ELSEVIER

Biochimica et Biophysica Acta 1279 (1996) 164–168

BBA Biochimica et Biophysica Acta

The transverse location of the fluorescent probe *trans*-parinaric acid in lipid bilayers

Miguel Castanho [a,b], Manuel Prieto [a,*], A. Ulises Acuña [c]

[a] *Centro de Química-Física Molecular, Instituto Superior Técnico, 1096 Lisboa Codex, Portugal*

[b] *Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, R. Ernesto Vasconcelos, Bloco C1-5° andar, 1700 Lisboa, Portugal*

[c] *Instituto de Química-Física 'Rocasolano', C.S.I.C., Serrano 119, 28006 Madrid, Spain*

Received 30 June 1995; revised 27 September 1995; accepted 16 October 1995

Abstract

The transverse location of *trans*-parinaric acid in spherical vesicles made up from dipalmitoylphosphatidylcholine has been investigated by the differential quenching of the probe fluorescence by 5- and 16-doxylstearic acid derivatives. The quenching data are interpreted in terms of a local fluorophore concentration factor. In this way it was found that the polyene of *t*-PnA is located within the inner part of the bilayer (presumably aligned with the bilayer lipids), both in the gel and in the liquid crystalline phases.

Keywords: Fluorescence; Lipid bilayer; *trans*-Parinaric acid

1. Introduction

The natural lipid *trans*-parinaric acid (*t*-PnA), Fig. 1, is a fluorescent 18-carbon tetraene that is widely applied to probe the structure and dynamics of lipid bilayers, biological membranes and lipophilic proteins [1–4]. The basis of these applications are the sensitivity of several spectral parameters of *t*-PnA to the micro-environment, the similarity of the probe with the membrane lipids and the expected well defined location of the fluorophore in the bilayer.

The emission decay of *t*-PnA in membranes is relatively complex and at least two or three exponential components are usually observed. In addition, *t*-PnA shows a preferential solubility in the most structured parts of the membrane (e.g., the gel phase), that is accompanied by the appearance of a new, long lifetime component. Although the molecular origin of this solubility behaviour is largely unknown this has not precluded the use of the probe in a host of different biological processes [4]. Quite on the contrary, some properties as, e.g., the generation of a density-dependent long emission lifetime component have been exploited with success to reveal the presence of lipid domains and heterogeneity in synthetic bilayers and biological membranes [5–8].

Nevertheless, a detailed knowledge of the complex decay kinetics of *t*-PnA would facilitate considerably the development of further biological applications. One possi-

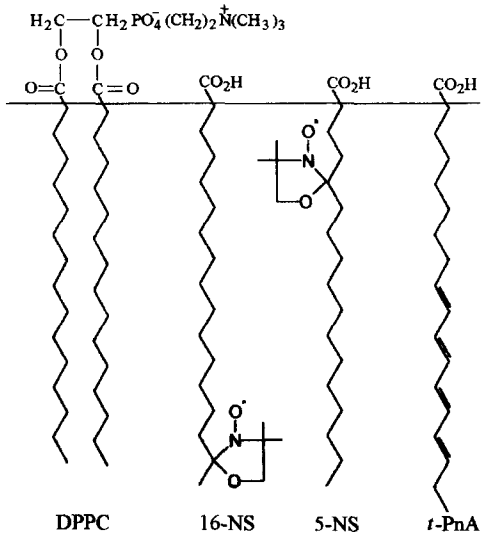

Fig. 1. A model representing a lipid membrane (DPPC) with incorporated 5-doxylstearic acid (5-NS), 16-doxylstearic acid (16-NS) and *trans*-parinaric acid (*t*-PnA).

* Corresponding author. Fax: +351 1 3524372; e-mail: prieto@alfa.ist.utl.pt.

