Photochemical dimerization of parinaric acid in lipid bilayers

(membranes/photochemistry/diffusion)

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ABSTRACT Parinaric acid (9,11,13,15-octadecatetraenoic acid), a conjugated tetraene fatty acid, undergoes a second-order photochemical reaction in phospholipid bilayers. The reaction results in the loss of the characteristic absorption of this chromophore and the development of new absorption demonstrating the presence of a triene chromophore. The progress of this reaction is easily monitored by measurement of the decrease in the fluorescence intensity from a uniformly illuminated sample. The reaction rate measured in this way is sensitive to the thermal phase transition of the bilayer and to the presence of cholesterol. The relationship of the second-order rate constant to the lipid diffusion coefficient is discussed. This relationship differs from that previously used for the analysis of similar photochemical processes.

Parinaric acid (9,11,13,15-octadecatetraenoic acid)[¶] is a naturally occurring conjugated tetraene fatty acid whose absorption, fluorescence, and structural properties make it a unique probe for lipid bilayer and lipid-protein interaction studies (1-8). The spectroscopic and partitioning properties of parinaric acid have been explored in detail. The photochemical behavior of this probe has not previously been discussed. One of the objectives of this paper is the presentation of kinetic data which demonstrate that the primary photochemical decomposition reaction of parinaric acid is irreversible dimerization involving a ground state and an excited state species.

Because of the bimolecular nature of this reaction, its rate constant depends on the diffusion of the reactants in the bilayer. This dependence was demonstrated by making measurements of the photochemical reaction rate as a function of temperature through the range of a bilayer phase transition. The variation of the rate with addition of cholesterol at fixed temperature was also determined.

In order to analyze our results in a more quantitative fashion it was necessary to reexamine the general question of the relationship between a second-order photochemical rate constant and the reactant diffusion coefficient. This question has previously arisen in a similar context in studies in which a bimolecular photoprocess was proposed as the basis of a method for the determination of diffusion coefficients in bilayers (9-15). The analysis presented here emphasizes the contribution of excitation events in which the excited species has a reaction partner within a reaction radius at the time of excitation. The final expression differs in important ways from those used previously. By inverting this expression, an equation is obtained for the probe diffusion coefficient in terms of the photochemical rate constant. In this respect, parinaric acid offers a considerable advantage compared to pyrene excimer formation (11, 12, 14) or 12-(9-anthroyloxy)-stearate photodimerization (15, 16). These bulky aromatic chromophores appear to aggregate in gel phase lipids and in certain fluid lipids formed by natural lipid mixtures (11, 12, 15, 16).

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MATERIALS AND METHODS

Phospholipids were obtained from Sigma Chemical Co. and used without further purification. Cholesterol was purchased from Steraloids, Wilton, NH, and purified by recrystallization three times from methanol. Stock solutions (30 mM) of phospholipid and cholesterol were prepared in chloroform and stored at -20°C under argon. L- α -Stearoyl lysophosphatidylcholine was prepared from L- α -distearoyl phosphatidylcholine by using *Crotalus adamanteus* venom (17) supplied by the Miami Serpenterium. The probe molecule α -stearoyl- β -*trans*-parinaroyl phosphatidylcholine was prepared from the lysophospholipid by the method of Gupta *et al.* (18) under conditions that rigorously excluded oxygen and light. The phospholipid was purified on a Biosil A column (BioRad) eluted with a gradient of freshly distilled argon-deoxygenated methanol in chloroform to a final concentration of 60% methanol by volume. The phospholipid was evaporated under reduced pressure and taken up in a mixture of chloroform, methanol, and water (5:4:1 by volume). This solution was passed down a column of Rexyn I-300 (Fisher) to remove residual dimethylaminopyridine catalyst from the mixture. The purified compound was stored as a solution in ethanol under argon at -20°C in the dark. *trans*-Parinaric acid used in the preparation of the phospholipid was prepared and purified as described (4). It was stored under the same conditions as the labeled phospholipid.

