Temperature-Jump Studies of Merocyanine 540 Relaxation Kinetics in Lipid Bilayer Membranes†

A. S. Verkman* and M. P. Frosch

Cardiovascular Research Institute, University of California, San Francisco, California 94143, and Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115

Received April 11, 1985

ABSTRACT: The temperature-jump technique was used to study the rapid kinetics of merocyanine 540 (M-540) interactions with single-walled phosphatidylcholine (PC) vesicles. The absorption spectrum of M-540 in PC vesicles has an isosbestic point at 560 nm at low [PC]/[M-540], where solution M-540 and membrane-bound M-540 dimers are present, and an isosbestic point at 548 nm at high [PC]/[M-540], where membrane-bound M-540 monomers and dimers are present. In response to a 15-kV discharge across a solution containing M-540 and PC vesicles (2.5 °C temperature increment), there was a rapid increase in absorbance at 575 nm (<5 μs) followed by a slower (~1 ms), monoexponential relaxation process of opposite sign and approximately equal amplitude to the initial rise. The amplitude of the slower process was wavelength-dependent and reversed sign at ~540 nm. The slower relaxation time constant decreased as [PC] was increased at constant [M-540]. A proposed model for the potential sensitivity of M-540 involves intramembrane reorientation of dye molecules and dimerization. The results obtained here suggest that reorientation of dye molecules is the rate-limiting step, with a rate constant for reorientation from parallel to perpendicular to the plane of the membrane of 1340 ± 200 s^{-1} at 23 °C.

The electrical potential is an important modulator of ion transport, nerve conduction, and nutrient uptake in biological membranes. A number of dyes have been characterized that undergo changes in optical absorbance or fluorescence properties in response to a change in membrane potential. Merocyanine 540 (M-540) is one of the fastest such dyes and has been used to measure membrane potential changes in conducting nerves (Davila et al., 1973; Salzberg et al., 1973), cardiac and skeletal muscle (Salama & Morad, 1976; Nakajima et al., 1976), and cell and vesicle suspensions (Haeyaert et al., 1980; Smith et al., 1984).

The mechanism by which M-540 responds to a change in membrane potential has been studied in several laboratories. Ross et al. (1974) first suggested that M-540 was a membrane-bound dye having a potential-dependent equilibrium between dye monomers and dye dimers of differing optical properties; subsequent measurements have supported this finding (Waggoner, 1976; Waggoner & Grinvald, 1977). Dragsten & Webb (1978) studied electrical field perturbations on M-540 fluorescence polarization in hemispherical bilayer membranes and found two kinetic processes: a slow reaction (~100 ms), due to dye partitioning into the membrane, and a submillisecond reaction, due to dye reorientation within the membrane coupled with dye dimerization. A precise definition of the mechanism and rate constants for the fast reaction was not possible because of the limited instrument resolution time.

In order to define the rapid kinetics for M-540 interaction with a pure lipid bilayer, we studied the effect of thermal perturbations on the optical absorbance of M-540 in phosphatidylcholine (PC) vesicles by using the temperature-jump technique. Our data support a mechanism of rapid intramembrane M-540 dimerization coupled with a rate-limiting dye reorientation. In terms of the model proposed by Dragsten & Webb (1978), the rate constant is 1340 ± 200 s^{-1} for dye movement from a position parallel to perpendicular to the plane of the membrane and 245 ± 80 s^{-1} for dye movement in the opposite direction. The rate of the dimerization reaction is >250 000 s^{-1}.

Materials and Methods

Chemicals. M-540 was obtained from Molecular Probes, Inc. (Junction City, OR). Egg phosphatidylcholine (PC) was obtained from Makor Chemicals Ltd. (Jerusalem, Israel) and gave a single spot after thin-layer chromatography on silica gel G (ICN Nutritional Biochemicals, Cleveland, OH) using chloroform–methanol–water (65:25:4). All other chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO).

Vesicle Preparation. Single-walled PC vesicles were prepared by sonication with the method of Huang & Thompson (1974). Lipid stored at −20 °C in ethanol was spun in a rotary evaporator to remove ethanol, lyophilized, and suspended in buffer (100 mM Tris–maleate, 100 mM KCl, pH 7.4) at a lipid concentration of ~25 mM. The suspension was sonicated at 4 °C under N_2 for 1 h in a Branson Model W 185 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY). The vesicles were then centrifuged at 40000*g* for 45 min to remove titanium particles and lipid debris in a Sorvall Model RC2-B refrigerated centrifuge (Du Pont Instruments-Sorvall, Newtown, CT). Vesicles were stored under N_2 at 4 °C and used within 48 h of preparation. Lipid concentration was assayed by the method of Gomori (1942).

