salt, with equimolar concentrations of cytochrome

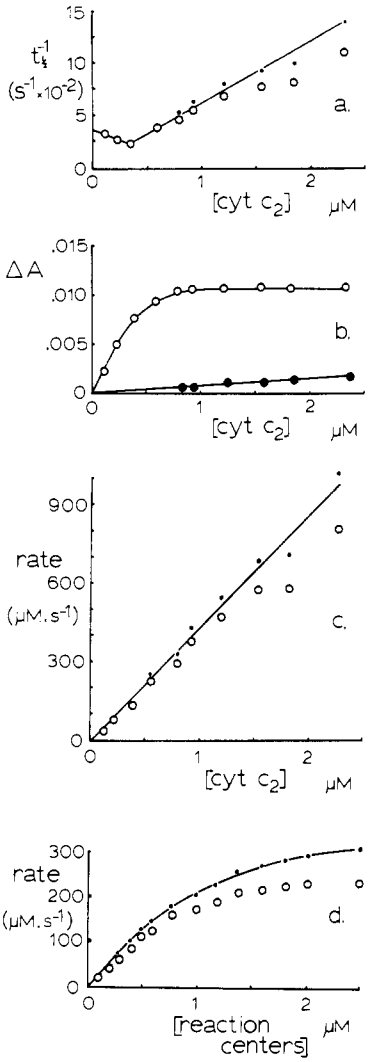

FIGURE 2: Kinetic characteristics of the slow phase of cytochrome c_2 oxidation by reaction centers in egg PS vesicles: (a, b, and c) reaction centers (0.4 μM) titrated with cytochrome c_2; (d) cytochrome c_2 (0.9 μM) titrated with reaction centers in PS vesicles. All abscissas are total concentrations. The lines in a, c, and d are best fit to the data (points) after correction for complexation of cytochrome and reaction centers (see text). In b, both the total extent of cytochrome oxidation (○) and the fast phase (●) are shown. Buffer as in Figure 1.

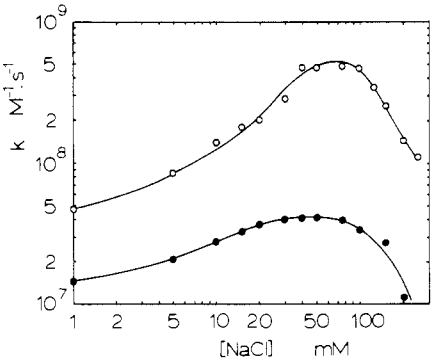

FIGURE 3: Salt dependence of cytochrome c and c_2 oxidation. Reaction centers (1.0 μM) in egg PS vesicles and cytochrome c (●) or c_2 (○) (1.0 μM) in 10 mM Tris, pH 8.0, and 20 μM 1,4-naphthoquinone.

and reaction centers, a saturating, single turnover flash elicited the oxidation of only 60% of the cytochrome c_2 and 10% of the cytochrome c present. At high ionic strength, all the cytochrome was oxidized.

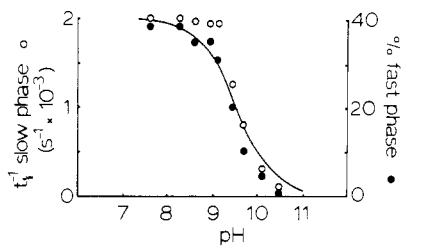

FIGURE 4: pH dependence of the biphasic kinetics of cytochrome c_2 oxidation. Reaction centers (0.4 μM) in crude PS vesicles, cytochrome c_2 (5.6 μM) in 0.1 M NaCl, 20 μM 1,4-naphthoquinone, and 3 mM each Mes, Mops, Tricine, Tris, and Caps; pH was adjusted in the cuvette by additions of HCl and NaOH. The curve is drawn for a single titratable group with pK = 9.6.