SSDI 0005-2736(95)00251-0

ble explanation of the multiplicity of lifetimes might be that a significant fraction of probe molecules are not located within the membrane core. In fact, the presumed location of *t*-PnA is based in common chemical intuition and indirect evidence, rather than in direct observations. The purpose of this work is to provide such evidence from fluorescence quenching experiments, using lipophilic spin-probes that place the deactivating nitroxide group in a defined position within the membrane [9]. It is shown here that the emitting part of *t*-PnA is indeed buried in the lipid bilayer – as depicted in Fig. 1 – both in the gel and in the liquid-crystal thermal phases. Therefore, the processes giving rise to the complex decay kinetics had to be mostly intramolecular.

2. Materials and methods

trans-Parinaric acid (*t*-PnA), from Molecular Probes (Eugene, OR), was checked by HPLC and absorption and emission spectroscopy. The lipophilic nitroxides, 5-doxylstearic acid (5-NS) and 16-doxylstearic acid (16-NS), were also from Molecular Probes (Eugene, OR), and the saturated phospholipid L-α-1,2-dipalmitoyl-3-*sn*-phosphocholine (DPPC) from Sigma (St. Louis, MO), were used as received.

Small unilamellar vesicles (SUV) of DPPC were prepared by sonication (Branson, 40 W) of multilamellar lipid vesicles, until no significant decrease in the scattered light intensity of the suspension was observed. The SUV were then annealed for 10 min at 50°C, to decrease structural defects in the bilayer which could induce its fusion [10]. The final concentration of lipid in the aqueous Tris buffer (50 mM, pH 7.4, NaCl 10 mM) was $1 \cdot 10^{-3}$ M.

The incorporation of both the fluorescent probe (*t*-PnA) and the lipophilic quenchers (5- or 16-NS) into the lipid bilayer, was carried out by adding sequentially small aliquots from ethanol stock solutions, keeping the final concentrations of ethanol in the SUV dispersion below 2% (v/v). The final concentration of *t*-PnA was $1.76 \cdot 10^{-6}$ M (probe/lipid ratio of $\approx$ 1:600). The concentration of the stock solutions was determined from the absorption spectra (*t*-PnA, ϵ (299.4 nm) = $8.9 \cdot 10^4$ M^{-1} cm^{-1} [1]; nitroxide probes ϵ (430 nm) = 14 M^{-1} cm^{-1}, [11]).

The effective concentration of the nitroxide quencher in the DPPC lipid bilayer, $[Q]_L$ (mol of quencher per unit volume of lipid), is a linear function of the total quencher concentration, $[Q]_T$ (mol of quencher per unit volume of solution):

$$[Q]_L = C_L \cdot [Q]_T \quad (1)$$

The value of the conversion factor C_L can be determined from the quencher partition constant between the lipid and aqueous phases (K_Q), the lipid molar volume, v_L, and lipid molar concentration [L] as:

$$C_L = \frac{K_Q}{1 + (K_Q - 1) v_L [L]} \quad (2)$$

where

$$K_Q = [Q]_L / [Q]_W \quad (3)$$

($[Q]_W$ is the number of mol of quencher per unit volume of aqueous phase).

The values of the lipid/buffer partition constant K_Q for 5-NS and 16-NS were taken from Wardlaw et al. [12]. Lipid molar volumes for DPPC in the gel phase, $v_L = 0.82$ dm^3 mol^{-1}, and liquid-crystalline phase, $v_L = 0.95$ dm^3 mol^{-1}, were estimated from data described in Janiak et al. [13], Herbette et al. [14], and, Cornell and Separovic [15], Lewis and Engelman [16], respectively.

The contribution to the total fluorescence emission from the aqueous fraction of the fluorescent *t*-PnA is negligible, due to its small quantum yield and extremely low solubility in the buffer. Additional technical artefacts that can be present in stationary fluorescence experiments (geometry factor, inner filter effects), were accounted for in the data analysis by the usual methods as described elsewhere [17]. The experiments were carried out at 25°C (gel phase) and 50°C (liquid crystal phase) in 5×5 mm rectangular cuvettes, using excitation and emission wavelengths of 300 nm (stationary state) and 410 nm (stationary and time resolved), respectively.

