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FLUIDITY OF BACTERIAL MEMBRANE LIPIDS MONITORED BY INTRAMOLECULAR EXCIMERIZATION OF 1,3-DI(2-PYRENYL)PROPANE

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Intramolecular excimer formation of 1,3-di(2-pyrenyl)propane was used to study the fluidity of liposomes prepared from membrane polar lipids of *Bacillus stearothermophilus*. On the basis of spectral data, local polarity and polarizability parameters were established suggesting that the probe molecules are located well inside the membranes, but displaced towards the polar head groups of the phospholipid molecules. The excimerization rate is very sensitive to lipid phase transitions and pre-transitions of synthetic pure lipid bilayers. In bacterial lipids from cultures grown at 55 and 68 °C, thermal profiles of excimer to monomer intensity ratios (I'/I) show a broad transition which is displaced to higher temperatures in response to the increase of the growth temperature; these results correlate well with differential scanning calorimetry data and fluorescence polarization of diphenylhexatriene. Additionally, lipid bilayers of bacteria grown at 68 °C exhibit a decreased membrane fluidity[1], as monitored by both fluorescent probes.

Intramolecular excimer formation with 1,3-di(1-pyrenyl)propane [$1Py(3)1Py$] has been used successfully in our and other laboratories to probe membrane fluidity (1-4). However, studies about its fluorescence decay behaviour indicate that one monomer and two excimers are involved in the excimerization process (5). Conversely, a similar probe, 1,3-di(2-pyrenyl)propane [2Py(3)2Py], shows a simpler kinetics, with only one excimer and one monomer (5), offering, in principle, advantages to monitor fluidity changes in biological membranes.

Thermotropic changes of membrane lipid components have deserved increased interest to understand structural and functional aspects of biomembranes, including in thermophilic microorganisms. We assessed the usefulness of 2Py(3)2Py as a fluidity probe using models of synthetic phospholipids and lipid membranes of the thermophile *Bacillus stearothermophilus*. Thermal profiles of lipid models revealed that the

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[1]The term fluidity is used here in an operational sense and defined as being directly proportional to the rate of excimer formation by 2Py(3)2Py or to the fluorescence depolarization of DPH. This fluidity is related but not identical with the physical definition of fluidity.

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excimerization rate of 2Py(3)2Py is very sensitive to lipid phase transitions and pre-transitions, paralleling the results obtained by differential scanning calorimetry (DSC) and fluorescence polarization of diphenylhexatriene (DPH).

MATERIALS AND METHODS

Lipids were extracted as previously described (6) and the polar lipids were isolated by preparative thin layer chromatography developed in acethone. Liposomes were prepared from aliquots of pure phospholipids or bacterial polar lipid extracts in chloroform, evaporated to dryness in round bottom flasks. The lipid film was then hydrated with an appropriate volume of 50 mM KCl, 10 mM Tris-maleate, pH 7.0, to obtain a nominal lipid concentration of 0.6 mM. The lipid suspension was obtained by hand-shaking in a water bath set at a temperature well above that of the transition phase of the phospholipids. The suspension was then vortexed, for 1 min, and briefly sonicated in a water bath to disperse large lipid aggregates.

Probe incorporation was carried out by adding aliquots of a 2Py(3)2Py ethanolic solution ($4x10^{-5}$ M) or of a DPH tetrahydrofuran solution ($2x10^{-3}$ M), while vortexing, to the lipid suspensions to give a final probe concentration of $7x10^{-7}$ M or $3x10^{-6}$ M, respectively. The mixtures were then incubated, in the dark, overnight, with gentle stirring, at a temperature above the phase transition of the lipid components. Blanks, always prepared under identical conditions, receiving equivalent volumes of ethanol or tetrahydrofuran served as controls for the fluorometric measurements. These measurements were carried out with a Perkin-Elmer spectrofluorometer, model MPF-3, provided with a thermostated cell holder. The excitation wavelength was 338 nm (4 nm excitation and 6 nm emission slits) for 2Py(3)2Py fluorescence spectra. An emission cut-off filter of 350 nm was used. The signals at 480 nm (excimer) and of the first monomer peak (375 nm) were measured to calculate the excimer to monomer fluorescence intensity ratio (I'/I). For DPH fluorescence polarization measurements, the excitation was set at 336 nm and the emission at 450 nm (4 nm excitation and 7 nm emission slits). The fluorescence polarization was measured as reported elsewhere (7). All these measurements were always corrected for the contribution due to the light scattering by using appropriate blanks.