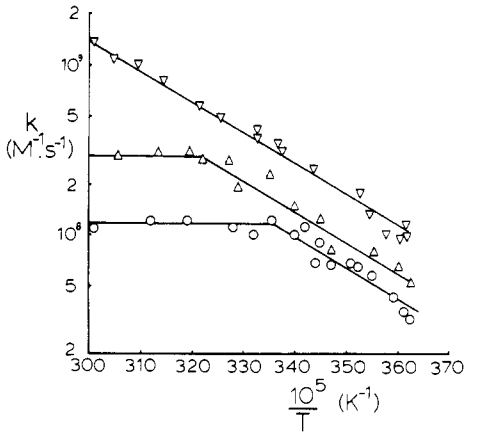

FIGURE 5: Temperature dependence of cytochrome c_2 oxidation by reaction centers in egg PS vesicles. Reaction centers (0.5 μM), cytochrome c_2 (1.0 μM) in 10 mM Tris, pH 8.0, and 20 μM 1,4-naphthoquinone: (○) no further additions; (△) plus 10 mM NaCl; (▽) plus 0.1 M NaCl.

The pH dependence of the rate of the slow phase of cytochrome c_2 oxidation for negatively charged vesicles, in 0.1 M NaCl (Figure 4), was similar to that observed for egg PC vesicles (Overfield & Wraight, 1980). Furthermore, the extent of the fast phase of oxidation, which is promoted by the negative surface charge, fell off as the pH was raised, in parallel with the rate of the slow, second-order phase. This suggests that the titration of groups that govern the association rate also affects the binding of the cytochrome to the reaction center.

Temperature Dependence and Cytochrome Mobility. In 0.1 M NaCl, the rate of oxidation of cytochrome c_2 by reaction centers in egg PS vesicles exhibited an apparent activation energy (E_A) of 8 kcal·mol^{-1} (Figure 5). This is very similar to that seen for PC vesicles and in solution (Overfield & Wraight, 1980). In low salt, however, the reaction rate was temperature independent at high temperature while exhibiting the same 8 kcal·mol^{-1} apparent activation energy at low temperature. The temperature at which this change in behavior occurred shifted to higher values as the salt concentration was increased.

The ability of the cytochrome to visit several reaction centers was determined by the flash saturation technique of van Grondelle et al. (1976). With twice as many reaction centers as cytochromes, the extent of cytochrome oxidation when only half the reaction centers are flash activated indicates the effective mobility of the cytochrome. If the cytochrome is free to diffuse away from an unactivated reaction center to an activated one during the lifetime of the oxidized reaction center (~0.1 s), then the full complement of cytochrome will be oxidized when only half the reaction centers are activated. On