Dispersions of phospholipids in buffer were prepared by evaporation of stock lipid solution under a stream of argon. In some cases, samples were stored under reduced pressure overnight to remove all traces of chloroform. Vacuum-degassed buffer was added and the mixture was heated to above the gel/liquid crystalline transition temperature under argon. This did not significantly affect the results. The lipid was dispersed by Vortex mixing and the samples were allowed to stand for several minutes at the elevated temperature. When the sample was to be labeled with the α -stearoyl- β -*trans*-parinaroyl phosphatidylcholine, this was co-evaporated with the phospholipid host and the above procedure was carried out with exclusion of light. Samples of phospholipid to be labeled with parinaric acid were prepared as above, protected from light, and the parinaric acid added as a small volume of concentrated ethanolic solution. The final concentration of ethanol did not exceed 1% by volume. The samples were placed in a heated bath and maintained above the phase transition temperature of the host lipid for approximately 1 hr to ensure complete binding and probe equilibration across bilayer membranes.

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[¶] There are two isomers of parinaric acid in common use. The naturally occurring material, α -parinaric acid, is the *cis*, *trans*, *trans*, *cis* isomer; β -parinaric acid is the *trans*, *trans*, *trans*, *trans* isomer. Only the β -isomer, also known as *trans*-parinaric acid, was used in this study.

Fluorescence measurements were performed by using a Spex Fluorolog fluorimeter interfaced to a Spexcomp computer. The fluorescence intensity versus time data were stored on a magnetic disc. Samples were contained in a quartz capillary of 1 mm internal diameter placed in a variable-temperature sample holder. The block temperature was varied with a regulated circulating water bath. A sample volume of about 5 μ l was used and totally illuminated. Data were stored for each sample at a given temperature in the presence and absence of fluorescence probe and the computer was used to correct for the background level. The data were next normalized to the maximum value of intensity ($\bar{I} = I/I(\text{max})$) and the second-order function $1/\bar{I} - 1$ was plotted with the array manipulation commands of the Spex operating system. An estimate of the absolute incident photon flux was obtained by replacing the sample compartment with the sensor of a (Coherent Radiation, Palo Alto, CA) laser power meter calibrated against a bolometer (Lasermetrics, Teaneck, NJ). In determination of the variation of photobleaching rate with incident light intensity, the light was attenuated after the excitation monochromator by using a glass filter. The fluorescence of a stirred standard sample of low absorbance was taken as a measure of the relative incident photon flux for such measurements.

In order to prepare larger quantities of the photoproduct of the parinaric acid photoreaction, a suspension containing dipalmitoyl phosphatidylcholine at 100 mg/ml was maintained at 70°C and illuminated with a high-pressure mercury arc for a period of 1 hr. The result of this treatment was subjected to thin-layer chromatography and stained with iodine. The progress of the photochemical reaction was also monitored by measuring the absorption spectrum in the 230- to 350-nm region after various periods of irradiation. For these experiments the lipid concentration was 1 mg/ml (2 mM) and the parinaric acid concentration was 6 μ M ($A = 0.5/\text{cm}$). The irradiation was performed at 60°C. Spectra were obtained at room temperature and stored in digital form. Scaled parinaric acid spectra were subtracted to obtain the spectrum of the reaction product.

RESULTS

The major results of this study are the observations presented in Figs. 1–3 that the photochemical decay of parinaric acid fluorescence in bilayers is second order in the parinaric acid concentration and that the rate constant for the reaction depends on the physical state of the bilayer.

The conditions used for the photochemical experiments of Figs. 1–3 are probe concentrations of the order of one molecule per 100 phospholipid molecules and an illumination intensity of approximately 0.1 mW. In all cases the lipid concentration was approximately 0.6 mM, so that at these probe-to-lipid ratios, essentially all of the *trans*-parinaric acid is bound to the bilayer. The fact that similar results were obtained for the free fatty acid and the labeled phospholipid indicates that the contribution of unbound phospholipid does not affect the form of the kinetics.