Lipid pellets for calorimetric studies were sealed in aluminium pans and heating scans, over the temperature range of 5 to 60 °C, were carried out on a Perkin-Elmer DSC-7 (Delta series) at a scan rate of 10 °C min^{-1} and a sensitivity of $2.4x10^{-3}$ mcal s^{-1}. The reference pan contained buffer used in lipid suspensions.

The phospholipids were quantified by measuring the amounts of inorganic phosphate (8) after hydrolysis of the extracts at 180 °C in 70% $HClO_4$ (9). The viscosity of liquid paraffin (Merck Uvasol, batch 8546239, used as received) was determined with a thermostated Hoppler viscometer at the desired temperatures. The density of paraffin was measured with a pycnometer (10).

RESULTS AND DISCUSSION

Fluorescence spectra of 1,3-di(2-pyrenyl)propane: Correlation with macroscopic viscosity

The fluorescence spectra of 2Py(3)2Py incorporated into membranes (synthetic phospholipids or bacterial lipid membranes) are similar to those in liquid paraffin (Fig. 1). The emission spectra consist of contributions from both monomer (I) and intramolecular excimer (I') emissions. These spectra are reproducible in different preparations of membrane systems and do not change by dilution of the probe in the lipid suspensions, suggesting the absence of intermolecular excimers.

The intramolecular excimerization rate increases with temperature (Fig.1) from 10 to 30 °C, with subsequent increase of the excimer to monomer fluorescence intensity ratio

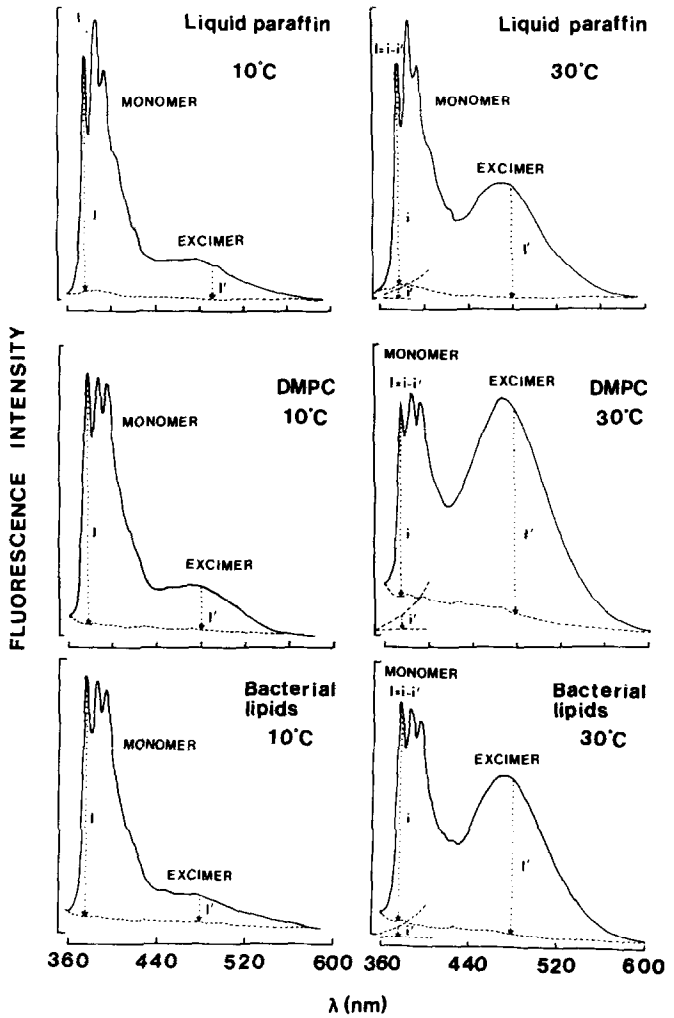

Figure 1. Fluorescence spectra of 2Py(3)2Py, solubilized in liquid paraffin and incorporated into DMPC or bacterial polar lipid bilayers, were recorded at 10 and 30 °C. Blank controls (without probe) are represented by the dashed lines. For high I'/I ratios, the monomer intensity is corrected by subtracting the contribution of the excimer (corrected monomer intensity, I = i - i').

(I'/I), either in liquid paraffin (from 0.15 to 0.54) or in dimyristoylphosphatidylcholine (DMPC) bilayers (from 0.18 to 1.12) or bacterial lipid membranes (from 0.11 to 0.74). At these temperatures, DMPC and bacterial lipid membranes are below (10 °C) or above (30 °C) the main phase transition temperature (see Figs. 3 and 4), corresponding to different membrane fluidity states, as reflected by the fluorescence intensity ratio (I'/I). This parameter is very sensitive to the fluidity of the probe environment in an isotropic medium. Indeed, a good correlation of I'/I with the macroscopic viscosity (cPoise) of liquid paraffin was observed, in agreement with observations for the similar probe, 1Py(3)1Py (1), although we realize that an isotropic solvent is not equivalent to the anisotropic medium of membranes.

