ELSEVIER

Biochimica et Biophysica Acta 1239 (1995) 213–225

BBA Biochimica et Biophysica Acta

Metastability of dimiristoylphosphatidylethanolamine as studied by FT-IR and the effect of α-tocopherol

Josefa Salgado, José Villalaín *, Juan C. Gómez-Fernández

Departamento de Bioquímica y Biología Molecular (A), Edificio de Veterinaria, Universidad de Murcia, Apartado Postal 4021, E-30080 Murcia, Spain

Received 28 March 1995; accepted 20 June 1995

Abstract

The metastability of dimiristoylphosphatidylethanolamine (DMPE) has been studied by means of Fourier transform infrared spectroscopy (FT-IR), both in the absence and in the presence of α-tocopherol. Two different methods of hydration were used to prepare the samples, poorly hydrated and well hydrated, and the results have been compared with anhydrous DMPE. Poorly hydrated DMPE gave place to a high-melting phase formed upon melting from gel to L_α at approx. 49°C, with a new transition to L_α at approx. 55°C. However, well hydrated DMPE incubated at 4°C for 49 days gave place to a subgel phase which was transformed by heating into a L_β phase at about 40°C and this into a L_α phase after further heating at 52°C. The subgel phase was more hydrated and less rigid than the high-melting phase. On the other hand, α-tocopherol, when included in poorly hydrated DMPE, stabilized a high-melting phase, which was transformed by heating directly into a L_α phase. However, when a sample of DMPE containing α-tocopherol was incubated for 49 days at 4°C a dehydrated solid phase different from the subgel and the high-melting phases was formed.

Keywords: Metastability; Dimiristoylphosphatidylethanolamine; α-Tocopherol; FTIR

1. Introduction

Vitamin E is known to be a very important component of biological membranes which in animals is predominantly found in the membranes of subcellular organelles and also in the plasma membrane. The most active and most abundant compound with vitamin E activity is α-tocopherol [1]. A role for α-tocopherol has been shown in the prevention and therapy of several conditions, together with its synthetic derivative α-tocopherol acetate, including aging [2], cancer [3], effects of pollution [4], sterility [5], muscular dystrophy [6], and anemia [7], among others. α-Tocopherol has been also suggested to act as a stabilizing agent in membranes [8]. It is widely agreed that α-tocopherol has a very important activity in biomembranes as a natural antioxidant [9,10]. Other biological activities have been suggested for α-tocopherol, related to its ability to stabilize biomembranes. Among them are stabilization against free unsaturated fatty acids [11–13] and an ordering effect on the membrane [11,14]. Moreover, it has been shown that α-tocopherol protected against the inhibitory effect of a lysophosphatidylcholine on 7-ethoxycumarin deethylase from rat liver microsomes [15]. Recently, we have demonstrated that α-tocopherol stabilized and formed bilayer structures in systems composed of single chain or very asymmetric phospholipids, and we suggested that the complementary shapes of those molecules might be the reason for the stabilization of the bilayer structure [16].

In order to better understand the function of α-tocopherol at the molecular level it is important to study its interaction with membrane components, and specifically with lipids. A variety of physical techniques, including differential scanning calorimetry (DSC) [17–21], electron spin resonance [22,23], nuclear magnetic resonance (NMR) [20,24–26], fluorescence [27–30] and Fourier transform infrared spectroscopy (FT-IR) [18,31] have been used to ascertain the location and interaction with phospholipids of α-tocopherol in the membrane. These studies concluded that α-tocopherol has its phenolic group located near the

Abbreviations: DMPE, 1,2-dimiristoyl-*sn*-glycero-3-phosphoethanolamine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; FT-IR, Fourier transform infrared spectroscopy; L_α, fully liquid-crystalline phase; L_β, fully hydrated gel phase; L_c, crystalline subgel phase; L_H, dehydrated high-melting solid phase.

* Corresponding author. E-mail: boullon@fcu.um.es. Fax: +34 68 364147.

SSDI 0005-2736(95)00150-6

polar moiety of the lipid matrix and posses a very high lateral diffusion. Moreover, when incorporated in model membranes with different phospholipid composition, α-tocopherol preferentially partitions into the most fluid domain [19].

Most of these studies were performed with phosphatidylcholines, but given the heterogeneous phospholipid composition of biological membranes it is very interesting to know whether α-tocopherol shows a similar effect on phospholipids different to phosphatidylcholines, such as phosphatidylethanolamines. These two types of phospholipids differ markedly in several physical properties which may be important in determining the bilayer organization of biomembranes. Phosphatidylethanolamines represent the second largest phospholipid component of animal membrane lipids after the phosphatidylcholines and they are characterized by their facility to adopt inverted hexagonal H_{II} phases [32]. Moreover, phosphatidylethanolamines, unlike phosphatidylcholines, do not get spontaneously hydrated when they are added to water [33]. We present in this report our studies on the effect of the incorporation of α-tocopherol in phosphatidylethanolamine model membranes by using a non-perturbing technique, Fourier transform infrared spectroscopy (FT-IR). It provides a useful structural information on both the fatty acyl chains and the polar head groups of phospholipids without the need of external probes. We have chosen 1,2-dimiristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE). DMPE, in the well hydrated state, presents a gel to liquid-crystalline transition temperature of approx. 50°C and its lamellar to hexagonal H_{II} phase transition temperature is well above 90°C. It is also known that DMPE presents metastability [34,35]. This study was also devised to extend and complete a previous study of the effect of α-tocopherol on phosphatidylethanolamines by DSC and NMR [21].

2. Materials and methods

2.1. Materials

α-Tocopherol and D_2O (99.9%) were obtained from Sigma (Madrid, Spain), 1,2-dimiristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) was obtained from Avanti Polar Lipids (Birmingham, AL, USA) and judged chromatographically pure by HPTLC. All other compounds were of analytical grade. Water was twice distilled and deionized in a Milli-Q apparatus from Millipore.

2.2. Sample preparation

Samples poorly hydrate

Samples of 3 μM DMPE and the appropriate amount of α-tocopherol in chloroform were dried under a stream of O_2-free N_2, and the last traces of solvent were removed by high vacuum for more than 3 h. Samples were dispersed in 500 μl of 0.1 mM EDTA, 10 mM Mops, 100 mM NaCl buffer (pH 7.4) and were submitted to three cycles, so that after being kept at 70°C during 60 min they were left at room temperature for 8 h between each heating cycle and sonicated shortly in a bath-type sonifier to ensure that the phospholipid was completely hydrated. Buffers were made either in H_2O or in D_2O. In the last case pH values shown were corrected for the isotope difference [36]. Subsequently the samples were spun for 30 min at 5000 × *g*.

Samples well hydrated

Mixtures of 3 μM DEPE and the appropriate amount of α-tocopherol were dried and dispersed in the same buffer that was described above. From this point the treatment was different, so that after freezing (−80°C) and thawing (70°C) five continuous times, they were heated (twice) from 20°C to 78°C with a heating rate of 0.5°C/min. Subsequently the samples were spun for 30 min at 5000 × *g*.

Long term incubation of samples

Apart from the analysis described above some well hydrated samples were incubated at 4°C during different time periods. In this way these samples were analyzed at 20 h, 2 days, 7 days and 49 days of incubation.

Anhydrous samples

3 μM of DMPE and the appropriate amount of α-tocopherol were mixed in a final volume of 100 μl of chloroform, and then pure spectroscopic quality KBr was added. Concentrations of lipid in KBr was in the range of 0.2 to 1.0% (w/w). The samples were dried under a stream of O_2-free N_2, and kept at high vacuum for more than 2 h in order to remove the last traces of solvent. Subsequently, the resulting powders were pressed into pellets of 12 mm diameter using a Quick Handi-Press (Spectra-Tech, Stanford, USA).

2.3. Infrared spectroscopy

Infrared spectra were obtained in a FT-IR Philips PU-9800 infrared spectrometer equipped with a DTGS detector continuously purged with dry air in order to remove water vapor bands from the bands of interest. The sample pellets were resuspended in 35 μl of either H_2O or D_2O buffer, and immediately studied by FT-IR. Samples were examined in a Thermostated Specac 20710 cell equipped with CaF_2 windows and using 25 μm teflon spacers (all from Specac, Kent, UK). Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using a sample shuttle accessory in order to average each consecutive scan. Samples were scanned between 20°C and 78°C at 2°C intervals with a water bath interfaced to the FT-IR computer. The equilibration time between different temperatures, taking into account the elapsed time needed to obtain the back-

ground spectrum, was 3 min whereas the time required to obtain the infrared spectrum of the sample was 1.5 min. Samples were equilibrated at 20°C for 15 min before acquisition. Underlying D_2O or H_2O bands were subtracted interactively by computation using the program Spectra-Calc (Galactic Industries, Salem, USA) prior to the measurements of frequencies and the application of resolution enhancement methods. Frequencies at the center of gravity were measured by fitting the top ten points of the curves to a Gaussian function. The spectra were subjected to Fourier deconvolution using the same software. Fourier deconvolution algorithm needs two values, F and γ [37]. We have used 0.2 and 5 respectively for optimum deconvolution.

