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LASER-RAMAN INVESTIGATION OF LYSOZYME-PHOSPHOLIPID INTERACTIONS

J.L. LIPPERT, R.M. LINDSAY and R. SCHULTZ

Department of Chemistry, Rochester Institute of Technology, Rochester, NY 14623 (U.S.A.)

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Summary

The interaction of lysozyme with mixed 1,2-dipalmitoyl-L-phosphatidic acid/1,2-dimyristoyl-L-phosphatidylcholine liposomes was investigated by laser Raman spectroscopy. Substantial changes were observed in the spectra of both the lipid and protein in the mixed liposomes over the range 10—62°C. At temperatures below 27°C, interaction with lipid appears to slightly increase the amount of helical structure in lysozyme at the expense of random conformation. At temperatures above 30°C, considerable β-sheet is irreversibly formed. Onset of β-formation appears to coincide with the formation of disordered lipid side-chains in the acidic component of the lipid.

At all temperatures, the O-P-O diester stretching mode at 782 cm^{-1} is much more intense in the lipid/protein mixture than in lipid alone. It is observed that the dimyristoyl phosphatidylcholine chain-disorder transition is lowered by 3°C, while that of the phosphatidic acid is lowered by 12°C, yet the post-transition conformation contains a significantly higher proportion of *trans*-segments in the presence of lysozyme.

These results are interpreted in terms of: (1) a polar interaction between acidic phospholipid and lysozyme at temperatures below either chain-disorder transition, in which lysozyme is essentially excluded from the hydrophobic portion of the lipid and (2) an interaction at higher temperatures which involves the lipid side-chains of dipalmitoyl phosphatidic acid in the disordered state and is manifested by a substantial conformational change.

Introduction

Raman spectroscopy has recently been shown to be an effective tool with which to study conformations of phospholipids [1—6], proteins [7—12], and

biomembranes [13—16]. The Raman active C-C and C-H_2 stretching modes of lipids have been used to monitor *trans-gauche* isomerism and lateral mobility in systems of synthetic phospholipids [1,3,5], as well as for the mixed systems, dipalmitoyl phosphatidylcholine-cholesterol [1], dipalmitoyl phosphatidylcholine-gramicidin A [17], and dimyristoyl phosphatidylcholine with poly(L-lysine), valinomycin, gramicidin A, and gramicidin S [18]. The Raman spectra of the phospholipid-polypeptide complexes have been interpreted in terms of (1) an extrinsic interaction which lowers the temperature of the gel-liquid crystal transition of the lipid and (2) a penetration of the protein into the hydrophobic region of the phospholipid, which broadens the transition [17,18].

Raman spectra of the conformationally sensitive amide I and amide III regions of proteins have been used to interpret and predict secondary structure [7—12]. The thermal denaturation of lysozyme has been followed by Raman spectroscopy [19,20], as have denaturation by lithium bromide, sodium dodecyl sulfate (SDS) and S-S bond reduction [21]. Observed Raman spectral changes were interpreted in terms of changes in secondary structure, C-S bond configuration, and tryptophan and tyrosine environments.

Binding of lysozyme to negatively charged phospholipids has been shown to decrease the lipid bilayer thickness [22], increase the permeability of liposomes [23] and increase lipid monolayer surface area [13]. The binding can be disrupted by high ionic strength before, but not after formation of a lipid-protein structure [25]. It has been suggested that these interactions represent initial electrostatic binding, followed by penetration into the lipid bilayer by protein.

This work presents a laser Raman spectroscopic study of the interaction of lysozyme with liposomes of the mixed phospholipid system, 1,2-dimyristoyl-L-phosphatidylcholine (DMPC) and 1,2-dipalmitoyl-L-phosphatidic acid (DPPA). The lipid system was chosen so that effects associated with binding to acidic lipids could be separated from those associated with phosphatidylcholine. This work presents a laser Raman study of the effect of lipid binding on protein conformation and the first direct evidence of a protein conformational change associated with a lipid gel-liquid crystal transition.

Experimental Procedure

Materials. Lysozyme from egg white (6 times crystallized; activity, 25 000 units/mg) was purchased from Miles Research Laboratories; high purity 1,2-dimyristoyl-L-phosphatidylcholine was purchased from Sigma Chemical Company, and 1,2-dipalmitoyl phosphatidic acid (recrystallized, white powder) was purchased from Serdary Research Laboratories, London, Ontario.