Fluorescence lifetimes were determined by the single photon timing technique. The instrument was previously reported [18], specific instrumental details being described by Castanho et al. [19]. The 316 nm nitrogen line of a ns flash lamp was used for exciting the probe fluorescence and, in addition to the monochromators, band-pass (excitation) and cut-off (emission) filters were used to decrease the scattered-light background. In all cases 10^4 counts were accumulated in the peak channel, over a background level of less than 5 counts. The numerical analysis of the decay curves was carried out by standard least-squares methods.

3. Data analysis

The complex emission decay of most fluorescent probes incorporated into micro-heterogeneous systems (as a lipid bilayer) can be approximated by a discrete sum of exponentials:

$$I_o(t) = \Sigma a_{0,i} e^{-t/\tau_{0,i}} \quad (4)$$

The introduction of a quencher in the bilayer may give rise, in the general case, to a reduction of the emission lifetimes (dynamic quenching) and pre-exponential factors (static quenching). Thus, neglecting the time-dependent terms of the diffusion mechanism, the new intensity decay is given by:

$$I(t) = \Sigma a_i e^{-t/\tau_i} \quad (5)$$

The Stern-Volmer analysis of the lifetimes ($\tau_{0,i}/\tau_i$ vs. [Q], where [Q] is the local quencher concentration in the

vicinity of the fluorophore, as described later) would produce the individual quenching constants, $k_{q,i}$. Alternatively, these bimolecular rate constants can be derived from the Smoluchowski diffusion equation as:

$$k_q = \gamma 4\pi N_A R_{PQ} D_{PQ} \quad (6)$$

where R_{PQ} is the collisional radius, D_{PQ} the combined probe-quencher diffusion coefficient, γ the quenching efficiency and N_A the Avogadro's constant. The local quencher concentration at each membrane depth, [Q], is related to the overall concentration in the membrane $[Q]_L$, by:

$$[Q] = \beta[Q]_L \quad (7)$$

The factor β is introduced to account for the fact that in the bilayer volume the quencher molecules are not distributed homogeneously relative to the position of the fluorophore.

On the other hand, the 'static' contribution to the quenching mechanism, that is contained in the decreased pre-exponential terms, can be described by two limiting models [20]: either the formation of a ground-state complex or the so-called 'sphere of action'. Since, in the present case, there is no evidence supporting the association of ground-state *t*-PnA with the NS-quencher molecules in the lipid bilayer, the static quenching contribution would be approximated by an expression [21], based on the sphere-of-action model:

$$a_i = a_{0,i} e^{-\gamma V N_A [Q]_L \beta} \quad (8)$$

where V represents the volume of the sphere of action (with radius R). In this expression (Eq. 8) the concentration of the quencher in the total lipid bilayer, $[Q]_L$, is multiplied by the factor β. Thus, if the quenchers are located preferentially at the same bilayer depth as the emitting chromophore, the quencher concentration 'sensed' by the probe would be higher than the average value and, therefore, $\beta > 1$. On the contrary, if the quenchers are excluded from the vicinity of the probe, $[Q]_L$ values would be decreased by a $\beta < 1$. In consequence, the experimental determination of β would inform on the relative position of the probe-quencher pairs within the bilayer.

The decay of the fluorescence of the probe studied here (*t*-PnA) in lipid bilayers requires from two to three exponential terms, depending on the lipid thermal phase [18]. In this case, an accurate measurement of the individual parameters appearing in Eqs. 4 and 5 could not be carried out, due to the strong correlation between pre-exponential factors and lifetimes. Therefore, the two contributions (static and dynamic) to the quenching mechanism were determined in the following way: the dynamic fraction of the quenching was computed as the ratio of the areas under the decay curves of the probe emission (Eqs. 4 and 5), in the absence and in the presence of quencher:

$$\frac{I_{0,d}}{I_d} = \frac{\sum_i a_{0,i,n}\tau_{0,i}}{\sum_i a_{i,n}\tau_i} \quad (9)$$

where $a_{o,i,n}$ and $a_{i,n}$ are now the normalised coefficients describing the best fit of the sum of the exponential functions to the observed decay.