The loss of fluorescence intensity, I , as a function of time t with constant illumination obeys an experimentally determined rate law of the form

$$-\frac{dI(t)}{dt} = k_i I^2(t). \quad [1]$$

This was established from the integrated form

$$\frac{1}{\bar{I}(t)} - 1 = I(o)k_i t \equiv St, \quad [2]$$

in which

$$\bar{I}(t) = I(t)/I(o). \quad [3]$$

FIG. 1. Photochemical decomposition of *trans*-parinaric acid and stearylparinaroyl phosphatidylcholine in dipalmitoyl phosphatidylcholine dispersions. (A) Curves of fluorescence intensity versus time for *trans*-parinaric acid at the indicated Celsius temperatures. The straight lines are the corresponding plots according to Eq. 2, demonstrating the second-order nature of the reaction. The concentration of parinaric acid was 1 molecule per 70 phospholipid molecules. (B) Same results for the parinaroyl-labeled phospholipid at a concentration of 1 molecule per 175 phospholipid molecules. The lipid concentration was approximately 0.6 mM.

Plots of the $[1/\bar{I}(t) - 1]$ versus t are given in Figs. 1 and 3. These were obtained by replotting the computer data file for each run. According to Eq. 2, the slope of these lines, S , is $I(o)k_i$. Because $I(o)$ is proportional to the initial probe concentration, Eq. 2 states that S should be proportional to the initial (two-dimensional) concentration. This was demonstrated by increasing the lipid concentration at fixed three-dimensional probe concentration. The observed second-order decay plots are essentially linear through at least 95% completion.

Eq. 1 is equivalent to the expression

$$-\frac{d[P]}{dt} = k_p [P]^2, \quad [4]$$

in which $[P]$ is the two-dimensional parinaric acid concentration (molecules/cm²). Eq. 4 follows from eq. 1 because

$$I = \Gamma[P], \quad [5]$$

in which Γ is proportional to the illumination intensity, the effective sample path length, the probe extinction coefficient, the fluorescence quantum yield, the lipid bilayer area per unit volume, and the instrumental collection efficiency. The relationship of k_i , k_p , and the experimental slope, S , is

$$k_p = \Gamma k_i = S/[P(o)]. \quad [6]$$

Because S is proportional to $[P(o)]$, k_p is independent of $[P(o)]$.

Experiments performed at different illumination intensities demonstrated that S , and therefore k_p , is proportional to the illumination intensity. This implies that the reaction being observed is of the form

FIG. 2. Second-order rate constant for *trans*-parinaric acid dimerization in dipalmitoyl phosphatidylcholine dispersions as a function of temperature. The relative rates are given on an arbitrary scale. All measurements were made at the same initial concentration and illumination intensity so the relative values of the slopes S are also the relative values of the rate constant k_p .

in which P and P^* represent ground and excited state parinaroyl chromophores. The fact that the reaction is accurately second-order essentially to completion means that the reaction is irreversible. It also means that neither P nor P^* is involved in any other irreversible reactions involving P , P^* , or X prior or subsequent to the above reaction.

The structure of the product X is not known, but several relevant factors have been determined. Irradiation of parinaric acid in a bilayer suspension at 315 nm results in a loss of the characteristic structured absorption of parinaric acid at 320, 305, 292, and 280 nm. As the reaction proceeds, there is a proportional increase in absorption with a peak at 274 nm and shoulders at 265 and 285 nm. The product spectra obtained by

