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SUBZERO TEMPERATURE STUDY OF THE INNER MITOCHONDRIAL MEMBRANE AND RELATED PHOSPHOLIPID MEMBRANE SYSTEMS WITH THE FLUORESCENT PROBE, *trans*-PARINARIC ACID

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Summary

The fluorescence intensity of *trans*-parinaric acid as a function of the temperature indicates a phase transition in bovine heart mitochondrial inner membranes below 0°C. The comparison of the dye fluorescence intensity in intact inner mitochondrial membranes and in vesicles from extracted phospholipids of mitochondria revealed a similar intensity increase with decreasing temperature. A synthetic phospholipid system of dioleoyl phosphatidylcholine was investigated because of its low phase transition temperature and showed a very definite intensity change at −25°C. *trans*-Parinaric acid in membrane systems probes an environment of intermediate polarity; this was found from the excitation and emission spectra and from fluorescence decay.

Introduction

Selected physical parameters of fluorescence and EPR probes have been used to characterize phases and phase transitions of mitochondrial membranes [1,2] and related but protein-free phospholipid systems [1,2]. Experiments with extrinsic fluorescence probes have been reported from the physiological temperature range and down to 0°C. Kinetic measurements of the mitochondrial enzyme activity have recently been extended to subzero temperatures using aprotic solvents to permit the evaluation of reactions and the identification of intermediate compounds which cannot easily be observed at room temperature. Since the presence of phospholipids is essential for enzymatic

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processes in the inner mitochondrial membrane phospholipid phase transitions and changes in mitochondrial functions have been related above 0°C but not below.

In this report we describe a change in the physical state of the mitochondrial lipid and membrane at subzero temperatures using the fluorescent probe *trans*-parinaric acid. Since the time-resolved fluorescence emission is multi-exponential in all the biologically relevant heterogeneous lipid systems discussed here we also studied the dye-solvent effect between *trans*-parinaric acid and a series of pure solvents and simple solvent mixtures.

Methods

Bovine heart mitochondria were isolated using the procedure of Low and Vallin [3] and the outer membrane was removed by osmotic shock treatment [4]. Mitochondrial phospholipids were extracted from the mitoplasts using ten vols. of chloroform/methanol 2 : 1 containing 5% of 28% NH_4OH [5] and homogenizing in a glass-teflon homogenizer after gasing the solutions with argon to remove oxygen. The residue was then extracted with deoxygenated chloroform/methanol 7 : 1 containing 5% of 28% NH_4OH and partitioned according to Folch et al. [6]. The chloroform phase containing the phospholipids was then flash-evaporated to dryness at 20°C, resuspended in benzene and stored under argon at −15°C until use. Liposomes of synthetic dioleoyl phosphatidylcholine (Sigma Chemicals) or isolated inner membrane phospholipids were prepared by adding medium containing 225 mM mannitol/75 mM sucrose/20 mM phosphate buffer (pH 7.4) and dispersed with a Branson sonifier for 1 min under argon. The suspension was diluted to 50% with ethylene glycol (Baker) to prevent freezing at subzero temperatures. *trans*-Parinaric acid (Molecular Probes Inc.) was added to deoxygenated suspensions of liposomes or mitochondrial membranes at 25°C from an ethanol stock solution (0.5 mg *trans*-parinaric acid/ml ethanol under argon). The probe to phospholipid ratio was approximately 1 : 100 in all systems.

The fluorescence decay of *trans*-parinaric acid was determined with a photon counting apparatus. A gated ns flashlamp (Photochem. Res. Assoc., 20 to 30 kHz repetition rate, pulse duration at 10% maximum, 6 ns) and fast electronic modules were used: Ortec 454 and 474 timing amplifiers, 463 and 473A constant fraction discriminators, 425 and 425A delay units, a 457 time-to-pulse-height converter and a LeCroy 3001 multichannel analyzer. The total number of detected fluorescence (stop) pulses was determined per 900 000 lamp (start) pulses in preset time ranges after the lamp peak intensity using a single-channel analyzer (Ortec 420A) and a dual counter (Ortec 715). The time range was typically from 50 to 500 ns and the integrated intensity was determined as a function of the temperature. The fluorescence detection probability (i.e. number of stop to number of start pulses ratio) was kept below 3% thus providing undistorted fluorescence decay curves [7]. The multi-exponential fits of the decay curves to determine decay times were performed by Laplace transform analysis and simulation. A Hewlett-Packard 9825A desk calculator was fed from the digital data output of the multichannel analyzer to permit the

transfer of lamp profile and decay curves [8]. The time base calibration was made with the nanosecond delay units.