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VASCULAR A10 CELL MEMBRANES CONTAIN AN ENDOTHELIN METABOLIZING NEUTRAL ENDOPEPTIDASE

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We have investigated the possible presence of endothelin-metabolizing neutral endopeptidase (NEP, EC 3.4.24.11) on A10 cell membranes using [^{125}I]-ET-1 binding and direct measurements of NEP. NEP activity of A10 cell membranes has been compared to that of solubilized rat kidney brush border membranes (KNEP). Specific [^{125}I]-ET-1 (50 pM) binding (defined with 100 nM ET-1) to A10 cell membranes was increased in a concentration dependent manner by the selective NEP inhibitors thiorphan, phosphoramidon, and SQ 28,603 {(±)-N-[2-(mercaptomethyl)-1-oxo-3-phenylpropyl]-ß-alanine} with EC_{50} values of 9.4, 28.4, and 5.7 nM respectively. At equilibrium (150 min), 70% more specific binding was apparent in the presence of these inhibitors. Phosphoramidon (2 µM) did not alter B_{max} values, but it decreased the apparent K_D for [^{125}I] ET-1 from 63 (±3) to 27 (±2) pM. Thiorphan, phosphoramidon, and SQ 28,603 inhibited A10 cell NEP activity with IC_{50} values of 5.3, 36.5, and 6.0 nM respectively, which was similar to values obtained with KNEP (3.6, 22.6, and 3.5 nM). ET-1 inhibited A10 cell NEP, and KNEP with IC_{50} values of 30 and 21.3 µM respectively. The order of inhibitory potencies: ET-3 > ET-1 = ET-2 ≥ sarafotoxin-6b was similar for both systems.These data suggest A10 cell membranes contain a NEP which has similar characteristics to NEP 24.11, and which actively metabolizes [^{125}I]-ET-1.

Endothelin (ET)-1 is a recently discovered 21-amino acid containing peptide with two intramolecular disulfide bonds which provide for significant structural rigidity. A structurally related peptide sarafotoxin is present in the venom of the Israeli burrowing asp, and this peptide exhibits most of the actions of ET-1 (1). ET-1 is a member of a family of endothelins (ET-2, ET-3) which have distinct distribution and may represent the agonists for a related family of ET receptors (2). ET-1 is the most potent vasoconstrictor peptide known, and its production and metabolism may represent important steps in the regulation of local levels of this vasoconstrictor peptide. ET-1 is synthesized from big-ET and constitutively released from vascular endothelial cells (3-

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intramolecular excimerization, a reasonable correlation was obtained between the intensity ratio I_{375}/I_{385} and the polarity-polarizability parameter, in several reference solvents (Fig. 2B). It should be pointed out that the inconvenience of excimerization was circumvented by correcting the excimer band contribution in the monomer peaks at 375 and 385 nm. A similar correlation has been obtained for pyrene and derivatives (4-11). However, as compared with these probes, the dependence of the monomer fluorescence spectrum on the solvent polarity, i. e., the Ham effect (12), is barely detectable with 2Py(3)2Py, as also reported for the similar probe 1Py(3)1Py (4,11); this fact makes these two probes more suitable for fluidity measurements, since I'/I ratios are not affected by the possible differences in polarity of different membrane systems.

Despite the low sensitivity to the surrounding medium polarity, 2Py(3)2Py incorporated into DMPC, DOPC (dioleylphosphatidylcholine) or bacterial lipid membranes reports a polarity parameter close to that observed for the polar solvents methanol and ethanol (Fig. 2B). Therefore, these findings agree with the results reported for other probe analogues (4,11), suggesting that 2Py(3)2Py is also located inside the membrane, not in the very hidrophobic core, but displaced toward the polar head groups of phospholipid molecules.

Intramolecular excimer formation in synthetic and bacterial lipid liposomes

The excimerization rate is very sensitive to lipid phase transitions as shown in the thermal profiles of dimyristoyl-, dipalmitoyl- and distearoylphosphatidylcholine (DMPC, DPPC, DSPC) (Fig. 3). In all cases, pre and main transitions are accompanied with sharp increases in I'/I (Fig. 3) or as discontinuities in Arrhenius plots (Fig. 3, inset) at temperatures very close to transitions reported in literature (13). At the same temperature, particularly below the main phase transition, the intensity ratio I'/I increases from DSPC to DPPC and DMPC indicating that the fluidity in the ordered low-temperature phase is much smaller in DSPC than in DPPC and DMPC (2). In addition to the main transition, pre-transitions, from $L\beta'$ to $P\beta'$, are also detected by changes in fluidity (Fig. 3) accompanying the change in geometry arrangement of lipid chains and consequent increase in *gauche* conformations (13).