FIG. 3. Second-order rate plots for *trans*-parinaric acid in phospholipid dispersions with added cholesterol. Curve 1, dilauroyl phosphatidylcholine at 22°C; curve 2, dilauroyl phosphatidylcholine with 20 mol % cholesterol at 22°C; curve 3, dipalmitoyl phosphatidylcholine with 40 mol % cholesterol at 28.5°C; curve 4, dipalmitoyl phosphatidylcholine at 28.5°C. The curve for dilauroyl phosphatidylcholine with 30 mol % cholesterol is midway between curves 2 and 3, whereas the result for 40 mol % cholesterol is identical to curve 4.

difference is strongly reminiscent of that of conjugated trienes. (Eleostearic acid has peaks at 264, 275, and 284 nm in *n*-decane with the relative intensities observed for the product.) The product of the reaction is apparently nonfluorescent even when irradiated at 275 nm. Linear conjugated trienes are also essentially nonfluorescent. By using the extinction coefficients for β -parinaric acid (80,000 liter mol⁻¹ cm⁻¹ at 275 nm) and β -eleostearic acid (51,000 liter mol⁻¹ cm⁻¹ at 275 nm) it is found that the loss of two tetraene chromophores results in the production of one triene chromophore. The product absorption spectrum also shows intensity in the 230- to 250-nm region in excess of that due to a triene chromophore. This is the region of diene absorption. Irradiation of the product mixture at 250 nm after 80% completion resulted in a decrease in the 275-nm component and a relative increase in the 230-nm component. Extensive broad band irradiation resulted in complete loss of absorption at wavelengths longer than 230 nm. The product showed only one spot on thin-layer chromatography with a mobility less than that of parinaric acid.

DISCUSSION

One hypothesis consistent with the data given above is that the photochemical dimerization of parinaric acid involves a 4+2 cycloaddition reaction leading to a substituted cyclohexene with triene and diene substituents. This is analogous to the product reported for the photodimerization of retinyl acetate (19). The only other reported study of a triene or higher polyene photodimerization reaction appears to be the solid-state reaction of all-*trans*-hexatriene-1,6-dicarboxylic acid (20). In this case a 2+2 cycloaddition reaction results in a substituted cyclobutane.

The rate-limiting step for this photoreaction appears to be the encounter of the reactants, because the reaction rate is influenced by factors that change the lipid diffusion coefficient. In order to analyze our data in a more quantitative fashion we must reexamine the relationship between the rate constant and the diffusion coefficient for a photochemical reaction. The two mechanistic parameters we wish to determine are the efficiency of reaction per encounter and the lifetime of the reactive species. The determination of these quantities, besides being of mechanistic interest, is relevant to the problem of the inversion of these measurements to obtain the lipid diffusion coefficient D .

We begin with a simple argument whose conclusions are verified by an alternative derivation, which is given in an appendix. We first define an encounter radius ρ and an intrinsic reaction probability α , such that when P and P^* are separated by a distance less than ρ there is a probability α of reaction. If the distance between reactants is greater than ρ the reaction probability is zero. When a molecule is excited it may have a reaction partner within its reactive area ($\pi\rho^2$). The diffusive motion of the reactants during the lifetime of the reactive species (τ) will result in an increase in the effective encounter radius by an amount $(4D\tau)^{1/2}$. If this diffusive distance is comparable to or less than ρ we may assume that the entire area with radius $\rho + (4D\tau)^{1/2}$ is sampled by the diffusive process. The rate constant will then be

$$k_p = 2\pi\alpha\Phi[\rho + (4D\tau)^{1/2}]^2, \quad [8]$$

in which Φ is the rate of absorption of photons.