Steady-state excitation and emission spectra were taken with a Perkin-Elmer MPF-2A fluorimeter with a Hamamatsu 928 photomuliplier and with a 650-10S fluorimeter.

The sample temperature was regulated using a low temperature cuvette holder and sample compartment (Photochem. Res. Assoc.) connected to a refrigerating circulating bath. At temperatures below room temperature the cuvette holder was flushed with dry nitrogen gas which was cooled by a liquid nitrogen heat exchanger.

Results

Effects of pure solvents on the trans parinaric acid fluorescence

trans-Parinaric acid absorption exhibits solvent polarity dependent spectral changes [9]. We asked the question whether the spectral qualities such as fluorescence excitation and emission spectra are sufficient to determine the probe environment polarity or polarizability in heterogeneous membranes. Therefore we analyzed the fluoresence in a series of homogeneous solvents with a broad range of known parameters (refractive index, dielectric constant, density, viscosity and molecular weight). A summary of the results is compiled in Table I. Red shifts in the excitation, blue shifts in the emission and increased fluoresence decay rates indicated dye-solvent interaction due to solvent

TABLE I

SOLVENT EFFECT ON *TRANS*-PARINARIC ACID STEADY-STATE AND TIME-RESOLVED FLUORESCENCE [a]

n, solvent refractive index.

	Solvent dielectric constant ϵ	
	Low ($\epsilon < 5$)	High ($\epsilon > 9$)
High ($n > 1.43$)		
Excitation spectrum [b] (nm)	295, 308, 322 nm	311, 324 nm
Emission spectrum [c] (nm)	405—415 nm, normal	405—415 nm, narrow
Fluorescence decay time ranges [d]	6—8 ns, 2 ns	6—8 ns, 1.6 1.9 ns
Number of solvents probed	6	3
Examples	Benzene, Toluene	*o*-Dichlorobenzene, ethylene glycol
Dye-solvent interaction	strong	very strong
Primary effect of solvent	Wavelength shifts	Wavelength shifts and decay changes
Low ($n < 1.43$)		
Excitation spectrum [b]	278, 289, 301, 314 nm	278, 289, 300, 314 nm
Emission spectrum [c]	410—420 nm, broad	415—425 nm, normal
Fluorescence decay time ranges [d]	6—12 ns, 3—4 ns	6—10 ns, 1.6—1.8 ns
Number of solvents probed	9	4
Examples	*n*-Hexane, 1,4-Dioxane	Methanol, *n*-Hexanol
Dye-solvent interaction	weak	strong
Primary effect of solvents	none	Decay changes

Summary of 17 pure solvents and 5 simple solvent mixtures at room temperature.
Wavelength positions of excitation maxima ± 1 nm.
Wavelength position of emission maximum, width determined at 10% of maximum intensity.
Double exponential fits with simulation.

polarity [10]. In low dielectric constant solvents the increase in the refractive index primarily affects the spectra but not the decay rates. In low refractive index solvents the increase in the dielectric constant primarily affects the decay rates. In highly polar and polarizable solvents (refractive index 1.43 and dielectric constant >9) both steady-state and time-resolved fluorescence are affected. The fluorescence decay rates are also solvent density (density range from 0.6 to 1.5 g/cm^3) and viscosity (from 0.3 to 0.7 cP) dependent.

Steady-state excitation and emission spectra of trans-parinaric acid in phospholipid and bovine heart mitochondria

The wavelength of maximum emission was 410 nm (excitation 320 nm) and did not change as the temperature was lowered from +20°C to —20°C. It was the same for all three lipid systems, mitochondrial membranes, extracted phospholipid vesicles and dioleoyl phosphatidylcholine vesicles. At 20°C the uncorrected emission spectrum had a width (full width at 50% of intensity maximum) of 80—90 nm and at subzero temperatures 60—70 nm. The excitation peaks at 310 and 320 nm did not change as the host system (lipid vesicles to intact inner membranes) was changed or by alteration of the temperature. However, we found a change in the relative excitation peak intensity 310—320 nm from 1.3 : 1 at +20°C to 0.9 : 1 at —20°C in intact inner membranes.