The static term can be obtained from the steady-state Stern-Volmer plot (I_0/I vs. $[Q]_L$), once the dynamic component is known:

$$\frac{I_0}{I} = \frac{I_{0,d}}{I_d} e^{\gamma V N_A [Q]_L \beta} \quad (10)$$

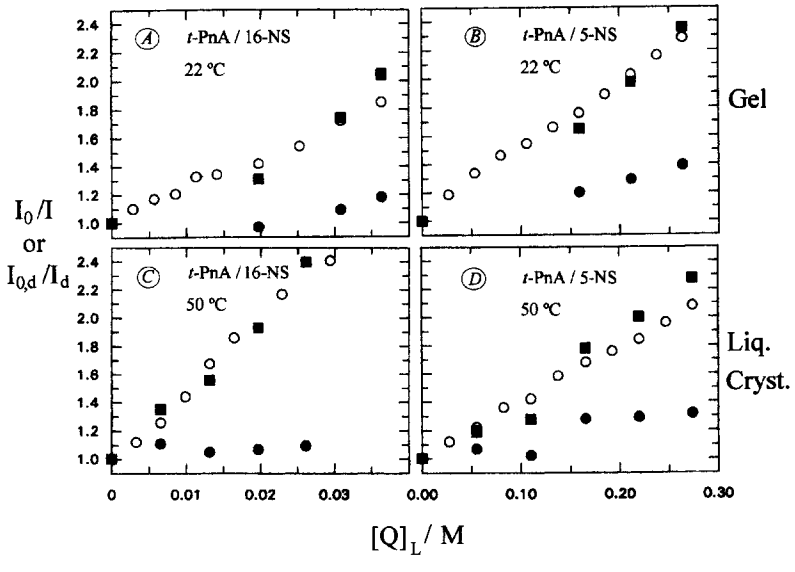

Fig. 2. The Stern-Volmer representation of the quenching of *t*-PnA fluorescence in SUV of DPPC as a function of 5-NS and 16-NS concentration, in the gel and liquid-crystalline lipid phases. ○, stationary emission I_0/I; ●, dynamic contribution $I_{0,d}/I_d$; ■, theoretical stationary ratio, computed with the sphere of action model (Eq. 10, $\gamma = 0.6$, $V = (4/3)\pi 12^3$ Å^3).

In this simple approach it is assumed that V and γ are identical for all probe sub-populations.

4. Results

The fluorescence spectrum and decay kinetics of *t*-PnA in SUV of the saturated lipid DPPC were similar to those reported before for large unilamellar vesicles of the same lipid [7]. Thus, at temperatures below the lipid thermal transition temperature ($T_m = 41°C$) where the bilayer is in the gel state, the fluorescence decay is described by the sum of three exponential terms ($a_1 = 0.27$, $\tau_1 = 1.49$ ns; $a_2 = 0.20$, $\tau_2 = 15.2$ ns; $a_3 = 0.53$, $\tau_3 = 39$ ns). These are reduced to two exponentials in the fluid, liquid-crystalline phase.

The addition of any of the two nitroxide quenchers 5-NS and 16-NS resulted in a decrease of the stationary fluorescence intensity and a less noticeable decrease of all lifetime components. On the other hand, neither the absorption nor the emission spectra of *t*-PnA showed significant changes.

When the lipids are in the gel phase the quenching by 16-NS of the stationary emission of *t*-PnA (I_0/I) is four times more effective than that produced by the 5-NS derivative (Fig. 2A–B). The difference between the two spin probes is even more apparent in the fluid phase (as seen from the difference in abscissa in Fig. 2C–D). It is also shown in Fig. 2 that the dynamic component of the quenching ($I_{0,d}/I_d$), determined by integration of the fluorescence decay as described above (Eq. 9), is negligible for 16-NS and very low for the 5-NS derivative.

5. Discussion

The data of Fig. 2 indicates that the nitroxide group is an efficient quencher of the fluorescence of *t*-PnA. The interaction mechanism is probably the enhanced intersystem-crossing mediated by the increased spin-orbital coupling via electron-exchange [22,23]. Due to the lack of a suitable common solvent and the low emission yield of the probe, the independent measurement of the intrinsic quenching efficiency (γ in Eq. 6) is not practical. Alternatively, it can be estimated from that of a water soluble pentaene (filipin) where a value of $\gamma = 0.5$ could be determined for the nitroxide quencher Tempol [23]. From the calculated ratio (1.2) of the exchange integrals [24] and the assumption that the quenching interaction is the same in *t*-PnA and filipin, a value of $\gamma = 0.6$ can be estimated [25] which points to a near unit quenching efficiency.