In bacterial lipid bilayers, the excimerization rate of 2Py(3)2Py detects a broad transition, extending from 14 to 36 °C (Fig. 4A). This is depicted in both thermal profiles of I'/I for liposomes prepared from polar lipid extracts of *B. stearothermophilus* grown at 55 °C (suboptimal temperature) and 68 °C (supraoptimal temperature for growth). Furthermore, these thermograms indicate that the fluidity, in terms of the fluorescence intensity ratio I'/I, is considerably lower in the higher temperature-lipid extract, either below or above the phase transition. Similar considerations can be drawn from thermograms of DPH fluorescence polarization (Fig. 4B).

By using differential scanning calorimetry of the same bacterial lipid extracts, we detected also a broad transition (Fig. 4C) wich is shifted to higher temperatures with the increase of the growth temperature. In these thermograms, four critical temperature values were defined: the lower boundary of the phase transition, T_i, the temperature of the onset

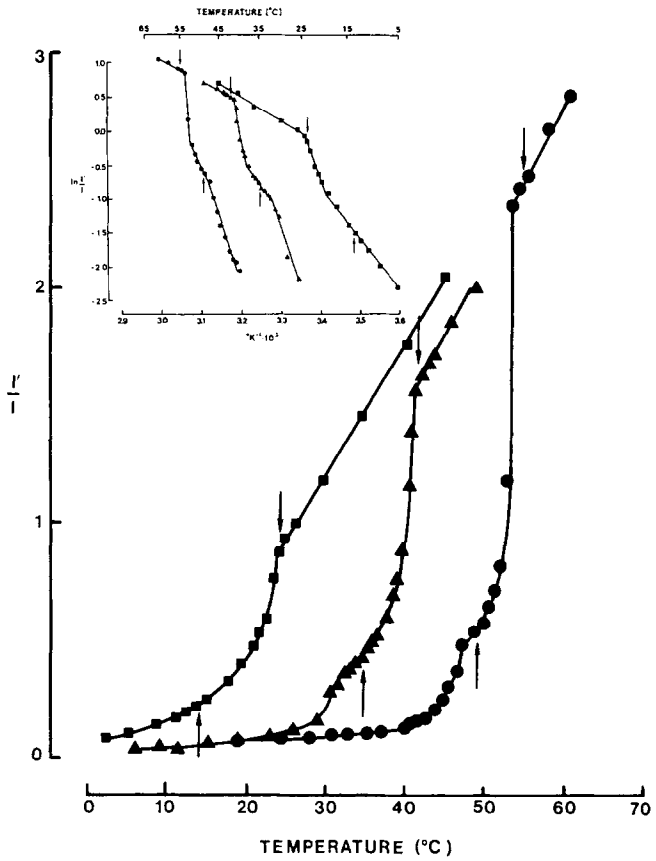

Figure 3. Excimer to monomer intensity ratio, I'/I, of 2Py(3)2Py incorporated in DMPC (■), DPPC (▲) and DSPC (●) membranes, as a function of temperature. The inset depicts the Arrhenius plots derived from the same data. The downward arrows indicate the main phase transition temperatures and the upward arrows, the pre-transition temperatures taken from ref.13.

of the transition peak, extrapolated to the baseline, T_o, the temperature at the endotherm peak, T_m, and the temperature determined by the intersection of the tangent to the decaying half of the peak to the baseline, T_f. These temperatures are also reflected in specific points in the thermograms obtained by fluorescence techniques (Fig. 4 and Table I). T'_i and T'_f spot the extreme temperatures where abrupt changes of the fluorescence parameter (I'/I ratio or polarization index) occur. T'_o can be noticed when the thermogram shows a two-step change of the I'/I ratio or polarization index, during the phase transition, and it corresponds to the intersection of two different slopes. T'_m reflects the midpoint of the main transition and it can be roughly estimated at the mid-distance between T'_f and T'_o. These characteristic temperatures determined by fluorescence techniques are reasonably coincident to those defined in DSC thermograms (Table I). It is of interest to note that T_m and T_i determined by DSC data correspond to the temperatures at which discontinuities of the Arrhenius plot of the excimer to monomer fluorescence intensity ratio, I'/I, are observed (Fig. 4A, inset). Therefore, either fluorescence or calorimetry denote a displacement of the main transition to higher temperatures, as the