Fluorescence decay measurements of trans-parinaric acid in phospholipid and mitochondrial membranes

The fluorescence decay curves were not single exponential as a function of the time in the range up to 800 ns. The curves were simulated by considering three components with different decay times, i.e. 3.0 ns, 9.5 ns and 50 to 70 ns depending on the temperature. Free *trans*-parinaric acid in the ethylene glycol/buffer solution proved to contain the fast and the intermediate component but not the slow component. The relevant information of *trans*-parinaric acid in mitochondrial membranes and phospholipid systems could only be obtained by consideration of the slow decaying component as a function of the temperature. At 30, 10, —10 and —30°C the decay time in inner mitochondrial membranes was determined to be 59, 62, 66 and 70 ns (±2 ns), respectively (Fig. 1). This increase in fluorescence lifetime is not sufficient to explain the intensity increase with lowered temperature found with continuous and pulsed excitation. The integrated intensity in the time range from 50 to 500 ns after the excitation pulse (see Methods) proved to be a useful parameter for probing the change in physical state of the lipid and membrane systems as a function of the temperature (Fig. 1).

trans-Parinaric acid probes a highly heterogeneous system. Its fluorescence decay time is sensitive to temperature changes and changes in the solvent system composition. The dominant effect, however, is the change in quantum yield or intensity due to the phase transition from the fluid phase [11] or disordered, liquid-crystalline state [12] to the lipid plus fluid eutectic phase [11] or ordered, quasi-crystalline state [12] (see below).

Temperature profiles of fluorescence in phospholipid dispersions and mitochondrial inner membranes