Temperature-Jump Measurements. Temperature-jump studies as described by Eigen & DeMaeyer (1967) were performed on an apparatus designed by Verkman et al. (1980), which is described in detail elsewhere. A 2.5 °C temperature increment was obtained within 6 μs by discharging a 0.1-μF capacitor charged to 15 kV across a 0.8-mL solution volume. The instrument resolution time is ~4 μs. There is no solution cooling during the time course of the experiments; cooling of solution to the surrounding temperature occurs over a >1-s

† Supported in part by NIH Grants AM27045 and AM35124 and by funds from the UCSF Academic Senate and MSC Clough Fund.

* Address correspondence to this author at the Division of Nephrology, University of California, San Francisco, CA 94143.

0006-2960/85/0424-7117$01.50/0

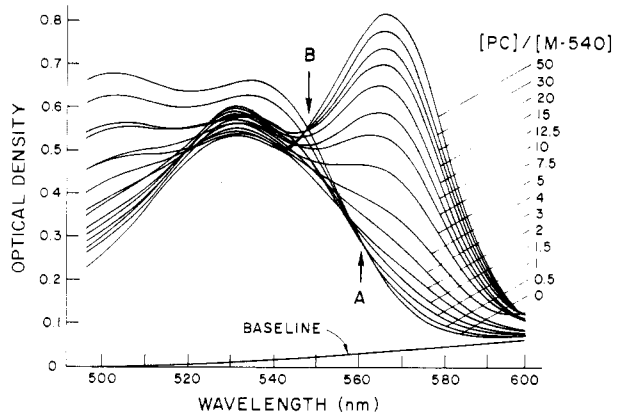

FIGURE 1: Titration of M-540 with PC vesicles. Absorption spectra were obtained on a dual-beam spectrophotometer. Sample solutions contained 11 μM M-540 and varying PC concentrations in buffer consisting of 100 mM Tris–maleate and 100 mM KCl, pH 7.4, at 23 °C; blank solutions were identical with corresponding sample solutions except that they contained no M-540. The [PC]/[M-540] ratios are indicated for each absorption spectrum. The arrows labeled A and B represent isosbestic points for equilibration between solution M-540 and membrane-bound dimer and equilibration between the membrane-bound monomer and dimer, respectively.

time course (Verkman et al., 1981). The absorbance of monochromatic light was measured by an RCA C31034 photomultiplier (Needham, MA), recorded on a Biomation 805 waveform recorder (Cupertino, CA), and transferred to a PDP 11/34 computer (Digital Equipment Co., Maynard, MA) for storage and analysis. All experiments were performed at 23 °C.

In studies of the voltage dependence of the absorbance signal, the charging voltage of the 0.1-μF capacitor was varied between 8 and 32 kV. The quantity of energy discharged into the solution is proportional to the square of the voltage in this range (Verkman et al., 1980). In studies designed to vary voltage and solution heating independently, the capacitor was discharged both into the solution chamber and into a parallel, identical solution chamber that was not in the light beam. Solution heating was thereby cut by 50% without altering the capacitor discharge voltage.

Calculations. The time course of transmitted light, $I(t)$, following the temperature-jump was fitted to a single-exponential function:

$$I(t) = A + Be^{-t/\tau} \quad (1)$$

where A is signal offset, B is signal amplitude, and τ is the exponential relaxation time constant. Relaxation times were fitted to the theoretical model in eq 14 by the nonlinear Newton's method (Bevington, 1969).

RESULTS

Figure 1 shows the absorption spectra of M-540 with varying concentrations of PC vesicles. There are two isosbestic points, labeled A and B, which require that there exist at least three components having different absorption spectra. At low [PC]/[M-540], there is an isosbestic point at 560 nm. Since the spectrum for unbound M-540 in solution passes through isosbestic point A (560 nm), the spectra for [PC]/[M-540] < 2 are composite spectra for solution M-540 and membrane-bound dimeric M-540 as discussed below.

At high [PC]/[M-540] there is an isosbestic point at 548 nm. As shown by Waggoner & Grinvald (1977), the predominant species present when [PC] is in great excess of [M-540] are membrane-bound monomeric and dimeric M-540. Because data for [PC]/[M-540] > 10 pass through an isosbestic point at 548 nm, which is distinct from the isosbestic point at 560 nm, relatively little solution M-540 is present under these conditions. In the intermediate region, 2 < [PC]/[M-540] < 10, the spectra are complex since solution M-540 and membrane-bound monomeric and dimeric M-540 components are present. Spectra in this region do not pass through an isosbestic point.