2.4. Differential scanning calorimetry

The pellets, prepared as stated above, were carefully transferred to small aluminum pans and scanned in a Perkin-Elmer DSC-4 calorimeter using a reference pan containing buffer. The instrument was calibrated using indium as standard. The samples were scanned with a heating and cooling rate of 4°C/min at 1 mcal/s in all experiments. The range of temperatures studied was from 30°C to 80°C.

3. Results and discussion

The experiments described in this work were designed to study the metastability of DMPE by infrared spectroscopy and explore the effect of α-tocopherol on the phase behavior of DMPE multilamellar vesicles. Complex lipid polymorphism, such as that presented by DMPE, and its modulation by distinct lipid molecules, may be important to help us to understand different important biological processes which take place in biological membranes. Several aspects have been studied in order to gain insight on the metastability of DMPE and its modulation by α-tocopherol.

3.1. DSC studies

Earlier experiments have shown that DMPE presents phase metastability depending on the thermal history of the sample. This has been observed by several authors studying DMPE prepared in excess water and in different forms [34,35,38]. By this way, it is known that DMPE presents two transitions, one from the gel L_β to the liquid-crystalline L_α phase at approx. 48°C and another one, from a dehydrated solid phase with a high-melting transition (that we will call L_H and which is different from the subgel phase, L_c, found in other phosphatidylethanolamines [35]) to the liquid-crystalline L_α phase at approx. 58°C. In poorly hydrated samples, it is possible to discern one exotherm, shortly afterwards the L_β to the L_α phase has occurred, indicating the transformation from the L_α phase to a dehydrated high-melting solid L_H phase. Therefore, DMPE, when poorly hydrated, presents three well characterized L_α, L_β and L_H phases [35].

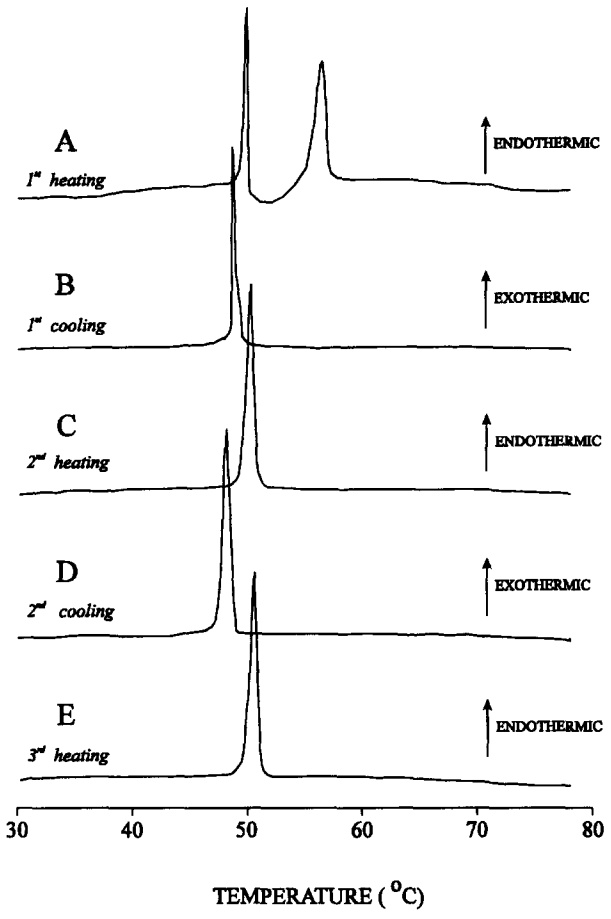

Fig. 1. Calorimetric thermograms for DMPE in different states of hydration. The first scan was obtained with the poorly hydrated state of DMPE. See text for details.

For this reason, and in order to compare these previous results with the FT-IR measurements, and to correlate the changes observed in the infrared vibration bands with the thermotropic behavior of DMPE [21,35], we have studied the thermotropic behavior of DMPE by DSC upon different hydration processes (Fig. 1). Therefore, we have prepared the samples in two different ways, as stated in Section 2: poorly hydrated and well hydrated. Poorly hydrated samples of DMPE showed two endotherms at 49.4°C and 55.3°C, with an intervening exotherm (Fig. 1A). When the sample was subsequently cooled down only one exotherm at 49.4°C was found (Fig. 1B). Afterwards, this sample showed only one peak at this temperature either by heating or cooling several times and at several heating and cooling rates (Fig. 1C–E). These results indicate, as was pointed out previously [35], that DMPE can exist in two distinct low temperature phases, low (L_β) and high-melting (L_H) phases. DMPE relaxes slowly from a low-melting to a high-melting solid phase, but the high-

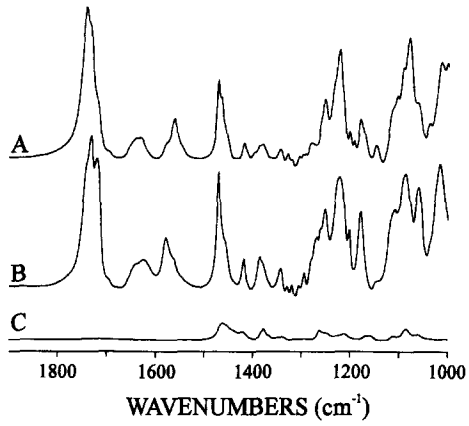

Fig. 2. Infrared spectra of the 1900–1000 cm^{-1} region of (A) pure anhydrous DMPE, (B) pure anhydrous DMPE in the presence of 20 mol% of α-tocopherol and (C) pure anhydrous α-tocopherol. The spectra have been normalized taking into account the intensity of the $-CH_2-$ and $-CH_3$ stretching region of the different spectra.

melting solid phase forms rapidly when the sample is subsequently heated through the transition temperature of the low-melting gel phase. The observed transition at approx. 50°C signals a transition from the gel (L_β) phase to the liquid (L_α) phase, whereas the endotherm at approx. 55°C indicates a transition from the dehydrated high-melting solid (L_H) phase to the liquid (L_α) phase [35]). On the other hand, the well hydrated sample showed only one endothermic transition upon heating at 49.5°C from L_β to L_α.

When the well hydrated sample was incubated for 8 h at room temperature, and then scanned by heating, again a pattern of one endotherm (49.5°C), followed by one exotherm and then another endotherm (55°C) transitions were observed (not shown) with a pattern identical to that observed in the poorly hydrated sample. Furthermore, when the well hydrated sample was preincubated at 4°C during 49 days, and then scanned by heating, the first scan showed only one endothermic transition at 55°C which corresponded to a L_c to L_α phase transition, according to the FT-IR results shown below. The subsequent cooling scan showed an exotherm at 49.5°C (from L_α to L_β). Upon immediate heating, a single transition was observed at 49.5°C (endothermic), indicating a L_β to L_α transition. The above results have been interpreted as showing that the L_H phase is more stable than the L_α or the L_β in a small range of temperatures, from 49.5°C to 55°C [34]. This L_H phase has been called 'high-melting phase' and it has been showed to be different to 'subgel phases' of other phosphatidylethanolamines [35]. On the other hand, as observed by us, this phase only forms when DMPE is poorly hydrated or incubated at low temperature after being well hydrated. This suggest that the formation of the L_H phase may be 'catalyzed' by the presence of some molecules of DMPE still not well hydrated. This interpretation is on line with recent observations that have shown that the mechanism for the gel to subgel phase transformation is based on nucleation of subgel domains followed by growth of the domains [39]. Nevertheless, after a long term incubation of well hydrated DMPE a L_c phase and not a L_H phase was formed (see below).

3.2. FT-IR results

In order to explore the effect of α-tocopherol on DMPE, we have used FT-IR, taking spectra of the three different types of samples used in this work.