The fluorescence of *trans*-parinaric acid in liposomes of dioleoyl α-L-phos-

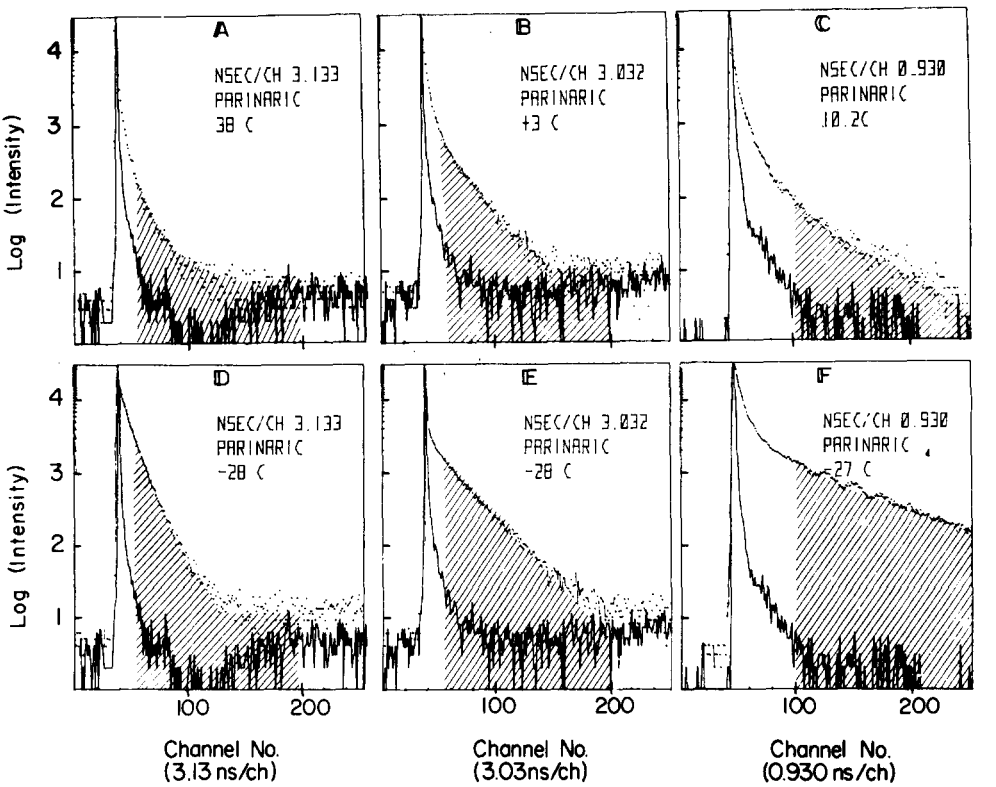

Fig. 1. Fluorescence decay of *trans*-parinaric acid in homogeneous and heterogeneous membrane systems at different temperatures. The shaded areas under the curves show the time domain of intensity integration after the excitation pulse from 50 to 500 ns. Dioleoyl L-phosphatidylcholine vesicles at 38°C (curve A) and at −28°C (curve D); extracted phospholipid vesicles from bovine heart inner mitochondrial membranes at 3°C (curve B) and at −28°C (curve E); intact bovine heart inner mitochondrial membranes at 10°C (curve C) and at −27°C (curve F). Dye to phospholipid ratio 1 : 100. Excitation filter, Perkin-Elmer UV2; emission filter, Schott KV399; photomultiplier, RCA 1p28 operated at −900 V.

phatidylcholine had a definite increase at −25°C (Fig. 2A). Similar to that observed for the transition from the disordered (fluid, liquid-crystalline) arrangement to the ordered (lipid plus fluid eutectic, quasi-crystalline) arrangement of phospholipids with saturated fatty acyl residues at higher temperatures [13]. The transition temperature for dioleoyl phosphatidylcholine is lowered by 3°C in the presence of ethylene glycol compared with differential scanning calorimetry data (−22°C, [14]) for the phospholipid in aqueous medium without the organic solvent.

Addition of *trans*-parinaric acid to 50% ethylene glycol/buffer solution without phospholipid showed a small temperature dependent increase in probe fluorescence at lower temperatures indicating minimal probe-solvent interaction (Fig. 2D).

Liposomes of extracted mitochondrial inner membrane phospholipids containing *trans*-parinaric acid showed an increase from 10 to 90% of the observed integral intensity (50—500 ns range) in the temperature range from +5 to −28°C with an apparent midpoint around −14°C. Both dioleoyl phosphatidylcholine and extracted mitochondrial phospholipids had a cooling and heating

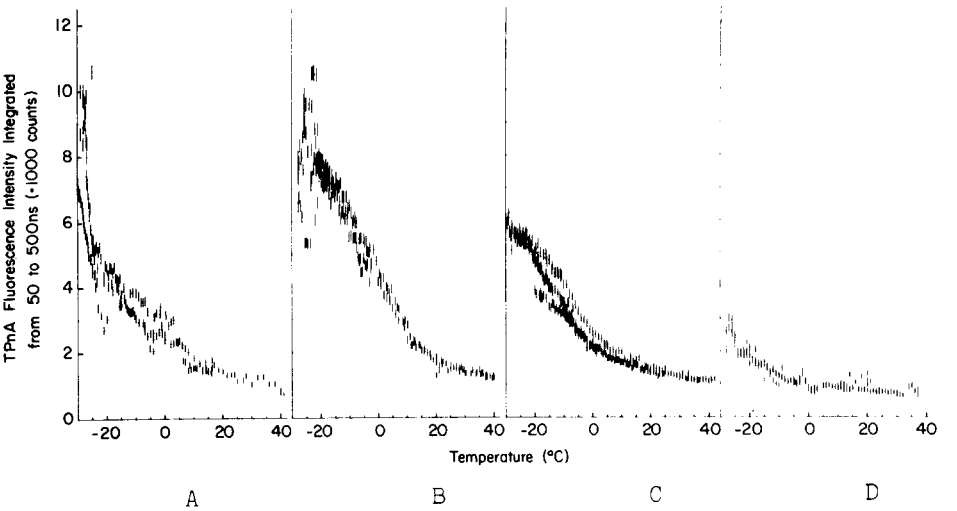

Fig. 2. Fluorescence intensity of *trans*-parinaric acid (TPnA) in homogeneous and heterogeneous systems, time domain 50 to 500 ns. L-α-dioleoyl phosphatidylcholine vesicle suspension (0.1 mg phospholipid/ml), curve A; same dye in extracted phospholipid vesicles from bovine heart inner mitochondrial membranes (0.1 mg/ml), curve B; intact bovine heart inner membranes (2 mg protein/ml), curve C; ethylene glycol/ buffer solution, curve D. Dye to lipid ratio 1 : 100, dye concentration 1.3 μM. Experiment description see Fig. 1 legend.

dependent hysteresis effect at temperature scan rates of more than 0.3°C/min (Fig. 2B).