Table I: Titration of M-540 in PC Vesicles[a]

$[M\text{-}540]_t$ (μM)	$[PC]_t$ (μM)	OD_{570} ($\times 10^3$ M^{-1})	$[C_M]$ (μM)	$[C_D]$ (μM)	$[C_M]^2/[C_D]$ (μM)
1.52	250	0.224	1.31	0.103	16.7
3.04	250	0.401	2.34	0.35	15.9
4.56	250	0.560	3.27	0.64	16.6
6.08	250	0.681	3.97	1.06	14.9
7.60	250	0.790	4.59	1.50	14.0

[a] OD_{570} is the measured optical density for a solution containing the specified $[M\text{-}540]_t$ and $[PC]_t$. $[C_M]$ and $[C_D]$ are calculated from eq 3 and 4 by assuming monomer ϵ_M = 170 000 M^{-1} and dimer ϵ_D = 6000 M^{-1} based on the work of Waggoner & Grinvald (1977).

The monomer–dimer equilibrium constant can be estimated from the optical density at 570 nm (Table I). It is assumed that there are only membrane-bound monomers, C_M, and dimers, C_D, present under the conditions of the titration:

$$2C_M \leftrightarrow C_D$$

where the equilibrium constant, K_T', is defined by

$$K_T' = [C_M]^2/[C_D] \quad (2)$$

The conservation condition is

$$[M\text{-}540]_t = [C_M] + 2[C_D] \quad (3)$$

where $[M\text{-}540]_t$ is total M-540. The concentrations of C_M and C_D can be inferred from eq 3 and the measured optical density at 570 nm:

$$OD_{570} = \epsilon_M[C_M] + \epsilon_D[C_D] \quad (4)$$

where ϵ_M and ϵ_D are the molar extinction coefficients of the membrane-bound M-540 monomer and dimer, respectively. On the basis of the titration given in Table I, K_T' = 15.6 ± 1 μM.

In response to a 2.5 °C temperature jump, a solution containing M-540 and PC vesicles undergoes a biphasic time course of light transmittance. As shown in Figure 2, there is a rapid decrease in transmittance occurring within the instrument resolution time (<4 μs) followed by a slower increase in transmittance on a 1-ms time scale. The slower increase in transmittance is a single-exponential, concentration-dependent process and has an amplitude approximately equal to the initial rapid decrease in transmittance. The amplitude of the slower process is wavelength-dependent as shown in the lower panel of Figure 2 and reverses sign at ~540 nm. The wavelength dependence of the relative signal is quite similar to the difference in M-540 monomer and dimer spectra in Figure 1 and suggests that the process of increasing transmittance at 570 nm represents a time-dependent shift in the monomer–dimer equilibrium to favor the dimer species. There was no change in the intensity of transmitted or scattered light (570 nm) in response to a temperature jump when the solution consisted of 1 mM PC vesicles in the absence of M-540.

The rapid decrease in transmittance at 570 could represent either an instantaneous temperature-induced change in M-540 absorbance or a true process of dimer dissociation. The OD_{570} of the solution used for the experiments in Figure 2 changed by <10% over the temperature range 15–35 °C, indicating that the overall dimerization process has a low reaction enthalpy and that there is little dependence of M-540 absorbance

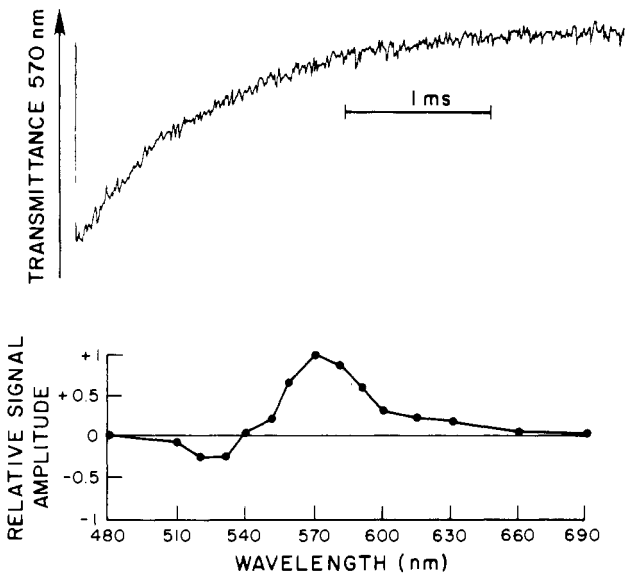

FIGURE 2: Relaxation kinetics of M-540 in PC vesicles. The upper curve shows the time course of transmitted light (570 nm) for a solution containing 8 μM M-540 and 250 μM PC vesicles following a 2.5 °C temperature increment. The initial decrease in transmittance occurs within the instrument resolution time (<5 μs). The slower increase in transmittance has a single-exponential time constant of 940 μs. The amplitude of this process expressed in units of relative transmittance $[(I_t - I_0)/I_0$, where I_t and I_0 are the intensities of the transmitted and incident light, respectively] is 0.08. The lower graph shows the relative signal amplitude, obtained from the fitted amplitude of the phase of increasing transmittance, as a function of wavelength. The curve has not been corrected for the intensity profile of the tungsten lamp or for the sensitivity characteristics of the photomultiplier.