The methodology of using a family of quenchers placed at different depths in the bilayer to obtain information on the position of a fluorophore is based on the approximation that all the quenchers show identical diffusion coefficient (D) and intrinsic efficiency (γ). In consequence, the observed differences in the fluorescence quenching (either stationary or dynamic) can be traced back to differences in the local concentration of the quencher.

The nitroxide quenchers 16-NS and 5-NS are known to be located at the interior and surface respectively of the bilayer [26]. Although the spin labelled positions appear to lie closer to the exterior of the membrane than the analogous unlabeled acyl chain positions, 5-NS probes the membrane interface region while 16-NS probes the inner core region [26]. Even in fluid phase membranes and using the nitroxide group near the methyl end of the acyl chain (in both conditions a broader location distribution of the quencher is favoured [26,27]), the average position of the quencher moiety is clearly shifted towards the bilayer centre when compared to the average position of the nitroxide group in 5-NS [26]. According to Ellena et al. [26], the distance between the aqueous-hydrocarbon interface and the most likely position of the 16 nitroxide group is 2/3–1/2 times the distance between the interface and the 16 position of an unlabeled chain. Moreover, those workers estimated that a 30% decrease in the distance between any two of the spin label groups used for the determination of the fluorophore depth would result in a ≤ 2.5 Å change in the calculated depth. Such error does not affect the semi-qualitative information aimed in this work (*t*-PnA fluorophore main transverse location). Therefore, an inspection of Fig. 2 indicates that the *t*-PnA polyene should be in close vicinity to the 16-NS nitroxide group, both on the gel and fluid phases of the lipid bilayer and this conclusion is not hampered by the fact that 16-NS has a broader transversal distribution in the membrane than 5-NS [26,27]. The approach described by Eq. 9 gives a quantitative estimation of the dynamic contribution to the quenching reaction. Accordingly, for the 16-NS the quenching takes place entirely by a static process, while in the 5-NS this mechanism is not dominant.

With these data and the sphere-of-action model discussed before (Eq. 8) the β factors can be determined. Thus, by taking $\gamma = 0.6$ and a typical radius for the exchange interaction of $R = 12$ Å [20] it is found that for the fluid phase ($T = 50°C$), $\beta = 11$ for 16-NS and $\beta = 0.7$ for 5-NS. This large difference in the actual quencher concentration interacting with the fluorophore shows that the polyene system is located in the inner part of the membrane as depicted in Fig. 1. The corresponding β values for the gel phase (22°C) are 6 for 16-NS and 0.8 for 5-NS, indicating that for the fluorescent probe its location is almost invariant. The difference between the β values for 16-NS in both phases is significant, and is probably due to the opposed solubilities in the gel phase of the quencher and probe. As mentioned in the Introduction, *t*-PnA would be preferentially incorporated into the solid lipid domains while the decreased solubility of the 16-NS derivative in that medium [28] would tend to exclude the quencher that would accumulate at the interstitial bound-

aries and defects. This would be observed as an apparent decrease of the local concentration factor β.

6. Conclusion

A method to determine the transverse location of fluorescent probes in lipid bilayers from the quenching effect of nitroxide spin-probes is presented. This method has been applied to the quenching of *t*-PnA fluorescence by the 16-NS and 5-NS derivatives. It is shown that the polyene emitting part of *t*-PnA is essentially located in the central region of the bilayer, both in the gel and liquid-crystalline phases of the lipid.

Acknowledgements

This work was supported by 'Acção Integrada Luso-Espanhola' HP 48B (Portugal and Spain), and Projects STRD/SAU/257/92 (JNICT, Portugal) and PB93-126 (DGICYT, Spain).