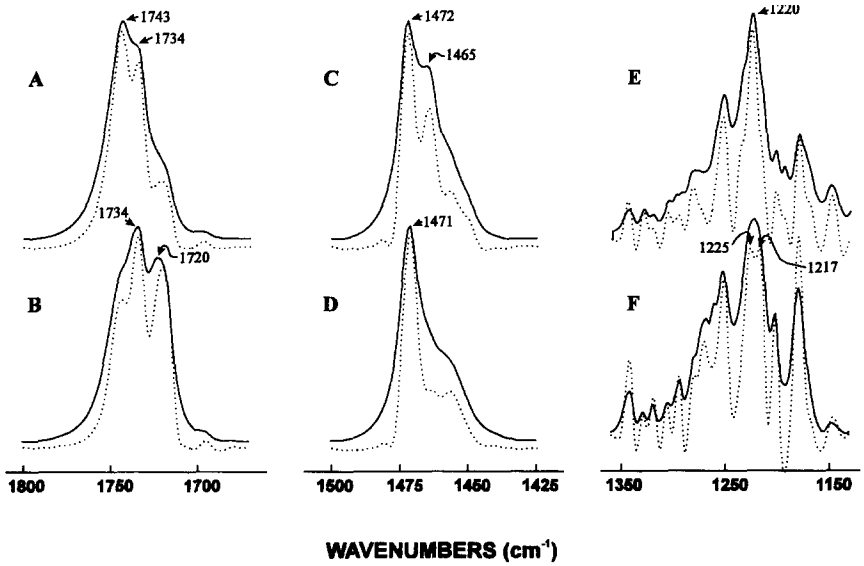

Fig. 3. Infrared spectra of DMPE in the dry state (anhydrous), either in the pure state (A, C and E) or in the presence of 20 mol% of α-tocopherol (B, D and F). The 1800–1700 cm^{-1} region (ester C = O stretching vibration mode region), the 1500–1400 cm^{-1} (CH_2 deformation, scissoring band region) and the 1350–1150 cm^{-1} region (headgroup PO_2^- phosphate band region) are shown. The original spectra are presented as a solid line whereas the deconvoluted spectra are represented as dotted lines.

3.3. Anhydrous samples

In order to understand the behavior of the different vibration bands of DMPE, either in pure form or in the presence of α-tocopherol and in the hydrated state, we made a previous study of the samples in the dry state. These samples were studied as KBr pellets.

The 1900–1000 cm^{-1} infrared region of pure anhydrous DMPE is shown in Fig. 2A. Anhydrous phosphatidylethanolamines may present different polymorphs depending on the solvent used for crystallization [40]. In this way, and as it has been shown before [41], DMPE recrystallized from ethanol exhibit crystal field splitting of the CH_2 scissoring band (characteristic of an orthorhombic packing of the acyl chains) whereas DMPE recrystallized from chloroform present a single CH_2 scissoring band (characteristic of an hexagonal packing of the acyl chains). All of our samples, as stated in Section 2, have been prepared from chloroform solutions. As observed in Fig. 2A, the infrared spectra of the anhydrous pure DMPE used in this work is similar to type II DMPE crystals [41].

The spectra of pure anhydrous DMPE including 20 mol% and pure anhydrous α-tocopherol can also be observed in Fig. 2B and C, respectively. The spectrum of pure anhydrous α-tocopherol has been scaled to that of DMPE plus 20 mol% of α-tocopherol taking into account its relative content by normalizing the intensity of the stretching vibration region of the $-CH_2-$ and $-CH_3$ groups of the two spectra. It can be observed that the vibration bands of α-tocopherol do not contribute significatively to the observed vibration bands of DMPE (compare Fig. 2A and C with Fig. 2B).

Fig. 3A shows the spectrum of pure DMPE in the C = O stretching carbonyl region whereas Fig. 3B shows the spectrum of the sample containing DMPE and 20 mol% of α-tocopherol, (original spectra, solid line) as well as the corresponding deconvolutions (dotted line). The C = O stretching vibration mode of pure DMPE is wide and with a maximum at 1743 cm^{-1}. Although it was clear that the original spectrum was formed by more than one component, deconvolution was used in order to enhance the resolution of this band and different components at 1743, 1734 and 1720 cm^{-1} were observed, indicating that the C = O groups of DMPE participate in hydrogen bonding, either of intra or intermolecular origin [42,43]. The presence of 20 mol% of α-tocopherol modified the relative intensities of the different components. The maximum of the C = O vibration band appeared at 1734 cm^{-1} (Fig. 3B), so that the intensity of the components due to hydrogen bonded C = O groups (1734 and 1720 cm^{-1}) increased whereas the intensity of the component due to non-hydrogen bound groups (1743 cm^{-1}) decreased. This result might be taken to indicate that α-tocopherol produces a rearrangement of the DMPE molecules, enhancing the hydrogen bonding of the ester C = O groups and suggesting that possibly α-tocopherol itself participates directly in hydrogen bonding with DMPE through its phenolic OH-group.

Another interesting band is that corresponding to the acyl chain out-of-plane CH_2 scissoring vibration mode, which appears in the region 1450–1480 cm^{-1}. Factor group splitting and the specific frequencies of the CH_2 scissoring band have been used before to distinguish the acyl chain packing of the fatty acyl chain of the phospholipids [44]. It can be observed that pure DMPE presented a splitting with component bands at 1472 and 1465 cm^{-1}, with a shoulder at 1456 cm^{-1}, being the first one the most intense (Fig. 3C). This splitting is very similar to that previously observed for saturated phosphatidylcholines, such as DPPC, in L_c phases which are produced after prolonged incubations at low temperatures [45]. In these cases, two sharp bands centered at 1472 and 1466 cm^{-1} where found. In infrared spectroscopic studies of solid hydrocarbons [46], this factor group splitting has been correlated with the assembly of the polymethylene chains into an orthorhombic subcell. In such systems, the CH_2 scissoring band tends to split into components of comparable integrated intensity when the zigzag planes of the polymethylene chains are perpendicular to each other. Therefore, our data suggest that in this phase, the zigzag planes of the hydrocarbon chains are not parallel. In the presence of 20 mol% α-tocopherol, the 1471 cm^{-1} band was practically the only one remaining, the other two being observed as shoulders of the main band (Fig. 3D). Therefore, whereas in pure DMPE there is a clear unequivalence of the acyl chains of the phospholipid, so that the zigzag planes of the hydrocarbon chains were not parallel, in the presence of 20 mol% of α-tocopherol this unequivalence disappeared almost completely, although it should be noted that the frequency which remains as predominant is notably high.

The antisymmetric PO_2^- double bond stretching band has been shown to be useful to monitor the hydration state of the polar head group of the phospholipids [42,47]. A frequency of approx. 1220 cm^{-1} characterizes a fully hydrated PO_2^- group, whereas its dehydration makes it appear at higher wavenumbers (approx. 1230–1240 cm^{-1}) [42,48–50]. The region of the PO_2^- antisymmetric vibration group of pure DMPE showed a narrow band which is a pattern characteristic of rigid samples, with a maximum at 1223 cm^{-1} (Fig. 3E). Phosphatidylethanolamines have been found to present a single PO_2^- band at 1240 cm^{-1} in CCl_4, where there are no H-bonds [50]. Therefore, this frequency suggests that this group is engaged in hydrogen bonding with other acceptor groups of DMPE, either intra or intermolecular. However, and very remarkably, in the presence of 20 mol% of α-tocopherol this band was widened and two components were visible by deconvolution, appearing now at 1225 and 1217 cm^{-1} (Fig. 3F). This result means, as deduced from the carbonyl bands observed above, that either α-tocopherol produces a rearrangement of the DMPE molecules, enhancing the hydro-

gen bonding of the ester C = O groups and/or that α-tocopherol itself participates directly in hydrogen bonding with the PO_2^- group of DMPE through its phenolic OH-group.

3.4. Poorly hydrated samples

These DMPE/α-tocopherol mixtures were prepared by hydrating at 70°C and incubation at room temperature, as described in Section 2.

As it can be observed in Fig. 4, the behavior of the ester C = O stretching vibration band showed the presence of two transitions, in accordance with the thermotropic transitions observed by DSC (see Fig. 1). The ester C = O stretching region of pure DMPE narrowed at about 48°C, indicating that the phase transition from the L_β phase to the L_α phase was taking place (Fig. 4A). Increasing the temperature, and at about 58°C, the band broadened, indicating the presence of another transition, most probably the transition from the L_H phase to the L_α phase (compare Figs. 1 and 4A). As it was commented above, this result suggests that this sample contained some dehydrated lipid domains, so that at temperatures above approx. 48°C these domains act as nuclei for the formation of the L_H phase (the observed exotherm in the DSC first heating scan, Fig. 1, and the narrowing of the C = O band, Fig. 4A). At 55.3°C, the transition from the L_H phase to the L_α phase then took place, as seen in the second endotherm in the DSC heating scan (Fig. 1), and in the broadening of the C = O band (Fig. 4A). Therefore, the narrow C = O vibration band observed between 48°C and 55°C is characteristic of the dehydrated high-melting solid L_H phase [51]. Another feature to be remarked in the spectra is the presence of several components underlying the envelope of the C = O vibration band which can be better resolved by deconvolution (see below).