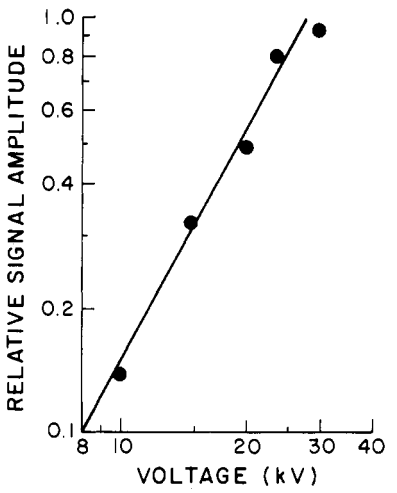

FIGURE 3: Effect of discharge voltage on the relaxation signal amplitude. The relative signal amplitude, obtained from the amplitude of a fitted exponential, is plotted against capacitor charging voltage in a log–log plot. The composition of the solution was the same as that in Figure 2. The fitted line has a slope of 1.8 ± 0.3.

on temperature. Although the overall reaction process may have a low enthalpy, the individual reaction steps must have significant nonzero enthalpies in order to observe the relaxation process. The individual reaction step enthalpies could compensate to give little overall temperature dependence of the OD_{570} under the experimental conditions used. In general, fluorescence quantum yields are quite sensitive to temperature, whereas extinction coefficients are temperature-independent.

It is interesting to explore the mechanism for the rapid dimer dissociation induced by the temperature jump. The driving force for dissociation can either be the temperature increment or a membrane-potential jump associated with the capacitor discharge. The initial electrical field applied to the sample is ~15 kV across a 1-cm sample thickness. As given by Farkas et al. (1984), this external electric field can induce a transmembrane potential of 56 mV in the membrane surface perpendicular to the direction of the electric field. It is therefore possible that the M-540 dimerization equilibrium could be perturbed by the membrane potential induced by the capacitor discharge.

In order to distinguish between a temperature-induced and an electric field induced relaxation process, two types of experiments were performed. The amplitude of an electric field induced relaxation process is proportional to voltage, whereas the amplitude of a temperature-induced relaxation process is proportional to $(\text{voltage})^2$, since the energy stored in a capacitor of capacitance C is $C(\text{voltage})^2/2$. Figure 3 shows a log–log plot for the voltage effect on the relaxation signal amplitude. The slope is 1.8 ± 0.3, indicating that the relaxation process is driven by temperature, not voltage.

A second line of evidence is based on experiments in which the solution temperature increment was varied without changing capacitor charging voltage. This was accomplished by placing a second temperature-jump cell in parallel with the first cell. When the second cell is in place, the solution temperature increment is decreased 2-fold without alteration of the capacitor charging voltage. Under these conditions, the relative temperature-jump signal amplitude for a solution containing 8 μM M-540 and 300 μM PC decreased to 44% of the signal amplitude measured without the second cell. Therefore, although the overall reaction enthalpy for dimerization is small, the individual steps of the overall dimerization process are temperature dependent.[1]

Dragsten & Webb (1978) have shown that the overall dimerization process is the result of reorientation of M-540 in the membrane followed by dimerization. On the basis of fluorescence polarization measurements in hemispherical bilayers, they concluded that monomers could be oriented parallel to or perpendicular to the plane of the membrane and that the dimers were parallel to the plane of the membrane:

$$2D_\perp \underset{k_{-r}}{\overset{k_r}{\rightleftharpoons}} 2D_\parallel \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} D_2 \qquad (5)$$

where k_r, k_{-r}, k_d, and k_{-d} are rate constants defined by equations A2 and A3 in the appendix and the equilbrium expressions

$$K_r = k_{-r}/k_r = [D_\perp]/[D_\parallel] \qquad (6)$$

$$K_d = k_{-d}/k_d = [D_\parallel]^2/[D_2] \qquad (7)$$

$D_\parallel$ and $D_\perp$ represent M-540 oriented parallel to and perpendicular to the plane of the membrane, and D_2 is a M-540 dimer. Because temperature-jump experiments are performed under conditions where the concentration of solution M-540 is negligable, it is possible to define $D_\parallel$, $D_\perp$, and D_2 as dimensionless concentrations of M-540 in the membrane that are related to C_M and C_D by the equations

$$[D_\parallel] + [D_\perp] = [C_M]/[PC] \qquad (8)$$

$$[D_2] = [C_D]/[PC] \qquad (9)$$

[1] The reaction enthalpy for the individual dimerization step in eq 5 can be roughly estimated from the temperature-jump signal size. Under the conditions of the experiment in Figure 2 (top), the transmittance change of 8% corresponds to a change in K_d by ~12%, assuming that $D_\parallel$ and $D_\perp$ have the same absorbance characteristics. Since the experiment was carried out with a 2.5 °C temperature increment, the reaction enthalpy for the K_d step is ~8 kcal/mol.