This expression assumes that, at the time of excitation of a particular molecule, the distribution of reactants is uniform. This is valid for low illumination intensities when there is sufficiently rapid diffusion. The illumination intensity determines the rate of absorption of photons, Φ , whose reciprocal is the time

interval between excitations of a given molecule. The photochemical reaction perturbs the random distribution of reactants so that it is deficient in near-neighbor pairs. If this perturbation is not completely relaxed it will result in a decreased reaction rate (10, 13, 21). Under our conditions the value of Φ is approximately $1 \text{ sec}^{-1} \text{ molecule}^{-1}$ and the diffusion coefficient is at least $10^{-12} \text{ cm}^2 \text{ sec}^{-1}$. A simulation of reaction and diffusion in a two-dimensional lattice was performed; it showed that even for $D = 10^{-14} \text{ cm}^2 \text{ sec}^{-1}$ and $\Phi = 1000 \text{ sec}^{-1} \text{ molecule}^{-1}$ the diffusive motion is sufficient to relax the spatial perturbation and results in a time-independent reaction rate. (There is a short-term transient variation of the reaction rate after the excitation event. This is included in Eq. 8 as shown in the appendix.)

The relationship given in Eq. 8 differs from the relationships given previously (9–13, 15) primarily because of its emphasis on the contribution of pairs that are reactive due to proximity at the time of excitation. These pairs are not important in the description of excited state diffusive quenching decay data (14) because they have a short lifetime. However, such pairs contribute to the decreased fluorophore quantum yield, as can be seen from the hypothetical limiting case of complete dimerization.

The values of the quantities appearing in Eq. 8 will now be discussed for the reaction of parinaric acid in dipalmitoyl phosphatidylcholine bilayers. In particular, we will use the data of Fig. 1B for the reaction of the labeled lipid stearoylparinaroyl phosphatidylcholine at 43°C and 21°C , where the rates are $k_p(43^\circ\text{C}) = (3.2 \pm 0.3) \times 10^{-14} \text{ cm}^2 \text{ sec}^{-1}$ and $k_p(21^\circ\text{C}) = (0.88 \pm 0.1) \times 10^{-14} \text{ cm}^2 \text{ sec}^{-1}$. The limits given refer to the total spread of duplicate runs. The diffusion coefficients for these two bilayer phases are known to be approximately $D(43^\circ\text{C}) = 7 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$ (22–24) and $D(21^\circ\text{C}) = 4 \times 10^{-12} \text{ cm}^2 \text{ sec}^{-1}$ (25). We assume that ρ is 3–6 Å because the chain separation is 5 Å in solid-phase lipid bilayers. We also assume that α and ρ are the same for both phases.

With these assumptions we may test the hypothesis that τ corresponds to the excited singlet state lifetime. This assignment of the singlet state as the reactive species is reasonable in view of the photochemical (26, 27) and photophysical (28, 29) data, which demonstrate that trienes and longer polyenes do not appreciably intersystem cross to the triplet state. However, it has been reported (30) that the photochemical dimerization of retinol proceeds through its triplet state. For this reason we must use our kinetic data and Eq. 8 to estimate τ . This can be done as follows. The observed triplet lifetime for a conjugated triene has been reported as $0.3 \mu\text{sec}$ in fluid solution at 28°C (28). The triplet lifetime decreases with increasing chain length (29) but may be longer in solid-phase bilayers than in fluid solution. A reasonable upper limit for the triplet lifetime is $1 \mu\text{sec}$ for parinaric acid in the solid-phase bilayer. Because D is about $4 \times 10^{-12} \text{ cm}^2 \text{ sec}^{-1}$, $(4D\tau)^{1/2}$ is $4 \times 10^{-9} \text{ cm}$ or approximately $1/10$ th of ρ (3–6 Å). Therefore, as a first approximation we may neglect the diffusive contribution in the low-temperature phase and set $k_p(21^\circ\text{C}) = 2\pi\alpha\Phi\rho^2$. The ratio $k_p(43^\circ\text{C})/k_p(21^\circ\text{C})$ therefore serves to determine $(4D\tau)^{1/2}/\rho$ at 43°C as 0.91 and, because $D = 7 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$, a value of $\rho = 4 \text{ Å}$ corresponds to τ at 43°C of 4.7 nsec. The measured fluorescence lifetime of parinaric acid under these conditions is 5 nsec. We therefore conclude that the excited singlet is probably the reactive state.