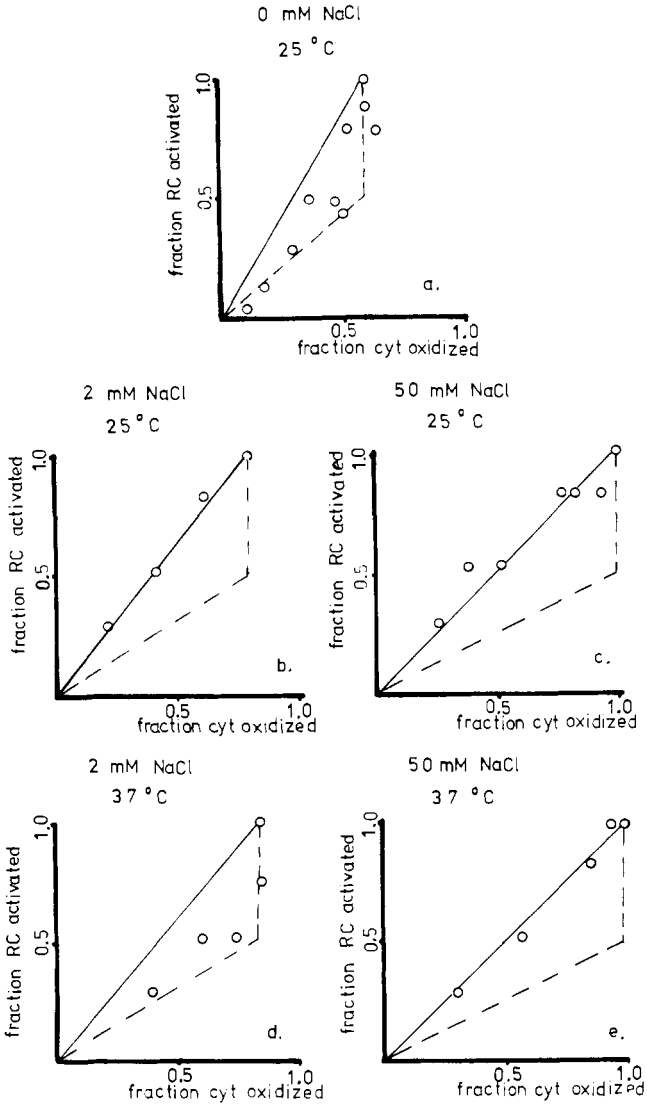

FIGURE 6: Flash saturation curves for cytochrome c_2 oxidation. Reaction center (1.0 μM) in egg PS vesicles, cytochrome c_2 (0.5 μM) in 10 mM Tris, pH 8.0, and 20 M 1,4-naphthoquinone; NaCl added as indicated. The fraction of reaction centers (RC) activated in an attenuated laser flash is plotted against the fraction of cytochrome c_2 oxidized. Solid lines indicate the expected behavior in the limit of no cytochrome mobility; dashed lines indicate the limit of fully effective mobility.

the other hand, if the cytochrome and unactivated reacton center form a long-lived complex, then the proportion of cytochrome oxidized will follow, linearly, the proportion of reaction centers activated. Figure 6 shows that the effective mobility of cytochrome c_2 is dependent on both temperature and ionic strength. Thus, the dissociation of reduced cytochrome c_2 from unactivated reaction centers in PS vesicles was slow at 25 °C in both 2 and 50 mM NaCl, but possibly fast in the absence of salt. However, at 37 °C, the cytochrome was freely mobile in 2 mM NaCl although still restricted in 50 mM NaCl. As noted above, the maximum extent of cytochrome c_2 oxidation was incomplete in low salt.

Discussion

The rate law for cytochrome c_2 oxidation by reaction centers incorporated into egg PC vesicles was found to be second order (Overfield & Wraight, 1980). In egg PS vesicles, however, in 0.1 M NaCl, the reaction appears to be second order only at high cytochrome to reaction center ratios and is first order in cytochrome alone at low ratios. The dependence of the inverse half-time on cytochrome concentration is as expected for a second-order process, indicating that the reaction is collisional. However, the rate of oxidation as a function of reaction center (vesicle) concentration did not continue to accelerate indefinitely but reached a limit at a small excess of reaction centers. This suggests that the diffusion of the cytochrome is limited to a space that contains only a small, fixed number of reaction centers. Since the negatively charged membrane itself has been shown to bind the cytochrome more strongly than the reaction centers, we suggest that, at moderate or low ionic strength, the cytochrome is limited to the surface of the vesicle and that the cytochrome–reaction center association proceeds by two-dimensional diffusion. Confinement of the cytochrome to the surface of the vesicle would result in independence from the reaction center concentration when the concentration of cytochrome became less than that of the vesicles. In the experiment of Figure 2, the average number of reaction centers per vesicle was determined, from the protein to lipid weight ratio and the average vesicle diameter, to be about 3. This is in reasonable agreement with Figure 2d, which shows that the rate with 0.9 μM cytochrome c_2 becomes independent of reaction center concentration between 2 and 3 μM. The agreement is even better when the random distributions of reaction centers and cytochromes are taken into account (see below). Thus, at low cytochrome to reaction center ratios, the effective ratio is fixed by the number of reaction centers per vesicle.

The positively charged heme cleft face of the cytochrome c_2 can interact ionically with negative phospholipids, as shown for mammalian cytochrome c (Dutton et al., 1970; Kimelberg et al., 1970; Vanderkooi et al., 1973; Birrell & Griffith, 1976). For cytochrome c, the oxidized form binds more strongly than the reduced form, causing a small drop in the redox midpoint potential, E_m. This effect is most pronounced in low salt, supporting the ionic nature of the interaction (Dutton et al., 1970; Kimelberg et al., 1970). The same is true for cytochrome c_2 (unpublished observations; J. Pachence and P. L. Dutton, personal communication). The lowered E_m in low salt makes the oxidation reaction more thermodynamically favorable and is unlikely to contribute significantly to the kinetic changes observed, although it is probably reflecting the same underlying phenomena, i.e., alterations in ionic binding interactions. In Figure 3, the retarded oxidation rate in low salt may indicate that the interaction between the positive heme cleft face and the negative membrane is sufficiently strong to severely limit the rate of diffusion and hence the rate of reaction. As the salt concentration is raised, mobile counterions shield the electrostatic interaction and allow the cyctochrome to diffuse more rapidly, although it is still largely restricted to the surface of the vesicle. At relatively high ionic strength (greater than 0.1 M NaCl) the shielding becomes so effective that the association with the negative membrane fails, and the cytochrome becomes free in solution. The rate then falls, as it does in solution and in PC vesicles (Overfield & Wraight, 1980), because the binding regions (positive heme cleft, negative reaction center) are oppositely charged.