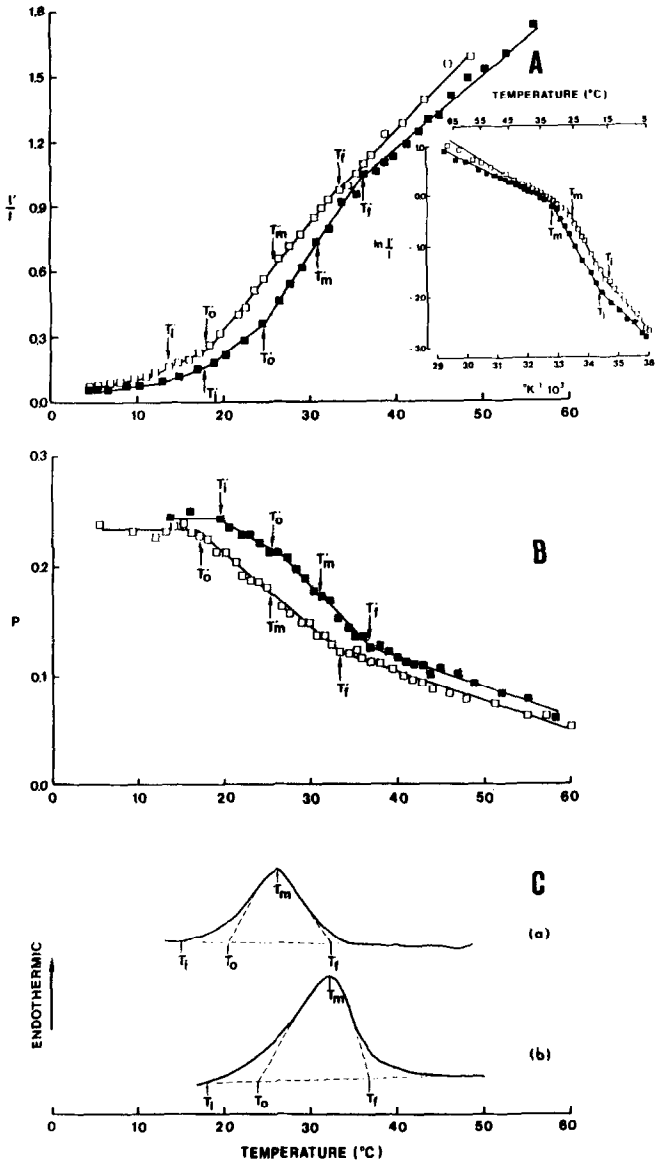

Figure 4. Excimer to monomer intensity ratio, I'/I, of 2Py(3)2Py (**A**) or fluorescence polarization of DPH (**B**), as a function of temperature, in liposomes from polar lipid extracts of *B stearothermophilus* grown at 55 °C (□) and 68 °C (■). Arrows indicate temperatures T'_i, T'_o, T'_m and T'_f (see Table I and text) and the inset depicts the Arrhenius plot of I'/I values, where the arrows indicate the phase transition temperatures, T_m and T_i, as determined by DSC in **C**. **C**. DSC of liposomes obtained from 55 °C (a) and 68 °C (b) lipid extracts. Arrows denote temperatures T_i, T_o, T_m and T_f (see Table I and text).

growth temperature increases from 55 to 68 °C. These findings are significant to the thermoadaptation mechanism of *B. stearothermophilus* and will be explored in detail in a following paper.

In conclusion, intramolecular excimerization of 2Py(3)2Py has shown to be a useful tool to probe thermotropic changes and fluidity of membrane lipids of *B.*

Table I - Characteristic temperatures of the phase transition in liposomes obtained from the polar lipids of cells grown at 55 and 68 °C. The table presents temperatures described in text, estimated by three different techniques: differential scanning calorimetry (DSC), DPH fluorescence polarization and 2Py(3)2Py intramolecular excimerization.

Growth Temp (°C)	DSC				DPH				2Py(3)2Py			
	T_i	T_o	T_m	T_f	T'_i	T'_o	T'_m	T'_f	T'_i	T'_0	T'_m	T'_f
55	15.2	19.5	25.7	32.7	-	17.8	25.6	33.5	13.8	18.3	25.9	33.5
68	17.5	24.0	32.0	36.9	19.5	25.8	31.1	36.5	17.8	25.3	30.8	36.3

stearothermophilus. Differential scanning calorimetry data provide important information about the gel to liquid phase transition but, additionally, intramolecular excimerization indicate changes in the organization of the membrane lipids expressed by the fluidity parameter I'/I.

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