A broad C = O stretching band contour centered at approx. 1735 cm^{-1} has been previously observed for other phospholipids, like dipalmitoylphosphatidylcholine (DPPC), for both the gel phase and the liquid-crystalline phase. This C = O vibration band could be resolved into, at least, two components after enhancing the resolution by methods such as deconvolution. These components appeared at frequencies of approx. 1740 and 1727 cm^{-1}, which were originally thought to arise from the stretching vibrations of the *sn*-1 and *sn*-2 ester carbonyls groups respectively of the phospholipid [42,45,52,53]. However, some recent studies [43,54–56] have shown that each one of the two component bands present in the C = O stretching band contour of the phosphoglycerolipids is in fact the

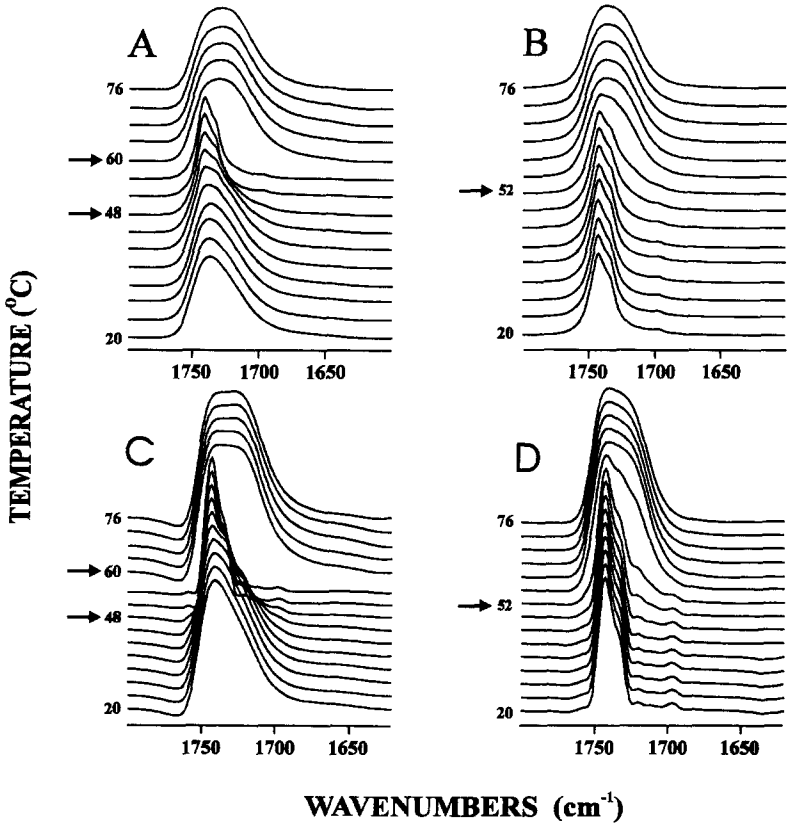

Fig. 4. Ester C = O stretching vibration band region for poorly hydrated samples of pure DMPE (A) and DMPE containing 20 mol% α-tocopherol (B). The corresponding deconvoluted spectra are shown in C for pure DMPE and D for DMPE containing 20 mol% α-tocopherol. The transition temperatures of the different found phase transitions are marked in the figure with arrows.

summation of nearly equivalent contributions from both the *sn*-1 and *sn*-2 carbonyl groups, so that the component appearing at the lowest frequency corresponds to a population of hydrogen bonded (hydrated) C = O groups and the component appearing at the highest frequency corresponds to a population of free (non-hydrated) C = O groups. The deconvolution of the C = O vibration band of pure DMPE in the poorly hydrated state is shown in Fig. 4C. It can be observed that the C = O band contour of DMPE at 20°C presents, after deconvolution, a single maximum at 1740 cm^{-1}, although given the high asymmetry of the band it seems reasonable to assume that at least one more component is also present. It should be remarked that the parameters used for deconvolution were purposely chosen in order to avoid the overdeconvolution of the spectra, as evidenced by the absence of negative side lobes. In any case the shape and frequency of the band indicates a high proportion of dehydrated C = O groups of DMPE at this temperature (Fig. 4C). At temperatures between approx. 48°C and 58°C it is possible to discern two very narrow bands, together with two components of very low intensity, with frequencies of 1744 and 1734 cm^{-1} for the intense components and 1722 and 1697 cm^{-1} for the less intense components (Fig. 4C). At temperatures above the L_H to L_α phase transition, two broad components appear through deconvolution, located at approx. 1741 cm^{-1} and 1724 cm^{-1}, a situation similar to what has been described before for DPPC [43].

However, and in the presence of 20 mol% of α-tocopherol, a totally different pattern was found below of the temperature of the L_β to L_α phase transition (Fig. 4B, original spectra, and D, deconvoluted spectra). For example, and at 20°C, the C = O vibration band was very narrow, presenting a bandwidth at half-height of 19.8 cm^{-1} to be compared with the bandwidth of 40.5 cm^{-1} found for pure DMPE at the same temperature. This is an illustration of the immobilization of these C = O groups by the presence of α-tocopherol, i.e., the induction of the presence of a dehydrated high-melting solid phase (see below). The maximum of the band is located now at 1743 cm^{-1} (Fig. 4B) and upon deconvolution components at 1743, 1734 and 1724 cm^{-1} are revealed together with a minor component, already visible in the original spectrum, at 1697 cm^{-1} (Fig. 4D), similarly to what was found for pure DMPE in the L_H phase (Fig. 4C). This complex pattern of bands must be a reflection of major changes in the hydrogen-bonding interactions between the solvent and the interfacial C = O ester groups of the lipid. Since the components located at the higher frequency have a higher intensity, the predominance of dehydrated over hydrated C = O groups is clear. Above of the phase transition it is possible again to observe two components, which by de-

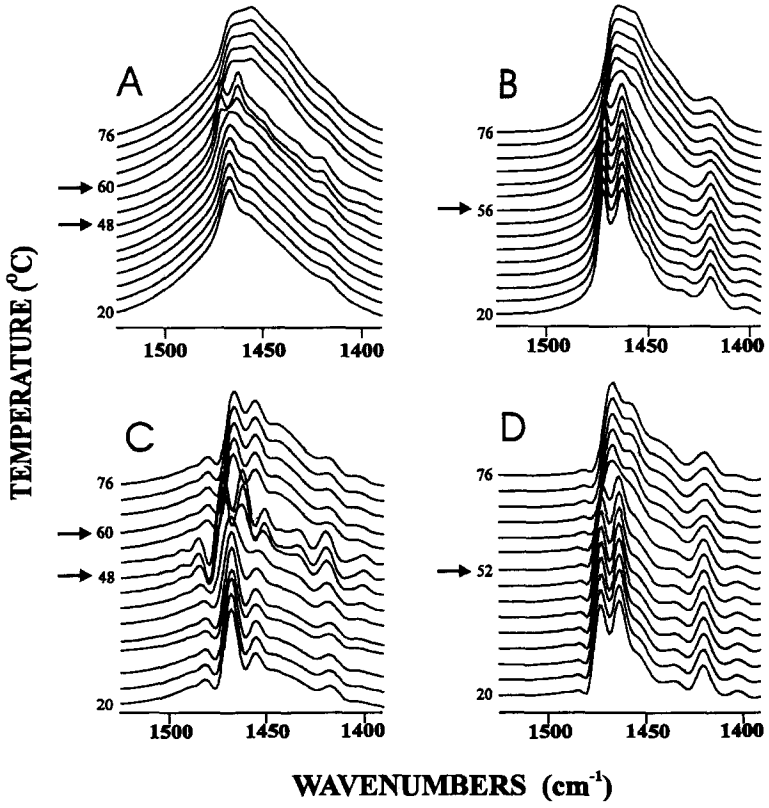

Fig. 5. CH_2 deformation vibration band (scissoring) region of poorly hydrated samples of pure DMPE (A) and DMPE containing 20 mol% α-tocopherol (B). The corresponding deconvoluted spectra are shown in C for pure DMPE and D for DMPE containing 20 mol% α-tocopherol. The transition temperatures of the different found phase transitions are marked in the figure with arrows.

convolution appear at frequencies of 1743 cm^{-1} and 1729 cm^{-1}. These frequencies are higher than those of pure DMPE in the L_α phase. The maximum of the composite band appeared, in fact, at 1737 cm^{-1} (DMPE/α-tocopherol) instead of at 1730 cm^{-1} (pure DMPE). It is also interesting to note the different bandwidth (bandwidth at half height) of the ester C = O band in the different conditions studied. Whereas for pure DMPE, as commented above, the bandwidth in the L_β phase was 40.5 cm^{-1}, in the L_H phase it was 17.9 cm^{-1} and in the L_α phase 48.8 cm^{-1}. In the presence of α-tocopherol, and at low temperature (crystalline phase, to be compared with the L_H phase of DMPE) the bandwidth was 20.5 cm^{-1}, but in the L_α phase it was 43.6 cm^{-1}, i.e., a difference of about 5 cm^{-1} with respect to the L_α phase of pure DMPE. These changes in frequency and bandwidth reflect the higher frequency and different intensity of the components.