Under conditions where the reaction is diffusion controlled, the probability of reaction on an encounter is unity, and we may equate the half-time of cytochrome oxidation with "the time to capture" (T) of Adam & Delbrück (1968), who first considered the effects of dimensionality on biological reactions. Using their approximate relationship for two dimensions (see Appendix)

$$T = b^2/D_2$$

where b is the space size (in cm),[2] we may calculate D_2, the

net two-dimensional diffusion coefficient for the cytochrome–reaction center encounter. For three reaction centers per vesicle, in low salt and with equimolar amounts of cytochrome and reaction centers, the half-times for cytochrome oxidation can be obtained from the second-order rate constants in Figure 3. With the vesicle diameter (400 Å) as an upper limit to the space size,[2] the calculated diffusion constants are 10^{-9} cm^2·s^{-1} for cytochrome c_2 and 2×10^{-10} cm^2·s^{-1} for cytochrome c. Using an average space size for three reaction centers per vesicle (230 Å) gives even lower values of 3×10^{-10} and 6×10^{-11} cm^2·c^{-1}, respectively, for cytochrome c_2 and c. As the salt concentration is raised, these values increase at least as fast as the ks in Figure 3. Thus, in 0.1 M NaCl, probable values for the diffusion coefficients are $0.3–1 \times 10^{-8}$ cm^2·s^{-1} for cytochrome c_2 and $0.3–1 \times 10^{-9}$ cm^2·s^{-1} for cytochrome c.

In previous discussions of surface diffusion of peripheral proteins, diffusion coefficients have generally been assumed to lie between the limits of isotropic diffusion in free solution (about 10^{-6} cm^2·s^{-1}) and those for lipids in a fluid membrane (10^{-8} cm^2·s^{-1}). Our calculations show that only under optimal conditions does the lateral mobility of cytochrome c_2 on a negative membrane approach the lower of these two values and that, at low ionic strength, the calculated diffusion coefficient is closer to estimates for integral proteins (Frye & Edidin, 1970; Poo & Cone, 1974). Cytochrome c has been shown to interact quite strongly with the head groups of negative phospholipids so as to cause clustering and phase separation of the lipids (Birrell & Griffith, 1976). It may be, therefore, that lateral diffusion of the cytochrome is limited by the coordinated diffusion of a significant cluster of lipids, yielding a much smaller diffusion coefficient than for single lipids.

In the flash saturation experiments, incomplete oxidation of the cytochromes was observed at low ionic strength. If reaction centers and cytochromes are distributed randomly and independently over the vesicles, their distributions can be described by Poisson's equations.[3] With an average of 3 reaction centers and 1.5 cytochromes per vesicle, approximately 40% of the cytochrome will fail to be oxidized for lack of an accessible reaction center. This is close to the figure observed for cytochrome c_2, in the absence of salt, when tight associations of the cytochrome with a single vesicle may be expected. For cytochrome c, considerably less than 60% goes oxidized at low ionic strength, and additional factors appear to be involved. The very low diffusion coefficient estimated for cytochrome c at low ionic strength (6×10^{-11} cm^2·s^{-1}) could account for this additional loss of oxidation. Not only must the diffusional approach be successful during the lifetime of an activated reaction center, but theoretical analyses of two-dimensional diffusion processes have shown that the apparent rate constant declines to zero with time (Noyes, 1961; Naqvi, 1974), an effect which can become noticeable for very slow diffusion.