References

[1] Sklar, A., Hudson, B.S., Peterson, M. and Diamond, J. (1977) Biochemistry 16, 813–819.
[2] Sklar, A., Hudson, B.S. and Simoni, R.D. (1977) Biochemistry 16, 819–828.
[3] Hudson, B.S., Harris, D.L., Ludescher, R.D., Ruggiero, A., Cooney-Freed, A. and Cavalier, S.A. (1986) in Application of fluorescence in the Biomedical Sciences (Taylor, D.L., Waggoner, A.S., Lanni, F., Murphy, R.F. and Birge, R., eds.), pp. 159–202, Alan R. Liss, New York.
[4] Hudson, B.S. and Cavalier, S.A. (1988) in Spectroscopic membrane probes (Loew, L.M., ed.), Vol. I, pp 46–62, CRC Press, Boca Raton.
[5] Ruggiero, A. and Hudson, B. (1989) Biophys. J. 55, 1111–1125.
[6] Mateo, C.R., Lillo, M.P., González-Rodríguez, J. and Acuña, A.U. (1991) Eur. Biophys. J. 20, 53–59.
[7] Mateo, C.R., Brochon, J.-C., Lillo, M.P. and Acuña, U. (1993) Biophys. J. 65, 2237–2247.
[8] Mateo, C.R., Acuña, A.U. and Brochon, J.-C. (1995) Biophys. J. 68, 978–987.
[9] Chattopadhyay, A. (1992) in Biomembrane structure and function – the state of the art (Gaber, B.P. and Easwaran, K.R.K., eds.), pp. 153–163, Adenine Press.
[10] Schmidt, C.F., Lichtenberg, D. and Thompson, T.E. (1981) Biochemistry 20, 4792–4797.
[11] Green, S.A., Simpson, D.J., Zhou, G., Ho, P.S. and Blough, N.V. (1990) J. Am. Chem. Soc. 112, 7337–7346.
[12] Wardław, J.R., Sawyer, W.H. and Ghiggino, K.P. (1987) FEBS Lett. 231, 20–24.
[13] Janiak, M.J., Small, D.M. and Shipley, G.C. (1979) J. Biol. Chem. 254, 6068–6078.
[14] Herbette, L.G., Napolitano, C.A. and McDaniel, R.V. (1984) Biophys. J. 46, 677–685.
[15] Cornell, B.A. and Separovic, F. (1983) Biochim. Biophys. Acta 733, 189–193.
[16] Lewis, B.A. and Engelman, D.M. (1983) J. Mol. Biol. 166, 211–217.
[17] Coutinho, A. and Prieto, M. (1993) J Chem. Ed. 70, 425–428.
[18] Farinha, J.P.S., Martinho, J.M.G., Xu, H., Winnik, M.A. and Quirk, R.P. (1994) J. Polymer Sci. Part B: Polymer Phys. 32, 1635–1642.
[19] Castanho, M.A.R.B., Coutinho, A. and Prieto, M. (1992) J. Biol. Chem. 267, 204–209.
[20] Eftink, M.R. (1991) in Topics in Fluorescence Spectroscopy (Lakowicz, J.R., ed.), pp. 53–127, Plenum Press, New York.
[21] Castanho, M.A.R.B. and Prieto, M. (1992) Eur. J Biochem. 207, 125–134.
[22] Hoytink, G.J. (1969) Acc. Chem. Res. 2, 114–120.
[23] Castanho, M.A.R.B. and Prieto, M. (1995) Biophys. J. 69, 155–168.
[24] Dexter, D.L. (1953) J. Chem. Phys. 21, 836–850.
[25] Weller, A. (1961) Prog. React. Kin. 1, 87–212.
[26] Ellena, J.F., Archer, S.J., Dominey, R.N., Hill, B.D. and Cafiso, D.S. (1988) Biochim. Biophys. Acta 940, 63–70.
[27] Davoust, J., Seigneuret, M., Hervé, P. and Devaux, P.F. (1983) Biochemistry 22, 3137–3145.
[28] Butler, K.W., Tattrie, N.M. and Smith, C.P. (1974) Biochim. Biophys. Acta 363, 351–360.