When the intact inner mitochondrial membrane sample was cooled from +25°C to −30°C there was an intensity increase from 10 to 90% in the temperature range from +10 to −22°C with a midpoint at −10°C (Fig. 2C). If

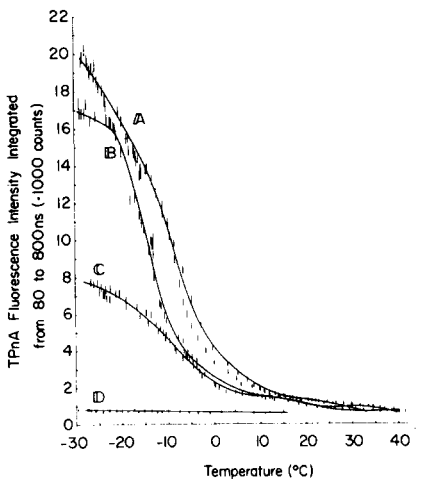

Fig. 3. Fluorescence intensity of *trans*-parinaric acid (TPnA) in bovine heart mitochondrial inner membranes as a function of the temperature. Data bars represent the number of detected fluorescence flashes per 900 000 excitation pulses within 80—800 ns after the excitation including the statistical error. The sample was cooled (curve A), heated (B) and cooled (C) at an average rate of 0.3° C/min, 2 mg protein/ ml, dye to lipid ratio 1 : 100, (D) solvent-buffer-dye control. Other experimental descriptions see Fig. 1 and 2 legends.

the sample was then heated to 42°C and subsequently recooled to −30°C, the temperature dependent fluorescence intensity increase did not exceed 50% of the initially observed maximum (Fig. 3). Since no such irreversible changes in probe response occur in the isolated mitochondrial phospholipid-vesicle suspension the high temperature heating effect may represent protein denaturation which in turn influences the probe environment.

Discussion

trans-Parinaric acid probes an environment of intermediate polarity (refractive index 1.42—1.44) and low polarizability (dielectric constant <5). This was found from the spectra and the decay in the time window up to 100 ns. The spectral shifts reflect changes of the accessible electronic states during excitation and emission. Very strong dye-solvent interaction which is found in highly polar solvents and which changes the transition probabilities such as decay rates is not observable in lipid or membrane systems.

The subzero temperature dependent changes in fluorescence intensity coincides with the enthalpy changes reported for rat liver mitochondria membrane as monitored by differential scanning calorimetry [15]. The alterations in membrane physical character appear to be related to the state of the membrane phospholipids since isolated phospholipids and intact inner membranes have the same temperature dependent change.

Other phase changes in the disordered (fluid or liquid-crystalline) arrangement of phospholipids at higher temperatures (10—42°C) are not detectable. Both scanning calorimetry and fluorescence detection with viscotropic probes [2] fail to reveal alteration in both membranes. In contrast to these techniques EPR nitroxide probes show definite alterations in probe motional parameters in intact membranes and isolated phospholipid suspensions [1]. We conclude that *trans*-parinaric acid fluorescence in the time range above 50 ns probes an environment which is different from the EPR detectable quality of the membranes discussed here.

There must be a tight relationship between the transition from disordered (fluid, liquid-crystalline) to ordered (lipid-fluid eutectic phase, quasi-crystalline) membrane arrangement and the function of mitochondrial enzymatic processes. Mitochondrial dehydrogenase activity is attenuated in this temperature range [16]. Other mitochondrial key functions including ADP-stimulated respiration, calcium uptake and membrane energization (Chance, B., Nakase, Y. and Itshak, F., unpublished data) undergo drastic rate changes in the 0 to −20°C range. Therefore, changes in enzymatic processes may be related to the phospholipid physical state which is probed by *trans*-parinaric acid.

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