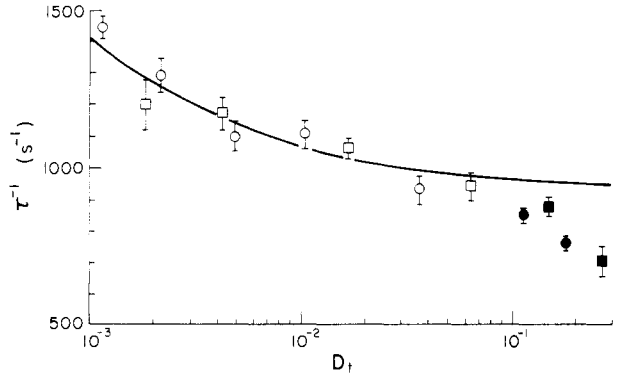

FIGURE 4: Kinetics of M-540 reorientation. Each τ^{-1} value is the average of three determinations; errors bars represent 1 SD. Circles correspond to data obtained at 500 μM PC, and boxes correspond to 250 μM PC. Filled symbols for $D_t > 10$ were not included in the fit. The fitted curve was drawn from eq 14 by a two-parameter weighted nonlinear least-squares fit. Fitted parameters are given in the text.

The overall dimerization constant, K_T, also becomes dimensionless:

$$K_T = ([D_\parallel] + [D_\perp])^2/[D_2] = K_T'/[PC] \quad (10)$$

K_T can be expressed in terms of K_d and K_r by using eq 6, 7, and 10:

$$K_T = K_d(1 + K_r)^2 \quad (11)$$

Using the dimensionless concentrations, the conservation condition in eq 3 becomes

$$D_t = [D_\perp] + [D_\parallel] + 2[D_2] \quad (12)$$

where $D_t = [\text{M-540}]_t/[PC]$.

In order to evaluate potential reaction mechanisms and to determine the values of rate constants by the temperature-jump technique, it is necessary to measure relaxation times as a function of reactant concentrations. For all concentrations tested, a single-exponential relaxation time was observed, suggesting that a single, rate-limiting reaction process is being probed. The range of D_t values that can be explored experimentally is limited by low OD for low values of D_t and by the requirement that the overall reaction be near a mide-quilibrium state in order to maximize signal amplitude (Czerlinski, 1966). The latter requirement stems from the fact that a temperature increment resets the values for K_r and K_d according the activation energies for the individual reaction steps. The induced changes in $[D_\parallel]$, $[D_\perp]$, and $[D_2]$, measured optically, are maximal when the reactant concentrations are maximally perturbed by small changes in K_r or K_d.

Figure 4 shows that τ^{-1} decreases from $\sim$1500 to $\sim$900 s^{-1} as D_t increases. The rate-limiting process for the reaction given in eq 5 could be either the reorientation or the dimerization process. If the dimerization process is rate-limiting, then τ^{-1} should increase as D_t increases according to (see Appendix)

$$\tau^{-1} = k_{-d}[(8D_t/K_T + 1)^{1/2} - 1] \quad (13)$$

If, however, the reorientation process is rate-limiting, then

$$\tau^{-1} = k_r + k_{-r}\frac{1 + 0.5(k_{-r}/k_r + 1)[1 - (8D_t/K_T + 1)^{1/2}]}{1 + (k_{-r}/k_r + 1)[1 - (8D_t/K_T + 1)^{1/2}]} \quad (14)$$

Equation 14 predicts that τ^{-1} decreases with increasing D_t as observed experimentally. In the limit of low D_t, $(\tau^{-1})_0 \sim k_r + k_{-r}$, and in the limit of high D_t, $(\tau^{-1})_\infty \sim k_r + k_{-r}/2$. k_r and k_{-r} can thus be estimated from these two limiting τ^{-1} values:

$$k_r = 2(\tau^{-1})_\infty - (\tau^{-1})_0 \quad (15)$$

$$k_{-r} = 2[(\tau^{-1})_0 - (\tau^{-1})_\infty] \quad (16)$$

Using the values given above, $k_r \sim 300$ s^{-1} and $k_{-r} \sim 1200$ s^{-1}.