Preparation of liposomes. Phospholipid liposomes were prepared by weighing out dimyristoyl phosphatidylcholine (typically 5 mg)/dipalmitoyl phosphatidic acid/water in a 1 : 1 : 20 ratio, mixing on a vortex mixer for 1—2 min and incubating for 30 min at 43°C. The pH was adjusted to 5.6, and the sample was remixed and reincubated. For systems containing lysozyme, a solution of lysozyme in water (1 : 5, w/w, pH 5.6) was added to the lipid suspension after the second incubation, to produce dimyristoyl phosphatidylcholine/dipalmitoyl

phosphatidic acid/lysozyme in a ratio of 1 : 1 : 3 w/w. This provides approximately 20 lipid molecules per protein. The mixture was vortexed for 1 min, and incubated at 43°C for 10 min. 10 μl samples were sealed in glass capillary tubes for Raman spectral analysis.

Raman spectrometer. The Raman spectrometer consists of a SPEX 1403 half-meter double monochrometer with a Spectra-Physics 164 argon ion laser typically operated at 300 mW at 514.5 nm. A Baird-Atomic interference filter is placed before the sample to remove plasma lines. The detection system consists of a cooled RCA C31034 photomultiplier coupled to an SSR photon counter which is interfaced to a Charles River Data Systems MF-11 computer (LSI-11 with 28K of core and dual floppy disk drives). All samples are contained in a cell holder regulated thermostatically to ±0.3°C. Temperatures reported are those of the sample holder, which is within 1°C of the sample temperature as measured by the gel-liquid crystal transition in dipalmitoyl phosphatidylcholine.

Spectral data are counted for 1 s × 1 cm^{-1} intervals over the spectral range and stored in the computer. Typically, eight repeated scans are averaged to provide high quality composite spectra. The composite spectra were subjected to a nine-point smoothing routine by the method of Savitsky and Golay [26] which does not skew the spectral bands. Those spectra which cover the frequency range 600—1800 cm^{-1} have had water subtracted to give a horizontal baseline 1690—1800 cm^{-1}. In certain cases as outlined in the text, differences between Raman spectra were obtained to analyze clearly spectral features of either lipid or lysozyme. The advantages of computer acquisition and presentation of Raman data as well as Raman difference spectra are more fully described in the literature [27].

Results and Discussion

Interaction with lipid in the trans-configuration

Fig. 1 shows the Raman spectra at 18°C, of lysozyme (1 : 1) DMPC/DPPA, and (1 : 1 : 3) DMPC/DPPA/lysozyme. At this temperature, below the gel-liquid crystal or chain-disorder transition of either lipid, the phospholipid side-chains are in the all-*trans*-configuration. A comparison of the spectrum of the lipid-protein system with the spectra of its components shows differences at 770—820 cm^{-1}, tentatively assigned to the phosphate diester stretch of the phospholipid, at 1240—1340 cm^{-1} the amide III region of the protein, at 1361 cm^{-1} assigned to a tryptophan mode sensitive to local environments and at 1650—1680 cm^{-1} the amide I region of the protein.

To amplify and interpret these differences further, Fig. 2 compares the Raman spectrum of lysozyme with the difference spectrum of (1 : 1 : 3) DMPC/DPPA/lysozyme minus (1 : 1) DMPC/DPPA at 18°C. In Fig. 2, the modes at 782 and 805 cm^{-1} are clearly observed in the difference spectrum, perhaps as part of a broad envelope from 740—830 cm^{-1}. Similar behavior has been observed following interaction of acidic lipids with di- and tripositive ions (Lipper, J.L., unpublished data). Koyama et al. [28], on the basis of normal coordinate analysis and model studies, assign a weak set of bands in L-DPPC at 830 cm^{-1} and 853 cm^{-1} to the -O-P-O- diester anti-symmetric stretch and at