Another region of the spectrum which was studied, and which is highly informative with respect to the organization of the lipids, was the CH_2 deformation region, i.e., the out-of-plane CH_2 scissoring vibration band which appear at about 1470 cm^{-1} and the α-methylene bending band which appear near 1418 cm^{-1}. As it was commented above, factor group splitting and the specific frequencies of the CH_2 scissoring band have been used before to characterize the acyl chain packing of the fatty acyl chains of the phospholipids [44,57].

Pure DMPE below of the L_β to L_α phase transition presents an asymmetric band with a maximum at 1467 cm^{-1} and a shoulder at 1455 cm^{-1} (Fig. 5A), similar to what has been found previously for saturated phosphatidylcholines such as DPPC [44]. As the temperature is increased, the L_β to L_α phase transition takes place, concomitantly with the formation of the L_H phase, and a completely different scissoring band is observed. In this case it is possible to note two peaks of similar intensity with frequencies appearing at 1471 and 1463 cm^{-1}. This splitting is very similar to what has been previously observed in phospholipids such as DPPC forming L_c phases [45] and it is also very similar to that shown here for pure DMPE in the dehydrated state (Fig. 2C). Therefore, this result suggests that in the L_H phase, DMPE is organized with the zigzag planes of the hydrocarbon chains in a not parallel arrangement. However, at higher temperatures and in the L_α phase, a different pattern was observed with the maximum of the band at 1455 cm^{-1} and a shoulder appearing at 1467 cm^{-1}. This can be better observed in the deconvoluted spectra, as shown in Fig. 5C. This behavior is similar to what has been found for other phospholipids in the liquid-crystalline phase [42].

In the presence of 20 mol% of α-tocopherol, and at low temperatures, it was possible to observe a clear splitting of the CH_2 scissoring vibration band, giving place to two bands appearing at 1473 cm^{-1} and 1463 cm^{-1} (Fig. 5B), similarly to what was found for pure DMPE in the L_H phase (see Fig. 5A). The magnitude of the splitting is correlated with the degree of interchain packing. In this way, a larger splitting reflects tighter interchain packing [58,59]. The splitting in pure DMPE is similar to the splitting in the presence of α-tocopherol indicating that α-tocopherol does not modify the interchain packing of DMPE. Increasing the temperature, and at approx. 58°C, a transition took place, whereby the scissoring band presented a maximum at 1467 cm^{-1} and a shoulder at approx. 1455 cm^{-1} (Fig. 5B). This behavior is also more clearly observed in the deconvolved spectra (Fig. 5D), indicating that above of 58°C a L_α phase has been adopted by the system.

There is also a significant change in the intensity of the peak appearing at 1418 cm^{-1}, which corresponds to the α-methylene bending band of the phospholipid. This band is weak and fairly broad in the L_β phase of pure DMPE but it gets sharper and considerably more intense in the L_H phase (Fig. 5C). In the sample of DMPE containing 20

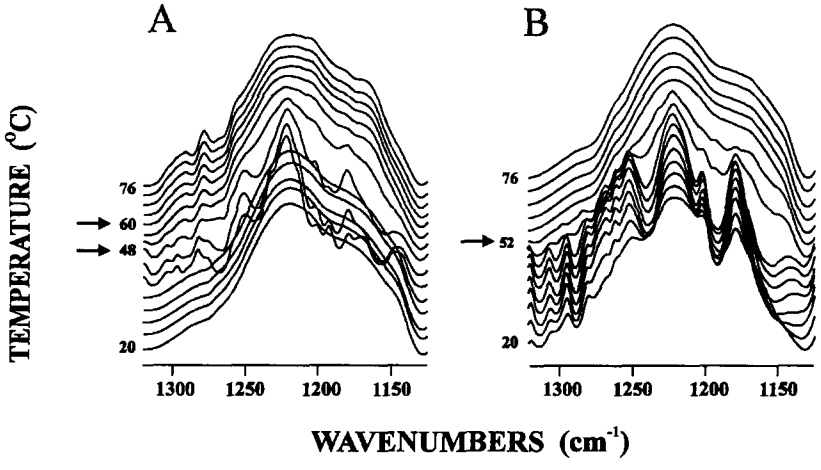

Fig. 6. Headgroup phosphate antisymmetric band region of poorly hydrated samples of pure DMPE (A) and DMPE containing 20 mol% α-tocopherol (B). The transition temperatures of the different found phase transitions are marked in the figure with arrows.

mol% of α-tocopherol it is possible to observe this band at lower temperatures (Fig. 5D). A similar behaviour was previously observed for this band in the L_c phases of saturated phosphatidylcholines [57]. Since a conformational unequivalence between the *sn*-1 and the *sn*-2 chains is to be presumed after the scissoring band behavior observed for the sample containing 20 mol% of α-tocopherol, it is notable that only one band is detected for the α-methylene group. A similar behavior was observed in the L_c phase of some phosphatidylcholines such as distearoylphosphatidylcholine, but not for DPPC [56]. Since this band was not resolved by deconvolution into more than one component (Fig. 5C and D), the interpretation must be that there is a considerable sharpening of one of the α-methylene bands produced by a conformational change with the consequence of reduction in the mobility of one of the α-methylene groups. This implies that the effect of α-tocopherol is not only seen in the reorientation of the polymethylene chains but also near the polar part of the membrane. Nevertheless, the appearance of a intense and narrow band coming from the α-methylene bending band of the phospholipid, as well as the appearance of the splitting in the CH_2 deformation band, is indicative of the presence of a crystalline phase.

The temperature dependence of the phosphate headgroup antisymmetric vibration band was also studied. Fig. 6A shows the asymmetric stretching band of pure DMPE. At low temperatures a visible broad band is visible with a maximum at approx. 1218 cm^{-1}. Increasing the temperature, and at the L_β to L_α phase transition, it is possible to observe a shift to higher frequencies of the maximum of the band (from 1218 cm^{-1} to 1223 cm^{-1}) and a narrowing of the band indicative of a great immobilization of the molecules (Fig. 6A). At increasing temperatures a shift to lower wavenumbers occurred (in this case the maximum of the band was 1219.1 cm^{-1}). However, at low temperatures and in the sample containing DMPE and 20 mol% of α-tocopherol, the phosphate antisymmetric band appeared very narrow, i.e., immobilized, but it got broadened at approx. 58°C (Fig. 6B) which is indicative of the L_H to the L_α phase. In the L_α phase, the samples showed broadened bands similar to those seen in other hydrated phospholipids in the liquid-crystalline state [42,45].

Taken together all of these results, they indicate that the inclusion of the α-tocopherol molecule between the DMPE molecules produced a rearrangement of the structure of the phospholipid molecule, with a change in the hydrogen bonding pattern, so that the dehydrated high-melting solid phase of DMPE (L_H) is stabilized at low temperatures.