Figure 4 shows a two-parameter fit to data obtained at two different values of [PC]. K_T was held constant at 0.062 on the basis of the value of K_T' measured at 250 μM PC (Table I). The analysis was restricted to data for which [PC]/[M-540] > 10 (open symbols in Figure 4), where virtually all M-540 resides within the membrane. Data corresponding to [PC]/[M-540] < 10 ($D_t > 0.1$, closed symbols) do not fall along the fitted curve since significant amounts of M-540 are in solution; the mathematical analysis necessary to fit data for $D_t > 0.1$ would require additional parameters (M-540 binding affinities), which are not justified on the basis of the data. The curve fitted to the open symbols was drawn from eq 14 with $k_r = 245 \pm 80$ s^{-1} and $k_{-r} = 1340 \pm 200$ s^{-1}. The calculated value for K_d is 0.01 $\pm$ 0.004.

Discussion

The change in optical absorbance and fluorescence of M-540 with membrane potential has been used widely to measure cell membrane potentials. While the relatively small changes in absorbance and fluorescence have limited the use of M-540 for steady-state membrane potential measurements, the fast response time of M-540 has been applied to measure action potentials in nerves (Davila et al., 1973) and membrane-potential changes associated with muscle contraction (Salama & Morad, 1976).

Several approaches have been used to explore the kinetics and mechanism of interaction of M-540 with lipid membranes. Using a rapid-mixing technique, Smith et al. (1980) found that M-540 bound to glyceryl monooleate suspensions with an apparent first-order rate constant of 66.7 s^{-1}. They suggested that M-540 binding to the membrane was diffusion-limited and occurred on a much slower time scale than the response to a change in membrane potential. On the basis of fluorescence intensity measurements, Aiuchi & Kobatake (1979) found that equilibrium binding of M-540 to liposomes follows a Langmuir isotherm, which was dependent upon the membrane surface charge. A number of studies have concluded that an M-540 monomer–dimer equilibrium exists within a bilayer lipid membrane (Waggoner & Grinvald, 1977). Dragsten & Webb (1978) used rapid electric field perturbations and fluorescence polarization to examine the orientation and kinetics of M-540 in hemispherical bilayer lipid membranes formed from a solution of oxidized cholesterol in decane. They concluded that M-540 dimers oriented parallel to the membrane surface were in equilibrium with two classes of monomers, oriented either parallel to or perpendicular to the membrane surface. In response to an electric field perturbation, they found both a slow response (0.1 s), due to dye partitioning into the membrane, and a fast, submillisecond response, possibly due to perturbation of the monomer–dimer equilibrium.

We report here the results of temperature-jump experiments for M-540 binding to single-walled PC vesicles using optical absorption to follow the reaction time course. Experimental conditions were selected to maximize M-540 binding to the vesicles so that solution to membrane partitioning was minimal. In response to a 2.5 °C temperature jump, there was a rapid decrease in transmittance at 570 nm (>250 000 s^{-1}) followed by a slower increase in transmittance ($\sim$1000 s^{-1}). On the basis of an analysis of the concentration dependence of the exponential relaxation times, we conclude that the rapid process corresponds to M-540 dimerization and that the slower

process corresponds to a rate-limiting reorientation within the membrane. From temperature-jump measurements alone, it is not possible to specify the nature of the unimolecular reorientation reaction; the terminology selected ($D_\parallel$ and $D_\perp$) is based on the parallel and perpendicular forms of the M-540 monomer described by Dragsten and Webb.

There are potential difficulties in applying the temperature-jump method to cell and vesicle suspensions. The relatively high electric field density may produce membrane damage or directly alter membrane properties. For example, Tessie & Tsong (1980) showed that voltage jumps can produce holes in red blood cell membranes. We do not, however, believe that the temperature-jump procedure damages unilamellar PC vesicle membranes. The relaxation time of a solution containing M-540 and PC vesicles is not affected by up to 10 repeated temperature jumps or by decreasing the discharge voltage from 15 to 10 kV. PC vesicles are uniformly small and are subject to a maximum induced transmembrane potential of <60 mV as explained under Results. PC vesicles are quite stable and are not lysed by exposure to a hypotonic solution. In addition, Verkman & Solomon (1980) have studied the interactions of phloretin with PC vesicles membranes by the temperature-jump technique and found no evidence for membrane damage resulting from the electrical discharge.

The rate of the dimerization reaction can be estimated from theoretical considerations. Assume that the membrane contains one M-540 molecule per 100 lipid molecules ($D_T = 0.01$). Since a PC vesicle contains ~3000 lipid molecules and has a 10^{-6} cm radius (Huang, 1969), there would be 30 M-540 molecules distributed over a 1.3×10^{-11} cm^2 surface area. Assuming that the M-540 molecules are equally spaced on a square lattice of 1.3×10^{-11} cm^2 surface area, the average distance between dye molecules is $\sim 2 \times 10^{-7}$ cm. The average time, t_0, it would take a monomer to diffuse a distance X is given by $t_0 = X^2/4D$, where D is the monomer diffusion coefficient in the membrane. Assuming $D = 10^{-6}$ cm/s, as is typical for small molecules in lipid membranes, and $X = 2 \times 10^{-7}$ cm, the calculated value for t_0 is 10 ns, 500 times faster than the temperature-jump instrument resolution time. As D_t is varied from 0.001 to 0.2, t_0 varies from 100 to 0.5 ns.