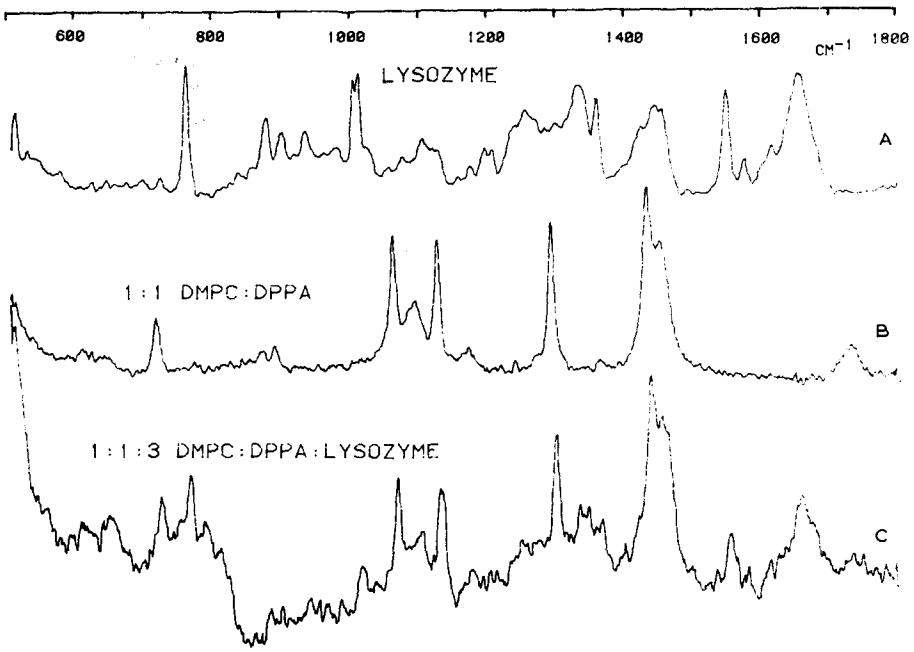

Fig. 1. Raman spectra of: A. Lysozyme (7% in aqueous solution); B. (1 : 1, w/w) mixture of 1,2-dimyristoyl-L-phosphatidylcholine (DMPC)/1,2-dipalmitoyl phosphatidic acid (DPPA) (10% aqueous suspension); C. (1 : 1 : 3, w/w) DMPC/DPPA/lysozyme in water. All spectra were obtained at 18°C and pH 5.6. In all cases, water has been subtracted from the spectra.

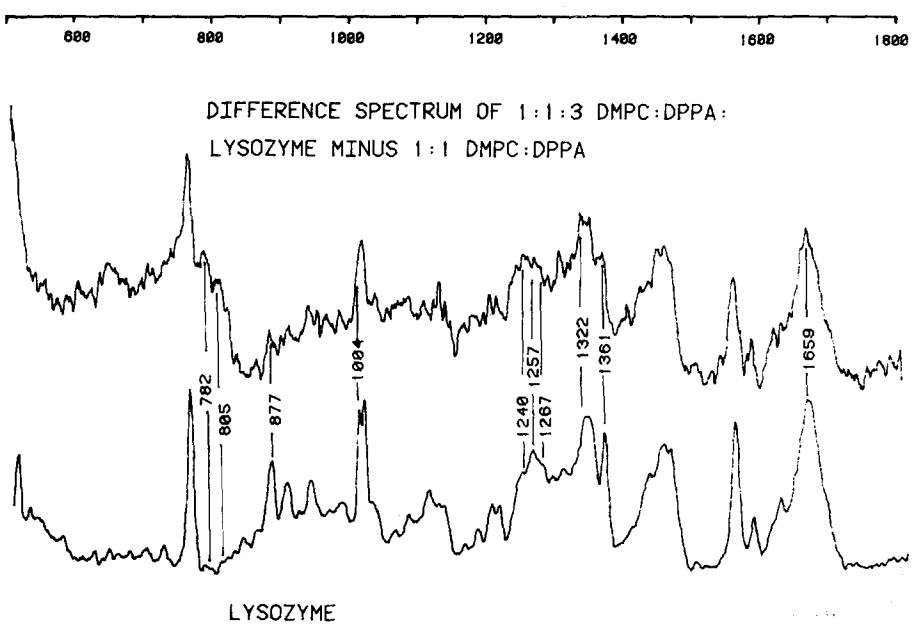

Fig. 2. Comparison of the Raman spectrum of lysozyme at 18°C with the difference between the spectrum of (1 : 1 : 3) DMPC/DPPA/lysozyme and (1 : 1) DMPC/DPPA, at 18°C.