In nonsaturating flashes, effective mobility of the cytochrome requires both adequate diffusion and rapid disengagement should the cytochrome first encounter an unactivated reaction center. We have previously shown that dissociation of reduced cytochrome c_2 and unactivated reaction centers (reduced primary donor, P), in solution, is slow (≤ 10 s^{-1}) and restricts the apparent mobility of the cytochrome regardless of ionic strength. However, the dissociation of reduced cytochrome c_2 and activated reaction centers (oxidized primary donor, P^+) appears to be rapid ($\sim 10^3$ s^{-1}). The association rate constants are similarly affected (Overfield et al., 1979). In the present work on reaction centers in PS vesicles, the flash saturation study (Figure 6) shows that the dissociation of reduced cytochrome c_2 and unactivated reaction centers was slow in 0.1 M NaCl. In low salt, however, above a critical temperature, the cytochrome was effectively mobile despite the lower diffusion coefficient. Thus, fast dissociation from unactivated reaction centers is favored under these conditions. It should be noted that cytochrome oxidation is still incomplete in low salt. Thus the flash saturation technique defines "effective mobility" between reaction centers on a given vesicle but does not imply rapid redistribution between vesicles. Conversely, in high salt, the cytochrome dissociates slowly from unactivated reaction centers but redistributes sufficiently rapidly to allow complete oxidation in saturating flashes.

It is possible that the same reaction center configuration that gives rise to fast dissociation from the oxidized (P^+) state in solution is stabilized in both the oxidized and reduced states in the negative membrane at low ionic strength and high temperatures. Similar experiments on cytochrome c_2 oxidation in chromatophores from *Rp. sphaeroides* have indicated the cytochrome to be effectively immobile (Prince et al., 1978). It is noteworthy, however, that chromatophores are normally prepared and used in high salt medium, while in the intact cell the cytochrome c_2 is in the periplasmic space, in a low salt (freshwater) environment.

The dual modes of cytochrome mobility are closely correlated with the overall temperature dependence of the reaction kinetics (Figure 5). Thus, at high ionic strength, cytochrome oxidation was an activated process, with $E_A \simeq 8$ kcal·mol^{-1}. This value is very similar to that observed for the reaction in free solution or with neutral vesicles. At low ionic strength the rate of oxidation with PS vesicles exhibited a sharp break from an activated process at low temperature ($E_A \simeq 8$ kcal·mol^{-1}) to one with a small apparent activation energy at high temperature. The conditions of high and low apparent activation energy correspond to the domains of slow and fast cytochrome dissociation, respectively. The latter case may reflect a truly surface diffusion-controlled reaction, strictly limited by the encounter frequency. As the temperature is lowered, however, the thermal energy necessary to overcome an 8 kcal·mol^{-1} barrier on close approach of the reactants becomes limiting.

At high reactant concentrations, cytochrome c_2 oxidation by reaction centers in solution exhibits a pronounced fast phase, and the slow phase reaches a pseudo-first-order limit (Overfield et al., 1979). This strongly suggests that a binding interaction between the cytochrome and reaction center occurs prior to the electron transfer. Silvius et al. (1978) have shown that temperature-dependent binding of substrate can significantly distort Arrhenius plots of enzyme activity and, under certain conditions, can yield more or less sharp breaks. A negative binding enthalpy will, in general, cause an underestimation

[2] The space size is a linear measure for any number of dimensions. In plane space it is the average distance that can be covered by diffusion during the residence time in the space (see Appendix). For diffusion on the surface of a sphere, the maximum space size is the diameter of the sphere (Adam & Delbrück, 1968; Richter & Eigen, 1974).

[3] If, on the average, there are m reaction centers and n cytochromes per vesicles, the probablility that a particular vesicle will have x reaction centers and y cytochromes is

$$P_{x,y} = \left(\frac{m^x}{x!}e^{-m}\right)\left(\frac{n^y}{y!}e^{-n}\right)$$

The vesicle population can then be described by summing over all possible values of x and y, from 0 to ∞. In practice, it is unnecessary to go beyond a few multiples of the average values, m and n.

of the activation energy at high temperature. The temperature dependence of the amplitude of the fast phase, over the range 5–45 °C, showed a negative binding enthalpy ($\Delta H_B \simeq -7$ kcal·mol^{-1}) for the cytochrome c_2–reaction center association in 0.1 M NaCl, both in free solution and in crude PS vesicles (R. E. Overfield and C. A. Wraight, unpublished observations). However, more extensive studies will be necessary to fully evaluate the contribution of this effect to the observed temperature dependence of the oxidation kinetics. Whatever the significance of this effect, the bimodal behavior of cytochrome mobility on reaction center-containing PS vesicles is clear and reveals a sensitivity of the cytochrome–reaction center association to its environment that is not evident in neutral membranes or in free solution. It also suggests that the ionic conditions widely used for kinetic studies in chromatophores might be fruitfully scrutinized.