Whereas the behavior, i.e., frequency and bandwidth, of the different bands for the L_β and L_α phases were similar to other respectively gel and fluid phases found in other

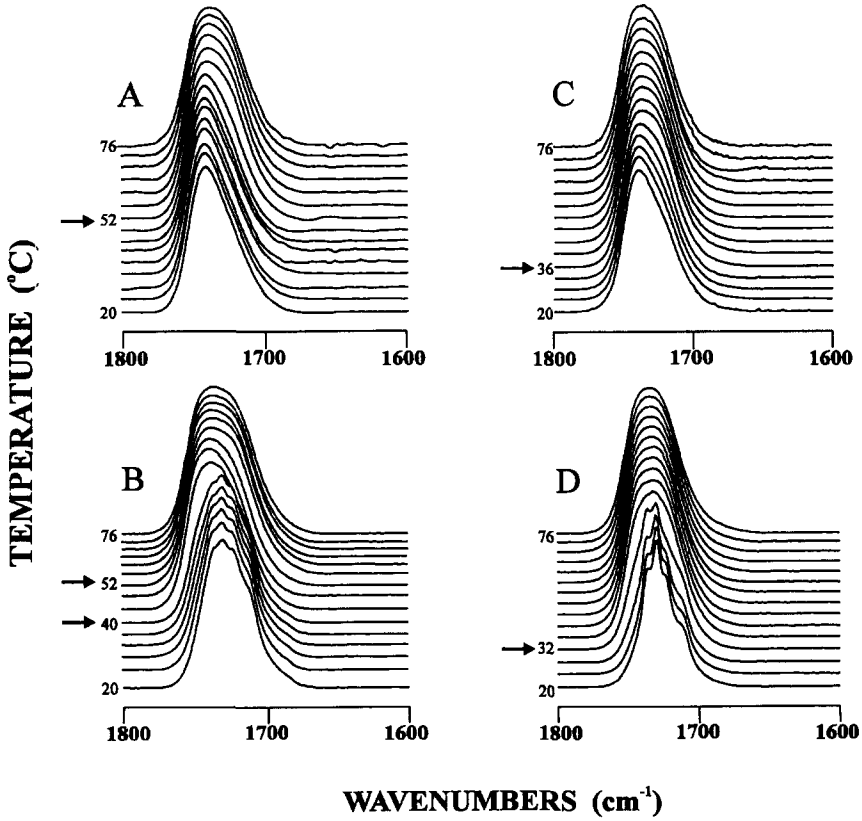

Fig. 7. Ester C = O stretching region band region of pure DMPE (A,B) and DMPE in the presence of 20 mol% of α-tocopherol (C,D) in the well hydrated state. Samples were scanned immediately after preparation, i.e., without incubation (A,C) or incubated at 4°C for 49 d (B,D). The transition temperatures of the different found phase transitions are marked in the figure with arrows.

phospholipids [42], the L_H phase was peculiar. In the L_H phase a dramatic narrowing of the C = O and PO_2^- stretching bands was found, indicating a very rigid type of motion of the molecules, as well as the appearance of several components in the C = O stretching vibration band indicating either the possible formation of hydrogen bonding between different groups of the DMPE molecules and/or the induction of different acyl chain rotational isomers. Moreover, the CH_2 scissoring motion modes presented in this phase a splitting reflecting a different acyl chain packing of the acyl chains respect to the L_β and L_α phases [53]. But, significantly, the appearance of this pattern might be taken as an indication of the formation of the dehydrated high-melting solid L_H phase.

3.5. Well hydrated samples

Samples of pure DMPE and their mixtures with α-tocopherol were prepared by repeated freezing and thawing, as described in Section 2, in order to obtain the well hydrated samples.

As observed in Fig. 7A, pure DMPE in the well hydrated state and scanned immediately after preparation, presented a completely different behavior to pure DMPE in the poorly hydrated state (compare with Fig. 4A). In this case, the C = O stretching vibration band presented only one transition at approx. 50°C, characteristic of the L_β to L_α phase transition [35]. At low temperatures, the asymmetric peak had a maximum at 1740 cm^{-1}, whereas above the phase transition it shifted to approx. 1738 cm^{-1}, at the same time that the bandwidth increased by approx. 5 cm^{-1}, indicating that the hydrated component of the C = O vibration band increased in intensity with respect to the unhydrated one. This behavior is similar to what has been observed for other phospholipids in the L_α phase [45]. The inclusion of 20 mol% of α-tocopherol (Fig. 7C) did not produce a significative change in the frequency of the maximum of the band, but it produced an increase in bandwidth at halfheight in all cases, from 35.7 cm^{-1} in pure DMPE to 37.9 cm^{-1} in the presence of α-tocopherol at low temperatures, and from 40.5 cm^{-1} in pure DMPE to 51.3 cm^{-1} in the presence of α-tocopherol at higher temperatures, as well as a shift of the phase transition to lower temperatures, down to approx. 38°C (Fig. 7C). These changes in bandwidth indicated an increase in the proportion of hydrated ester carbonyl groups due to the presence of α-tocopherol. The decrease induced in the phase transition is similar to that reported in a previous study by DSC [21].

The scissoring band region was also studied in these

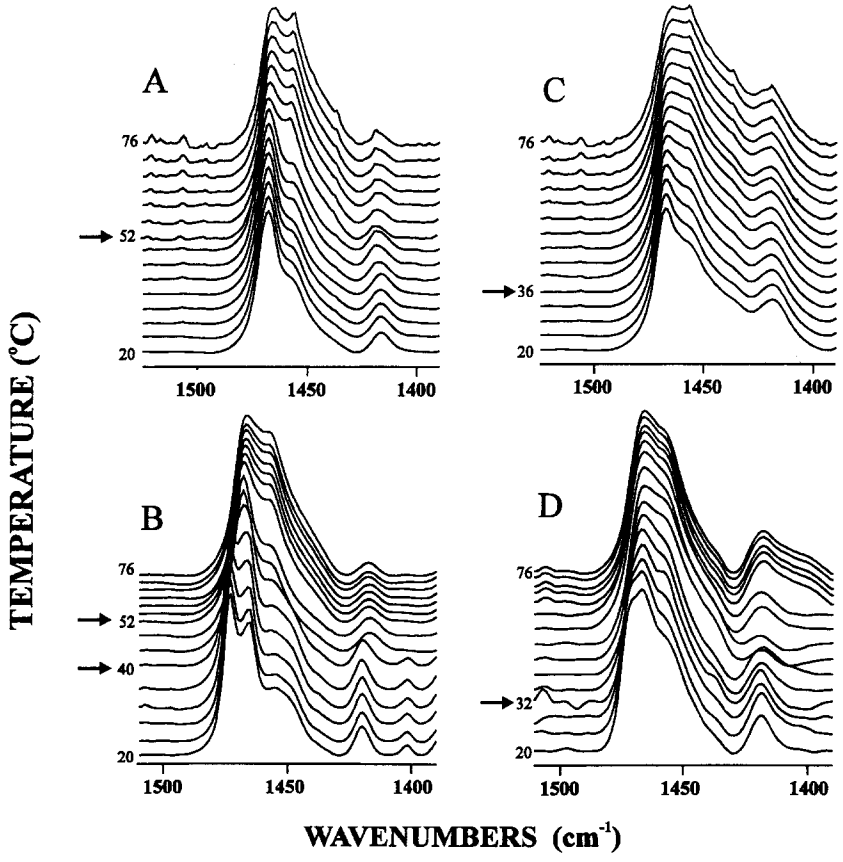

Fig. 8. CH_2 deformation vibration band (scissoring) region of pure DMPE (A,C) and DMPE in the presence of 20 mol% of α-tocopherol (B,D) in the well hydrated state. Samples were scanned immediately after preparation, i.e., without incubation, (A,B) or incubated at 4°C for 49 d (C,D). See text for details. The transition temperatures of the different found phase transitions are marked in the figure with arrows.

samples but no splitting of the scissoring could be observed here, with the maximum of the band centered at 1468 cm^{-1} both above and below of the phase transition, indicating hexagonal packing in all the samples, i.e., in DMPE both pure and containing 20 mol% of α-tocopherol (Fig. 8A and C, respectively).

The PO_2^- phosphate band was also studied for DMPE, pure and containing 20 mol% of α-tocopherol (Fig. 9A and C), but it did not show in any of the samples the type of rigidification seen for the samples in the poorly hydrated state (see Fig. 6).

3.6. Long term incubations of samples

It is known that aqueous DMPE samples may undergo metastability processes which depend upon long incubation at low temperatures [34,35]. Therefore and in order to monitor these processes by FT-IR and to ascertain the effect of α-tocopherol on DMPE metastability, well hydrated samples of DMPE, both pure and containing 20 mol% of α-tocopherol, were incubated at 4°C. Incubations ranged from 20 h to 49 days as stated in Section 2. It was only at this long term incubation time when significative changes were observed, and they will be commented below.

It is possible to observe the evolution of the C = O stretching vibration mode of DMPE, either in pure form or in the presence of 20 mol% α-tocopherol, without incubation and with incubation at 49 days (Fig. 7). There was a progressive widening of the band and also a shift of the maximum at increasing incubation times down to 1730 cm^{-1} (49 days incubation time) from the initial 1738 cm^{-1} (with no incubation).