765 cm^{-1} to the -O-P-O- diester symmetric stretch. Bands at the same frequencies are observed in anhydrous L-α-glycerophosphorylcholine (GPC), but hydrated GPC-$CdCl_2$ crystals show only a single mode at 785 cm^{-1}. This change was associated with differences in rotation about the O-C bond between the phosphate and the choline head group [28]. Such a detailed assignment cannot be made in the present case; however, we tentatively assign the new modes at 782 cm^{-1} and 805 cm^{-1} in the phospholipid-protein spectrum to a conformational change in the phosphate diester region of the lipid headgroup resulting from interactions with basic lysozyme.

The spectral changes in the amide I and amide III regions of lysozyme resulting from interactions with acidic lipid are quite subtle at temperatures below 30°C. The amide I region at 1659 cm^{-1} appears to be slightly broadened by the interaction but is not shifted. In the amide III region, the feature at 1257 cm^{-1} decreases slightly in intensity so that the bands at 1240 cm^{-1} and 1267 cm^{-1} appear more prominent, although their intensity does not change. At the same time, the band at 1322 cm^{-1} increases slightly in intensity.

The bands in the amide III region of proteins have customarily been interpreted in terms of α-helix, β-sheet, and 'random coil' content, with the mode at 1240 cm^{-1} associated with β-sheet, that at 1250—1260 cm^{-1} associated with random coil and that at 1280—1320 cm^{-1} associated with α-helix [7—12]. However, recently several workers [9,29] have suggested that the region at 1254—1280 cm^{-1} be assigned to β-turn. Therefore, we interpret the spectral decrease at 1257 cm^{-1} and increase at 1322 cm^{-1} to a small decrease in random or β-turn content and slight increase in α-helix when lysozyme interacts with DMPC/DPPA liposomes at temperatures at which the lipid hydrophobic region is in the all-*trans*-conformation.

Yu [30] has suggested that the 1361 cm^{-1} mode of tryptophan is a very sensitive indicator of the local environment around tryptophan. When the tryptophan residue is buried within the protein, the 1361 cm^{-1} mode is sharp and intense, but when it is exposed to hydrogen bonding interactions with solvent, the intensity diminishes. The change in 1361 cm^{-1} intensity seen in Fig. 2 is a bit puzzling, in that one would not expect the interaction with phospholipid to expose tryptophan to water. Possibly this represents a direct interaction of previously buried tryptophan residues with the polar lipid surface.

Temperature dependence of lipid conformation

The temperature dependence of the Raman spectra of (1 : 1) DMPC/DPPA at pH 5.6 is shown in Fig. 3A, and the ratio of intensities of the 1090 cm^{-1} *gauche* and 1129 cm^{-1} all-*trans*-C-C stretching modes is plotted in Fig. 4 as a function of temperature. This intensity ratio is proportional to the S_{trans} parameter suggested by Gaber and Peticolas [5] and is a measure of the all-*trans* or rigid lipid hydrocarbon chain content.

Fig. 4 shows marked decreases in chain order at 20—24°C and 57—59°C, corresponding to disordering of the 14 carbon DMPC side-chain and the 16 carbon DPPA side-chain, respectively. The lower temperature transition is more gradual than that observed for pure DMPC [18].

The temperature dependence of the 940—1400 cm^{-1} region of the Raman spectrum of (1 : 1 : 3) DMPC/DPPA/lysozyme at pH 5.6 is shown in Fig. 3B.