The essence of the quantitative argument given above is that diffusion is so slow at 21°C that the measured rate is dominated by the static contribution due to adjacent molecules. The participation of such species in the reaction can be demonstrated experimentally because the interaction of the chromophores

gives these pairs a distinct spectrum. This can be observed in the absorption spectra of concentrated samples. The excitation spectrum for the photochemical reaction differs from the absorption spectrum in a manner consistent with the high reactivity of neighboring pairs. The concentration of such pairs is roughly that expected on the basis of a random distribution.

The value of α in Eq. 8 may be estimated from a comparison of the absolute values of the calculated and observed rates. Using $\rho = 4 \text{ Å}$, $D = 7 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$, $\tau = 5 \text{ nsec}$, and the experimental value of $\Phi = (1 \pm 0.3) \text{ sec}^{-1}$, we find $\alpha = 0.7\text{--}1.1$. This reaction is apparently very efficient.

For fluid phase bilayers the quantity $(4D\tau)^{1/2}$ is approximately 4 Å. This means that the diffusive contribution to the rate constant is comparable to the static term. If Eq. 8 is solved for D and α set equal to unity, we have

$$D = (1/4\tau)[(k_p/2\pi\Phi)^{1/2} - \rho]^2; \quad [9]$$

it is possible to extract D from measurements of k_p , Φ , and τ without a critical dependence on ρ . Relative values of D can be obtained from kinetic and quantum yield measurements because τ is proportional to the quantum yield (3, 4). For solid phase bilayers the value of D obtained from Eq. 9 depends critically on ρ . However, the measurement of k_p can be made with considerable precision so that small changes in k_p can be determined.

The large change in k_p at the phase transition temperature shown in Fig. 2 reflects the change in lipid diffusion coefficient. The constancy of k_p above the transition is due to a compensation of the increase in D (23) by a decrease in τ (4). Addition of cholesterol to fluid dilauroyl phosphatidylcholine decreases D (23), but addition of cholesterol to solid dipalmitoyl phosphatidylcholine increases D (25). These effects are reflected in the rate data of Fig. 3. We therefore conclude that measurements of the photochemical reaction rate of parinaric acid can be used in at least a qualitative fashion to indicate changes in lipid diffusion coefficients.

APPENDIX

The relationship between the overall photochemical rate constant k_p and the self-diffusion coefficient can be derived by considering the following parallel reaction scheme:

The species (PP) is a pair of neighboring ground-state chromophores that have a separation and orientation such that they react with unit probability after excitation. (We assume that the quantity α defined in the text is unity.) If the rate constant for absorption of photons by monomers is Φ , then the rate constant for absorption by pairs is approximately 2Φ . The steady-state concentration of excited monomers P^* is $\Phi\tau P$. The rate constant for the last reaction, k_d , depends on the diffusion coefficient and time according to the expression given in refs. 10 and 13. The reaction rate constant is time dependent on the time scale of the excited state lifetime because the reaction process preferentially removes species that are separated by small distances at the time of excitation (21). When this time-dependent expression is integrated over the fluorescence lifetime the result is

$$k_d = 8\pi D[(R^2/2D\tau)^{1/2} + 1/2 - \dots], \quad [A1]$$

in which R is the reaction radius and D is the self-diffusion coefficient. The overall rate of decrease of the concentration of P is given by

$$-\frac{d[P]}{dt} = 4\Phi[(PP)] + 2k_d[P][P^*] \\ = 4K[P]^2 + 2k_d\Phi\tau[P]^2. \quad [A2]$$

So according to Eqs. 4 and A1

$$k_p = 4\Phi K + 2k_d\Phi\tau \\ = 4\Phi[K + 2\pi R(2D\tau)^{1/2} + 2\pi D\tau]. \quad [A3]$$

If the distribution of ground state species is purely random, then $K \approx \pi R^2$. With this approximation, Eq. A3 is the same as Eq. 8 if $R = \rho/\sqrt{2}$.

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