The thermal phase behavior of pure DMPE as seen through the carbonyl stretching vibration, changed after the incubation, since a first transition took place at about 40°C and a second one at about 52°C (Fig. 7B). Below 40°C, the C = O group vibration has a complex patterns with several components, in both pure DMPE and DMPE in the presence of 20 mol% of α-tocopherol. In pure DMPE, the deconvolved spectrum showed components at 1744, 1737, 1730, 1721 and 1712 cm^{-1} (not shown). The band as a whole is narrow, as it would be expected for a solid phase, although not so narrow as in the L_H phase described above. On the other hand, the frequencies are also higher than in the L_H phase (see Fig. 4). It can be deduced, then, that this phase formed after a long incubation is not a L_H phase. Apart from that, the transition which takes place at about 40°C yields a relatively dehydrated phase as it can be concluded from the maximum of the band (1739 cm^{-1}).

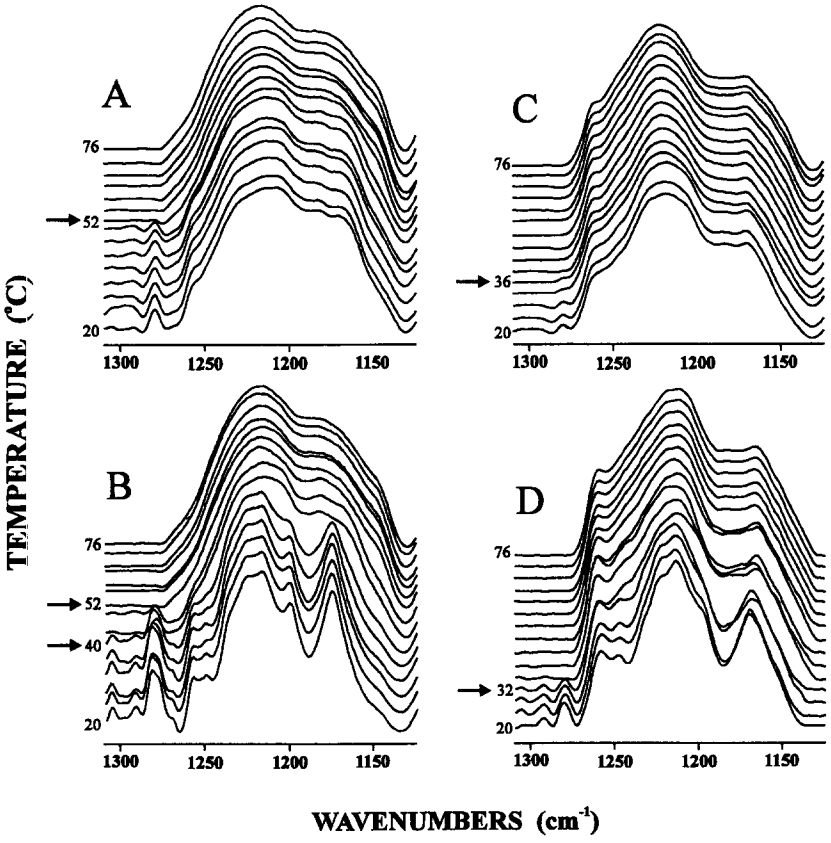

Fig. 9. PO_2^- headgroup antisymmetric stretching band region of pure DMPE (A,C) and DMPE in the presence of 20 mol% of α-tocopherol (B,D) in the well hydrated state. Samples were scanned immediately after preparation, i.e., without incubation, (A,B) or incubated at 4°C for 49 d (C,D). See text for details. The transition temperatures of the different found phase transitions are marked in the figure with arrows.

It is very asymmetric and similar to the L_β phase observed for other DMPE samples studied here (see Figs. 4 and 7A). The final transition at about 52°C produces a very broad band, with the maximum at approx. 1737 cm^{-1}, presumably corresponding to a L_α phase (compare again with Figs. 4 and 7A). All of this suggest clearly that this long incubation at 4°C has produced a subgel phase (L_c) which evolves into a L_β phase at 40°C and this into a L_α phase at about 52°C.

It should be remembered here that exactly this pattern of metastability has been observed before for DMPE after long-term incubation [38], so that a L_c phase was transformed at 41°C into a L_β phase and this into a L_α phase at about 50°C. It is not clear why other authors, after very similar incubations, ended up with high-melting L_H phases [34,35]. Silvius et al. [35] proposed that the lower temperature of incubation (−4°C) used by Mulukutla and Shipley [38] compared with the 4°C incubation temperature used by the other two groups could be the reason of the differences. However, we have used here 4°C, and nevertheless we have obtained a subgel phase rather than a high-melting phase. Some other subtle factors, such as the degree of hydration of the lipid previous to the incubation, must be involved in the determination of the final phase of DMPE.

The effect of α-tocopherol on the thermal behavior of incubated DMPE was also very interesting, since in this case only one transition took place at approx. 32°C, from a solid phase to what seems a L_α phase. Therefore, in the presence of α-tocopherol a direct transition takes place from a dehydrated solid phase to the L_α phase, without passing through a L_β phase as it happens with incubated pure DMPE. After deconvolution of the spectrum corresponding to 25°C, components at 1744, 1737, 1730, 1723 and 1712 cm^{-1} were observed (not shown), and this pattern is similar to the sample of pure and incubated DMPE commented above. Nevertheless, the C = O envelope was narrower at this temperature than in pure DMPE indicating a restricted motion of the phospholipid (Fig. 7D).

The scissoring band region of the well hydrated pure DMPE after 49 days of incubation at 4°C showed a splitting at low temperatures, i.e., two components discerned in the original spectra at 1472.8 and 1466 cm^{-1} (Fig. 8B), indicating that the packing of the acyl chains had changed from a hexagonal to an orthorhombic or monoclinic subcell, as it should be expected for a subgel phase (L_c). It is however interesting that α-tocopherol stabilized the sample in a hexagonal packing, so that after 49 days of incubation the 1468 cm^{-1} band was the mainly one which was observed although a small shoulder at approx. 1473 cm^{-1} could be distinguished (Fig. 8D). Therefore, in the presence of 20 mol% of α-tocopherol, a dehydrated solid phase different of the L_c phase is present.

Fig. 9 shows the thermal dependence of the PO_2^- antisymmetric stretching of the different samples. What it is interesting here is that the spectra of the L_c phase of incubated DMPE (Fig. 9B) are sharper than those of the L_β phase (Fig. 9A), but they do not show the sharpness of the L_H phase (Fig. 6), reassuring that the L_H phase is more rigid than the L_c one. Apart from that, it is also interesting that in the presence of α-tocopherol these spectra are changed only slightly.

4. Conclusions

DMPE has a very complex phase behavior depending on subtle conditions. It may form a high-melting phase (L_H) at relatively high temperatures in samples which are not exhaustively hydrated, probably because the small domains of crystalline DMPE which remain unhydrated may nucleate the formation of this L_H phase. This high-melting phase is quite rigid and dehydrated, as seen by the carbonyl and the PO_2^- bands. Apart from that, the scissoring band reveals that this L_H phase has a monoclinic or orthorhombic packing. However, well hydrated DMPE, after a very long incubation time at low temperatures, produce a subgel (L_c) phase which is less rigid and more hydrated than L_H, judging from the carbonyl band, and less rigid as seen through the PO_2^- band. But, this phase has also monoclinic or orthorhombic packing as deduced from the scissoring band. That L_c and L_H are not identical phases was already proposed by Silvius et al. [35].

On the other hand, α-tocopherol stabilized a dehydrated high-melting solid phase, probably L_H, in the poorly hydrated DMPE at low temperatures, giving place to a L_H to L_α transition. However, when α-tocopherol was included in DMPE incubated at 4°C for 49 days, it stabilized a dehydrated solid phase which was not presenting a monoclinic or orthorhombic pattern and which was different from the L_c phase produced by pure DMPE subjected to the same incubation, and also from the L_H phase. Nevertheless, this was a solid phase as indicated by the C = O band patterns and the PO_2^- antisymmetric stretching. Other authors, using DSC and FT-IR have concluded that DMPE, in the presence of alcohols, may form phases different to the subgel or to the high-melting phases [51], and these phases were not very different of those found here for DMPE incubated for 49 days at 4°C in the presence of 20 mol% of α-tocopherol. Complex lipid polymorphism and its modulation by different lipid molecules, such as α-tocopherol, may help us to understand the interactions between membrane lipids which is basic to understand the very complex nature of biological membranes.