Conclusions

Two-dimensional diffusion and diffusion-controlled reactions on the membrane surface have been considered in some theoretical detail by a number of workers (Noyes, 1961; Naqvi, 1974; Adam & Delbrück, 1968; Richter & Eigen, 1974; Berg & Purcell, 1977), although experimental support is rather meager. Studies on the structural basis of the cytochrome *c*–cytochrome oxidase (Smith et al., 1977; Ferguson-Miller et al., 1978) and cytochrome *c*–cytochrome b_5 (Ng et al., 1977) reactions have yielded considerable evidence for an interaction and have strongly supported the notion of cytochrome *c* as a mobile electron shuttle in the intermembrane space of mitochondria (Bücher 1953; Nicholls, 1976). Roberts & Hess (1977) have shown that the kinetics of oxidation of cytochrome *c* by lipid-supplemented aggregates of purified cytochrome oxidase are suggestive of a two-dimensional diffusive approach of the cytochrome *c*. However, for cytochrome oxidase supplemented with negative phospholipids (diphosphatidylglycerol) in low salt medium (10 mM cacodylate and Tris, pH 6.5), their choice of 10^{-8} cm^2·s^{-1} for the lateral diffusion coefficient of cytochrome *c* is far removed from the values we have obtained with PS vesicles ($D_2 \simeq 0.6$–2.0×10^{-10} cm^2·s^{-1} in 10 mM Tris, pH 8.0). A more appropriate choice may affect conclusions on the kinetic competence of cytochrome *c* as a mobile shuttle under these conditions. In the present study the extent of cytochrome oxidation by reaction centers in a negative membrane was incomplete at low ionic strength.

The enhancement of the rate of cytochrome oxidation as the salt concentration was raised to 0.1 M NaCl, together with the approach to zero-order kinetics with respect to reaction center–vesicle concentration, strongly suggests that the diffusion process is restricted to the vesicle surface for PS vesicles at low and moderate ionic strength. A marked stimulation of diffusion-limited rate processes can follow from such a reduction in dimensionality (Adam & Delbrück, 1968; Richter & Eigen, 1974), and this is partially borne out for cytochrome c_2, although not for cytochrome *c*. Compared to neutral vesicles (see Figure 5 of the preceding paper: Overfield & Wraight, 1980), the maximal acceleration on negative vesicles, in 0.1 M NaCl, was about eightfold for cytochrome c_2, while the rate for cytochrome *c* was unchanged. At low ionic strength, the rates on the negative membrane were retarded for both cytochromes. Thus, the suggestion that the turnover of membrane-bound enzymes could be accelerated by lateral diffusion seems to be borne out, for this system, only under limited conditions. The electrostatic attraction that confines the cytochrome to two dimensions, while providing the possibility for acceleration of the encounter rate, also causes a reduction in the diffusion constant. Only under certain circumstances will a careful balance of ionic forces yield a significant, net acceleration.

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Appendix

In discussing rate enhancement of diffusion-limited processes by reduction in dimensionality, Adam & Delbrück (1968) used a general theorem concerning diffusion toward a small target of diameter *a* within a large diffusion space of dimensionality *i* and diameter *b*; the mean time of diffusion to the target, or "time to capture", T_i, can be represented by

$$T_i = (b^2/D_i)\, f_i(b/a)$$

The function $f_i(b/a)$ is strongly dependent on dimensionality. For $b/a \gg 1$, it is linear in b/a for $i = 3$, log b/a for $i = 2$, and independent of b/a for $i = 1$. Thus, for a constant numerical value for D_i, there is a marked rate enhancement on going from three to two dimensions, but very little further enhancement on going from two to one dimension.