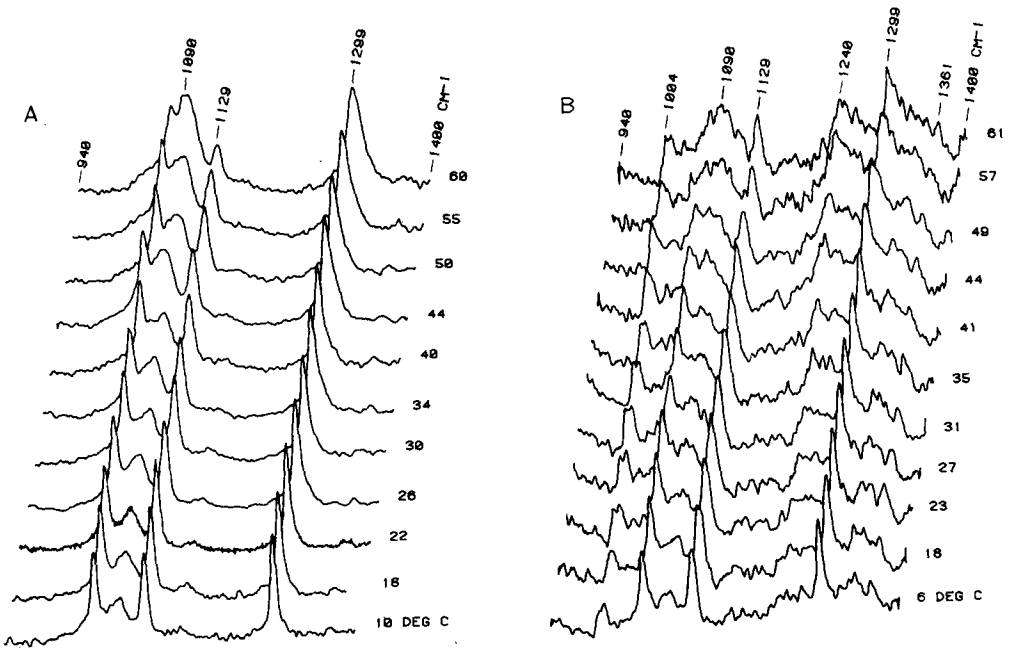

Fig. 3. A. Temperature dependence of (1 : 1) DMPC/DPPA Raman spectra in the region 940—1400 cm^{-1}, over the range 10—60° C. B. Same as A for (1 : 1 : 3) DMPC/DPPA/lysozyme.

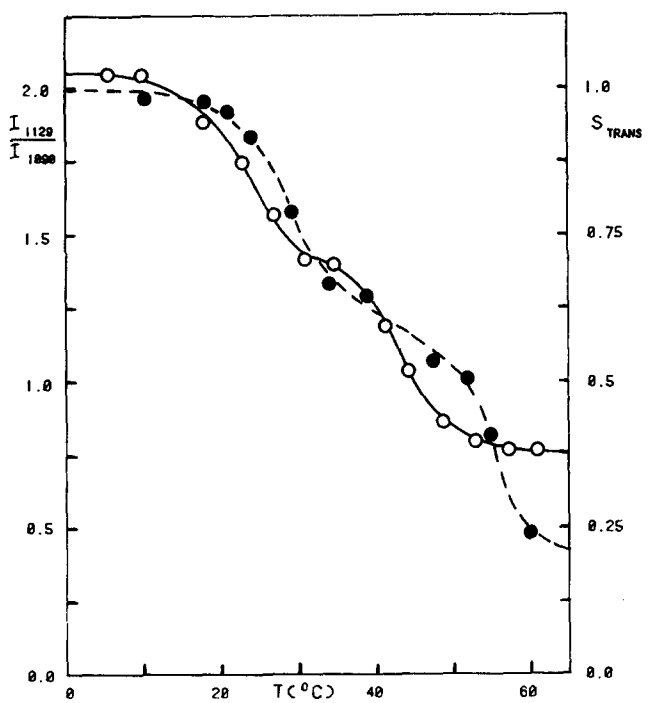

Fig. 4. Temperature dependence of the relative intensities at 1129 cm^{-1} and 1090 cm^{-1} and their relationship to the *trans* order parameter, S_{trans} for (1 : 1) DMPC/DPPA (●------●) and (1 : 1 : 3) DMPC/DPPA/lysozyme from which lysozyme has been subtracted (○———○).

As with the lipid system, the relative intensity of the 1129/1090 cm^{-1} couple decreases with temperature, although clearly not so much at higher temperatures. Fig. 4 shows the temperature dependence of I_{1129}/I_{1090} and S_{trans} for the phospholipid-protein complex. The ratios were obtained from difference spectra in which lysozyme, in its native conformation in water, was subtracted from the spectra of the phospholipid-lysozyme complex. At temperatures below the DMPC transition, S_{trans} is virtually identical for the phospholipid and lipid-protein adduct. Thus, the lysozyme-lipid interaction appears to have no effect on hydrophobic lipid side-chains in the all-*trans*, rigid conformation.

The lower temperature DMPC chain-melting transition is shifted approx. 3°C lower, but does not seem to be appreciably broadened by the interaction with lysozyme. Similar behavior has been observed for poly(L-lysine)-DMPC [17] and it has been suggested to be a manifestation of weak, polar lipid-protein interactions.