It is useful to make a limited comparison between the present results, obtained with phosphatidylcholine vesicles and the temperature-jump technique, with those of Dragsten and Webb, obtained with oxidized cholesterol/decane hemispherical lipid bilayers and a voltage-pulse technique. The present results define a rapid dimerization process (<6 μs) and a slower reorientation process (0.5–1 ms) for M-540 interaction with a PC vesicle membrane. Dragsten and Webb defined a very slow (100 ms) partitioning process and a fast potential-sensitive process. They present data obtained by using a voltage pulse having a rise time of ~0.5 ms and find no evidence for a process with a time constant between 1 and 100 ms. They do state that the response time of the voltage pulse instrument can be decreased to 2–6 μs by using 0.5 M salt, increasing pipet diameter, bulging the membrane, and adding capacitive lag circuitry. They state that the rise time for the fluorescence response under these conditions is under 6 μs; however, the data are not presented to examine the signal to noise characteristics.

In addition to the differences in lipid preparations and techniques, there are several reasons why the 0.5–1-ms process defined by the present experiments may not have been observed by Dragsten and Webb. The 0.5–1-ms process could not have been observed by using a voltage pulse with 0.5-ms rise time; the changes in technique required to decrease the response time of the voltage pulse instrument to 2–6 μs may have altered the membrane properties or greatly decreased the fluorescence signal to noise ratio. In addition, the amplitude of a fluorescence or absorbance response in a perturbation experiment is related to the degree to which individual reaction steps are perturbed by a temperature or voltage jump and to the relative contributions of the reacting species to the optical signal. In the temperature-jump experiment, a relatively large signal was observed because of a favorable reaction enthalpy of the reorientation step. In the voltage-jump experiments, it is possible that the signal arising from the reorientation step would be small if the voltage perturbation effected primarily the dimerization step. Thus, the signal could arise primarily from the faster dimerization process. Due to signal to noise limitations of the voltage-jump method, the reorientation process may have been obscured.

On the basis of the values for the fitted rate constants, k_r and k_{-r}, there is a 5:1 ratio of M-540 monomers that are oriented perpendicular and parallel to the membrane surface. A monomer oriented parallel to the membrane surface can undergo a rapid dimerization process. Our studies do not address the question of whether the reorientation and/or the dimerization reactions are driven by changes in transmembrane electrical potential. If the dimerization process is the potential sensitive reaction step, then the response rate of M-540 is >160 000 s^{-1}. On the basis of the thermally induced perturbation of the temperature-jump technique, it has been possible to examine the kinetics of a reorientation reaction coupled to the dimerization process. While the reorientation process is the rate-limiting process for overall intramembrane M-540 dimerization, it would not restrict the response rate of the $2D_\parallel \leftrightarrow D_2$ reaction step but act only to enhance the absorbance change for measurements performed on time scales >1 ms. Further characterization of the kinetics of the fast process will require techniques having nanosecond time resolution such as the measurements of M-540 fluorescence lifetimes in lipid bilayers.

ACKNOWLEDGMENTS

We thank Floyd C. Rector for his excellent advice and suggestions, A. K. Solomon, in whose laboratory the temperature-jump measurements were performed, and David Sullivan for expert technical support.

APPENDIX

Derivation of Equations 8 and 10. For the reaction mechanism given in eq 5 and the definition for K_T and D_t in eq 10 and 12, the concentration of monomeric M-540, $[D_\parallel] + [D_\perp]$, is

$$[D_\parallel] + [D_\perp] = (K_T^2/16 + K_T D_t/2)^{1/2} - K_T/4 \quad \text{(A1)}$$

The kinetic differential equations describing the reaction are

$$-d[D_\perp]/dt = k_r[D_\perp] - k_{-r}[D_\parallel] \quad \text{(A2)}$$

$$d[D_2]/dt = k_d[D_\parallel]^2 - k_{-d}[D_2] \quad \text{(A3)}$$

In a temperature-jump experiment, the concentrations of $D_\parallel$, $D_\perp$, and D_2 are perturbed slightly from equilibrium:

$$[D_\perp] = [D_\perp] + x \qquad [D_2] = [D_2] + y$$
$$[D_\parallel] = [D_\parallel] - x - 2y \quad \text{(A4)}$$

where x and y are small compared to dye concentrations. Substitution of equations A4 into A2 and A3 gives

$$dy/dt = -k_d(2x + 4y)[D_\parallel] - k_{-d}y \quad \text{(A5)}$$

$$-dx/dt = k_r x + k_{-r}(x + 2y) \quad \text{(A6)}$$

where the second-order terms (x^2, y^2, and xy) have been dropped in the limit of a small perturbation and terms that do not contain x or y cancel from the equilibrium relations in eq 6 and 7.