In two dimensions, the mean time to capture is, therefore

$$T_2 = (b^2/D_2)(1/Y_1^2)$$

where Y_1 is a rather insensitive function of the relative space and target sizes (the b/a ratio). For reaction centers in solution or in PC vesicles, the oxidations of cytochromes *c* and c_2 are diffusion controlled and can be adequately described by the von Smoluchowski equation using a net diffusion coefficient of about 10^{-6} cm^2·s^{-1} and a reaction distance of 10 Å. For the PS vesicles used in this work, the average separation of reaction centers was about 200–250 Å. For $b/a = 20$, Adam & Delbruck (1968) give $Y_1 = 0.93$, and we have chosen to omit it from the equation in the Discussion. It should be noted that Y_1 only ranges from 1.1 to 0.5 as b/a goes from 10 to 10^4.

A somewhat different approach was taken by Richter & Eigen (1974), starting with the von Smoluchowski equation to describe the encounter of a reactant molecule (A) with a target (B) in three dimensions:

$$k_{assoc} = (4\pi N/1000)(D_A + D_B)R_{AB}$$

where k_{assoc} is the association rate constant for A and B, N is Avogadro's number, D_A and D_B are isotropic diffusion coefficients for A and B (in three dimensions), and R_{AB} is the reaction distance. If, instead, the target molecule is fixed in an *i*-dimensional space and the reactant can interact with and diffuse in this space (e.g., on a membrane), R_{AB} can be replaced by an effective sink size, r, characteristic of the decreased dimensionality. The sink size is the average distance covered by a reactant molecule on an encounter with the membrane. It is obtained from the diffusion coefficient (D_2) and residence time of the reactant on the membrane surface:

$$r = (D_2/k_{diss})^{1/2}$$

where k_{diss} is the dissociation rate constant for the reactant and the membrane.

Under conditions giving rise to actual rate enhancement, these two approaches are essentially equivalent. However, the formulation of Richter & Eigen (1974) loses any straighforward physical sense if the reduced dimensionality results in a decrease in reaction rate, such as we observed for PS vesicles in low salt. We have, therefore, used the more intuitive ap-

proach of Adam & Delbrück (1968) to interpret our results both in low and high salt medium.

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Virotoxins: Actin-Binding Cyclic Peptides of *Amanita virosa* Mushrooms†

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ABSTRACT: Virotoxins are toxic peptides singularly found in *Amanita virosa* mushrooms. After purification and resolution by high-pressure liquid chromatography, the main component, viroisin, was selectively cleaved and submitted to Edman degradation. The structure could be completely elucidated and was in part found to be the same as in phallotoxins. Differing from the phallotoxins, however, virotoxins are monocyclic peptides and contain D-serine instead of L-cysteine. In addition, two amino acids were detected in virotoxins which thus far have not been found in nature: 2,3-*trans*-3,4-dihydroxy-L-proline and 2′-(methylsulfonyl)-L-tryptophan. The biological activity of viroisin is comparable to that of the phallotoxins: e.g., with 2.5 mg of viroisin per kg (white mouse), 50% of the animals die within 2–5 h by hemorrhagia of the liver. Also, on the molecular level, the virotoxins behave similar to the phallotoxins. Thus, viroisin binds to rabbit muscle actin as proved by difference UV spectroscopy. With an apparent equilibrium dissociation constant $K_D \sim 2 \times 10^{-8}$ M, the affinity of viroisin is very similar to that of phalloidin. However, the flexibility of the monocyclic structure and the presence of two additional hydroxy groups in the virotoxins suggest a different mode of interaction with actin. While there is proof that the bicyclic phallotoxins possess a rigid binding site, the virotoxins may adopt the biologically active conformation by an induced-fit mechanism upon contact with actin.

While the toxic cyclic peptides of the green death cap *Amanita phalloides* (Vall. ex. Fr.) Secr. have been extensively investigated during the past 4 decades by Wieland and coworkers [for a review, see Wieland & Faulstich (1978)], intensive research on the toxins of the white species *Amanita virosa* Lam. ex. Secr. was only recently initiated.

α-Amanitin was detected in *A. virosa* by Tyler et al. (1966). In a more rigorous investigation of this species, in two specimens from different locations in Europe, the α-amanitin content was measured to be 1.2–1.4 mg per g dry weight, this being slightly higher than that in the green species (Faulstich et al., 1974). However, in samples of *A. virosa* collected in

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