Acknowledgements

This work has been supported in part by grant No. PB92-0987 from DGICYT (Spain) and by grant PIB94/13 from Dirección General de Educación y Universidad, Re-

gión de Murcia, Spain. J. Salgado is a recipient of a pre-doctoral fellowship from 'Gobierno del País Vasco', Spain. We want to thank Drs. F.J. Aranda and V. Micol for their help with some preliminary experiments of this work.

References

[1] Burton, G.W. and Ingold, K.H. (1986) Am. Chem. Res. 19, 194–201.
[2] Wartanowicz, M., Panczenko-Kresowska, B., Ziemlanski, S., Kowalska, M. and Okolska, G. (1984) Am. J. Nutr. Metab. 28, 186–191.
[3] Watson, R.R. and Leonard, T.K. (1986) Am. Diet. Assoc. 86, 505–510.
[4] Savanian, A., Hacker, A.E. and Elsayed, N. (1986) Lipids 17, 269–277.
[5] Evans, H.M. and Bishop, K.S. (1972) Science 56, 650–653.
[6] Scott, M.L. (1978) in Handbook of Lipid Research (DeLuce, H.F., ed.), Vol. 2, pp. 133–197, Plenum Press, New York.
[7] Whitaker, J., Fort, E.G., Vimokesan, S. and Dinning, J.S. (1967) Am. J. Clin. Natr. 20, 785–789.
[8] Tappel, A.L. (1972) Ann. N.Y. Acad. Sci. 204, 12–28.
[9] Scott, M.L. (1980) Fed. Proc. 39, 2736–2739.
[10] Burton, G.W. and Ingold, K.H. (1989) Ann. N.Y. Acad. Sci. 570, 7–22.
[11] Diplock, A.T. and Lucy, J.A. (1973) FEBS Lett. 29, 205–210.
[12] Erin, A.N., Spirin, M.M., Tabidze, L.V. and Kagan, V.E. (1984) Biochim. Biophys. Acta 774, 96–102.
[13] Stillwell, W., Ehringer, W. and Wassall, S. (1992) Biochim. Biophys. Acta 1105, 237–244.
[14] Lucy, J.A. (1972) Ann. N.Y. Acad. Sci. 203, 3–16.
[15] Kagan, V.E. (1989) Ann. N.Y. Acad. Sci. 570, 121–135.
[16] Salgado, J., Villalaín, J. and Gómez-Fernández, J.C. (1993) Arch. Biochem. Biophys. 306, 368–376.
[17] Lai, M.Z., Düzgunes, N. and Szoka, F.C. (1985) Biochemistry 24, 1646–1653.
[18] Villalaín, J., Aranda, F.J. and Gómez-Fernández, J.C. (1986) Eur. J. Biochem. 158, 141–147.
[19] Ortiz, A., Aranda, F.J. and Gómez-Fernández, J.C. (1987) Biochim. Biophys. Acta 898, 214–222.
[20] Wassall, S.R., Thewalt, J.L., Wong, L., Gorrisen, H. and Cushley, R.J. (1986) Biochemistry 25, 319–326.
[21] Micol, V., Aranda, F.J., Villalaín, J. and Gómez-Fernández, J.C. (1990) Biochim. Biophys. Acta 1022, 194–202.
[22] Srivastava, S., Phadke, R.S., Govil, G. and Rao, C.N.R. (1983) Biochim. Biophys. Acta 734, 353–362.
[23] Severcan, F. and Cannistraro, S. (1988) Chem. Phys. Lipids 47, 129–133.
[24] Ekiel, I.H., Hughes, L., Burton, G.W., Jovall, P.A., Ingold, K.H. and Smith, I.C.P. (1988) Biochemistry 27, 1432–1440.
[25] Perly, B., Smith, I.C.P., Hughest, L., Burton, G.W. and Ingold, K.V. (1985) Biochim. Biophys. Acta 819, 131–135.
[26] Salgado, J., Villalaín, J. and Gómez-Fernández, J.C. (1993) Eur. Biophys. J. 22, 151–155.
[27] Fukuzawa, K., Ikeno, H., Tokumma, A. and Tsukatani, H. (1979) Chem. Phys. Lipids 23, 13–22.
[28] Gómez-Fernández, J.C., Villalaín, J., Aranda, F.J., Ortiz, A., Micol, V., Coutinho, A., Berberan-Santos, M.N. and Prieto, M.J.E. (1989) Ann. N.Y. Acad. Sci. 570, 109–120.
[29] Bisby, R.H. and Birch, D.J.S. (1989) Biochem. Biophys. Res. Commun. 158, 386–391.
[30] Aranda, F.J., Coutinho, A., Berberan-Santos, M.N., Prieto, M.J.E. and Gómez-Fernández, J.C. (1989) Biochim. Biophys. Acta 985, 26–32.
[31] Gómez-Fernández, J.C., Aranda, F.J. and Villalaín, J. (1991) in Progress in Membrane Biotechnology (Gómez-Fernández, J.C., Chapman, D. and Packer, L., eds.), pp. 98–115, Birkhäuser Verlag, Basel.
[32] Cullis, P.R. and De Kruijff, B. (1979) Biochim. Biophys. Acta 599, 399–420.
[33] Lis, L.J., McAlister, M., Fuller, N., Rand, R.P. and Parsegian, V.A. (1982) Biophys. J. 37, 657–662.
[34] Wilkinson, D.A. and Nagle, J.F. (1984) Biochemistry 23, 1538–1541.
[35] Silvius, J.R., Brown, P.M. and O'Leary, T.J. (1986) Biochemistry 25, 4249–4258.
[36] Glasoe, P. and Long, F. (1960) J. Phys. Chem. 64, 188–190.
[37] Griffiths, P.R. and Pariente, G.L. (1986) Trends Anal. Chem. 5, 209–215.
[38] Mulukutla, S. and Shipley, G.G. (1984) Biochemistry 23, 2514–2519.
[39] Tristan-Nagle, S., Suter, R.M., Sun, W.J. and Nagle, J.F. (1994) Biochim. Biophys. Acta 1191, 14–20.
[40] Dorset, D.L. (1976). Biochim. Biophys. Acta 424, 396–402.
[41] Mantsch, H.H., Hsi, S.C., Butler, K.W. and Cameron, D.G. (1983) 728, 325–330.
[42] Casal, H.L. and Mantsch, H.H. (1984) Biochim. Biophys. Acta 779, 381–401.
[43] Blume, A., Hübner, W. and Hessner, G. (1988) Biochemistry 27, 8239–8249.
[44] Cameron, D.G., Casal, H.L. and Mantsch, H.H. (1980) Biochemistry 19, 3665–3672.
[45] Cameron, D.G. and Mantsch, H.H. (1982) Biophys. J. 38, 175–184.
[46] Casal, H.L., Cameron, D.G. and Mantsch, H.H. (1983) Can. J. Chem. 61, 1736–1742.
[47] Dluhy, R.A.:, Cameron, D.G., Mantsh, H.H. and Mendelsohn, R. (1983) Biochemistry 22, 6318–6325.
[48] Fringeli, U.P. and Günthard, H. (1981) in Membrane Spectroscopy (Grell, E., ed.), pp. 270–332, Springer-Verlag, New York.
[49] Villalaín, J., Ortiz, A. and Gómez-Fernández, J.C. (1988) Biochim. Biophys. Acta 941, 55–62.
[50] Sen, A., Yang, P.W., Mantsch, H.H. and Hui, S.W. (1980) Chem. Phys. Lipids 47, 109–116.
[51] Centeno, J.A. and O'Leary, T.J. (1990) Biochemistry 29, 7289–7296.
[52] Levin, I.W., Mushayakarara, E. and Bitman, K. (1982) J. Raman. Spect. 13, 231–234.
[53] Mushayakarara, I. and Levin, W. (1982) J. Phys. Chem. 86, 2324–2327.
[54] Hübner, W. and Mantsch, H.H. (1991) Biophys. J. 59, 1261–1272.
[55] Lewis, R.N.A.H. and McElhaney, R.N. (1992) Biophys. J. 61, 63–77.
[56] Lewis, R.N.A.H., McElhaney, R.N., Pohle, W. and Mantsch, H.H. (1994) Biophys. J. 67, 2367–2375.
[57] Lewis, R.N.A.H. and McElhaney, R.N. (1990) Biochemistry 29, 7946–7953.
[58] Snyder, R.G. (1979) J. Chem. Phys. 71, 3229–3235.
[59] Snyder, R.G., Scherer, J.R. and Gaber, B.P. (1980) Biochim. Biophys. Acta 601, 47–52.