The higher temperature DPPA transition is shifted to lower temperatures by approx. 12°C and is slightly broadened. At temperatures above the transition, lipid in the complex contains substantially more *trans*-component than DMPC/DPPA alone. Clearly, the lipid-protein interaction has much more of an effect on the hydrophobic hydrocarbon chains of the phosphatidic acid than the phosphatidylcholine, suggesting that this is a polar interaction accompanied by penetration (apparently at temperatures above 30°C, see below) into the hydrophobic interior of the lipid bilayer. This supports the suggestion of Papahadjopolous and coworkers [23—25] that lysozyme interacts with phospholipid liposomes in a mixed polar-hydrophobic mechanism.

At 60°C, above both lipid transitions, lysozyme appears to increase the number of all-*trans*-segments in the lipid. This can be interpreted as evidence for either an immobilized, rigid phospholipid associated with the lysozyme, involving approx. 18—20% of the phospholipid or approx. four lipids associated with each lysozyme, or a more generalized effect which results in bulk lipid that is considerably more rigid than lipid alone at the same temperature.

Temperature dependence of the lysozyme conformation

The Raman spectrum of lysozyme in aqueous solution is temperature independent over the range 5—65°C [19,20], and is shown in Fig. 1. Above 80°C, the protein is irreversibly denatured and substantial changes are observed in the Raman spectrum. The most obvious of these are introduction of a broad amide III mode at 1247 cm^{-1} assigned to introduction of random conformation [19,20], an obvious broadening and decrease in peak intensity of the 509 cm^{-1} S-S stretching band, associated with S-S bond disruption, and a decrease in tryptophan intensity at 1361 cm^{-1}, associated with exposure of tryptophan to solvent. Similar changes were observed for chemical denaturation of lysozyme by 6 M LiBr [21].

Fig. 3B shows the effect of temperature on a limited region of the lipid-protein complex, while Fig. 5 shows the 500—1800 cm^{-1} spectral region of the complex at 18°C, 42°C, and at 18°C after heating to 62°C. Between 30°C and 53°C, a sharp band grows in the spectrum at 1239 cm^{-1} in the amide III region. A band at this frequency is characteristic of β-sheet, and is clearly different from the 1247 cm^{-1} mode that is observed in thermally and chemically

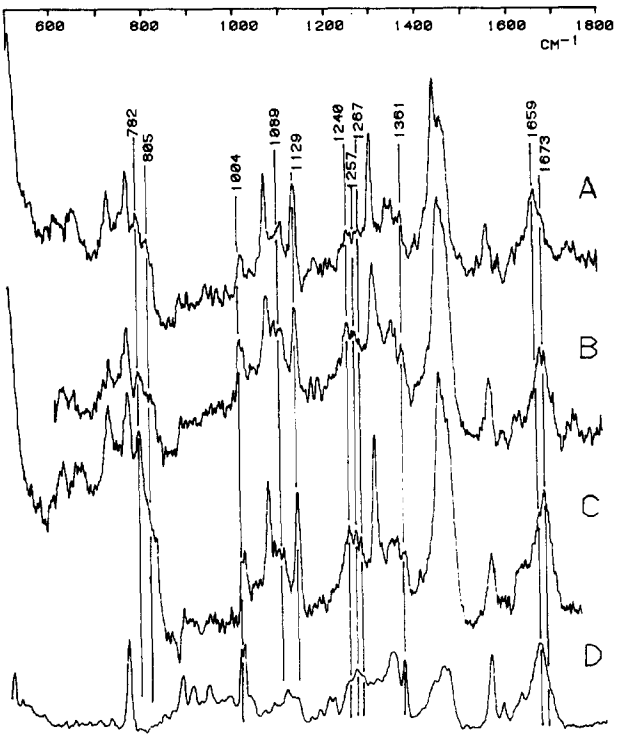

Fig. 5. Comparison of the Raman spectra of (1 : 1 : 3) DMPC/DPPA/lysozyme at A, 18°C; B, 42°C; C, 18°C after heating to 62°C, with D, lysozyme at 18°C.

denatured lysozyme [19—21]. As shown in Fig. 5, formation of the 1240 cm^{-1} amide III mode is accompanied by a shift in the amide I band from 1659 cm^{-1} to 1673 cm^{-1}, and both bands remain in the spectrum after the complex is heated to 62°C and cooled to 18°C. β-sheet has been shown to show an amide I band at 1673 cm^{-1}, which supports the suggestion of irreversible formation of β-structure above 40°C.