If the reorientation reaction is rate-limiting, then eq A5 simplifies, $dy/dt = 0$. Substitution of eq A5 into eq A6 then gives

$$-\frac{dx}{dt} = k_r + k_{-r}\left(\frac{K_d + 2[D_\parallel]}{K_d + 4[D_\parallel]}\right)x \quad \text{(A7)}$$

where $K_d = k_{-d}/k_d$. The inverse relaxation time, τ^{-1}, is given in the parenthesized term in eq A7. Equation 14 in the text follows from the parenthesized term, where $[D_\parallel]$ has been replaced by D_T by using eq A1 and 11. When K_d becomes infinite, eq A7 gives the familiar expression for the relaxation time of a monomeric interconversion: $\tau^{-1} = k_r + k_{-r}$ (Czerlinski, 1966).

When the dimerization reaction is rate-limiting, $dx/dt = 0$ in eq A6. Equation A5 becomes

$$-\frac{dy}{dt} = k_{-d} + k_d\left(\frac{4[D_\parallel]}{1 + K_r}\right)y \quad \text{(A8)}$$

where $K_r = k_r/k_{-r}$. Equation 13 in the text follows from the parenthesized term in eq A8 and eq A1, 7, and 11. When $K_r = 0$, eq A8 gives the familiar expression for the relaxation time of a dimerization reaction: $\tau^{-1} = k_{-d} + 4[D]k_d$ (Czerlinski, 1966).

Registry No. M-540, 62796-23-0.

REFERENCES

Aiuchi, T., & Kobatake, Y. (1979) *J. Membr. Biol. 45*, 233–244.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, pp 59–65, McGraw-Hill, New York.

Czerlinski, G. H. (1966) *Chemical Relaxation*, pp 180–202, Marcel Dekker, New York.

Davila, H. V., Salzberg, B., Cohen, L. B., & Waggoner, A. S. (1973) *Nature (London), New Biol. 241*, 159–160.

Dragsten, P. R., & Webb, W. W. (1978) *Biochemistry 17*, 5228–5240.

Eigen, M., & DeMaeyer, L. (1967) in *Techniques of Organic Chemistry* (Friess, S. L., Lewis, E. S., & Weissberger, A., Eds.) Vol. 8, pp 895–1054, Wiley, New York.

Farkas, D. L., Korenstein, R., & Malkin, S. (1984) *Biophys. J. 45*, 363–373.

Gomori, G. (1942) *J. Lab. Clin. Med. 27*, 955–960.

Haeyaert, P., Verdonck, F., & Wuytack, F. (1980) *Arch. Int. Pharmacodyn. Ther. 244*, 333–335.

Huang, C. (1969) *Biochemistry 8*, 344–352.

Huang, C., & Thompson, T. E. (1974) *Methods Enzymol. 32*, 485–489.

Nakajima, S., Gilai, A., & Dingeman, D. (1976) *Pfluegers Arch. 362*, 285–287.

Ross, W. N., Salzberg, B. M., Cohen, L. B., & Davila, H. V. (1974) *Biophys. J. 14*, 983–986.

Salama, G., & Morad, M. (1976) *Science (Washington, D.C.) 191*, 485–487.

Salzberg, B., Davila, H., & Cohen, L. (1973) *Nature (London) 246*, 508–509.

Smith, J. C., Frank, S. J., Bashford, C. L., Chance, B., & Rudkin, B. (1980) *J. Membr. Biol. 54*, 127–139.

Smith, J. Dc., Graves, J. M., & Williamson, M. (1984) *Arch. Biochem. Biophys. 231*, 430–453.

Tessie, J., & Tsong, T. Y. (1980) *J. Membr. Biol. 55*, 133–140.

Verkman, A. S., & Solomon, A. K. (1980) *J. Gen. Physiol. 75*, 673–692.

Verkman, A. S., Pandiscio, A. A., Jennings, M., & Solomon, A. K. (1980) *Anal. Biochem. 102*, 189–195.

Verkman, A. S., Dix, J. A., & Pandiscio, A. A. (1981) *Anal. Biochem. 117*, 164–169.

Waggoner, A. S. (1976) *J. Membr. Biol. 27*, 317–334.

Waggoner, A. S., & Grinvald, A. (1977) *Ann. N.Y. Acad. Sci. 303*, 217–241.