Between 23 and 27°C, and again at 42—53°C, the tryptophan mode at 1361 cm^{-1} decreases in intensity. This change represents a change in environment of at least some of the buried tryptophan residues in lysozyme. It is unfortunate that the very high light-scattering characteristics of the lipid-protein samples prevented our obtaining good spectra in the 509 cm^{-1} S-S stretching region. Because of this we were not able to obtain information about the effect of phospholipid-lysozyme interactions on the disulfide linkages.

The temperature dependence of the relative peak heights of the 1240 cm^{-1} β-sheet and 1361 cm^{-1} tryptophan bands are shown in Fig. 6. Interestingly, the onset of the decrease in the tryptophan 1361 cm^{-1} band appears to coincide with the chain-disorder transition of DMPC (and to a lesser extent the higher temperature transition of DPPA), although the lipid transitions are somewhat broader. On the other hand, the increase in β-structure, represented by the intensity at 1240 cm^{-1} appears to coincide with the chain-disorder transition

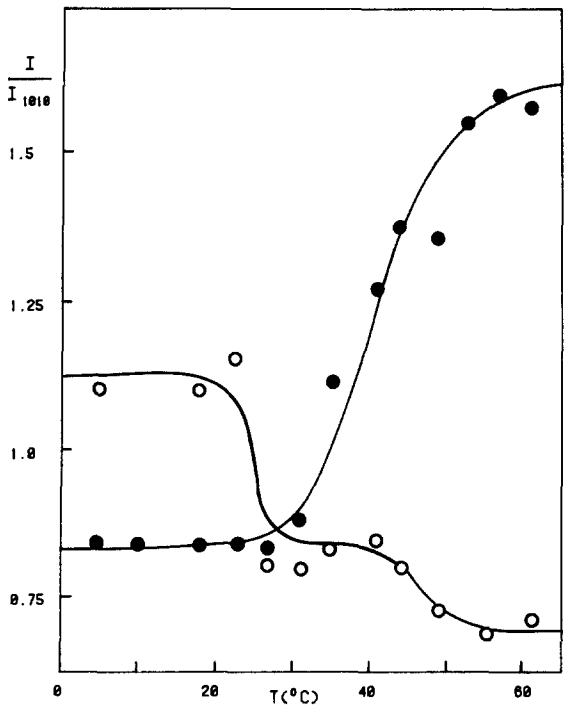

Fig. 6. Temperature dependence, relative to the intensity of the 1010 cm^{-1} doublet, of the 1240 cm^{-1} band assigned to β-sheet (●———●), and the 1361 cm^{-1} tryptophin band (○———○).

of the phosphatidic acid component of the mixed phospholipid. This complex behavior is extremely interesting and suggests that the interaction of tryptophan residues with lipid is enhanced by bilayer fluidity, while the changes in secondary structure appear to require fluidity in the acidic phospholipids that would tend to be associated with the basic lysozyme molecule. There is considerable evidence that the activity of a number of membrane-bound enzymes and presumably, their structure, are affected by phospholipid fluidity. However, this appears to be the first direct spectral evidence of a protein conformational change that is associated with a lipid conformational change in a protein-lipid system.

Concluding remarks

A small secondary structural change has been demonstrated for lysozyme subsequent to binding to acidic liposomes at temperatures at which the lipid is in the all-*trans*-configuration. Lysozyme has been shown to have only a minor effect on the fatty acid side-chain *trans*-to-*gauche* transition of the phosphatidylcholine component of mixed liposomes, but to markedly lower the transition temperature of the dipalmitoyl phosphatidic acid component and yet maintain a higher *trans*-content at temperatures above the DPPA transition.

A change in the environment of buried tryptophan residues is tentatively

associated with increased *gauche* content for either DMPC or DPPA, while a marked increase in β-sheet secondary structure in lysozyme parallels the *trans*-to-*gauche* transition of DPPA only. These appear to be unique spectral evidence of protein conformational changes tied to changes in lipid configuration in phospholipid-protein complexes.

It is not clear from the results of this work to what extent phase separations of various lipid-protein structures are related to the conformational changes noted for this system. We are pursuing a more detailed examination of the phase diagram and conformational relationships of lysozyme-DPPA-DMPC.

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