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Secondary Structure of the Hydrophobic Myelin Protein in a Lipid Environment as Determined by Fourier-transform Infrared Spectrometry*

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The secondary structure of a hydrophobic myelin protein (lipophilin), reconstituted with dimyristoylphosphatidylcholine or dimyristoylphosphatidylglycerol, was investigated by Fourier-transform infrared spectroscopy. Protein infrared spectra in the amide I region were analyzed quantitatively using resolution enhancement and band fitting procedures. Lipophilin in a phospholipid environment adopts a highly ordered secondary structure which at room temperature consists predominately of α-helix (approximately 55%) and β-type conformations (36%). The secondary structure of the protein is not affected by the lipid gel to liquid crystalline phase transition. Heating of the lipid-protein complex above approximately 35 °C results in a gradual decrease in α-helical content, accompanied by an increase in the amount of β-structures. Lipophilin dissolved in 2-chloroethanol is, compared to the protein in a lipid environment, richer in the α-helical conformation but still contains a sizable amount of β-structure.

The interaction between lipids and proteins determines many of the functional and structural properties of biological membranes. The effect of proteins on the molecular organization and structure of lipids has been studied extensively (1–3). Many aspects of the behavior of lipids in biological membranes are now well recognized. This is in contrast to a rather poor understanding of the conformational properties of proteins in a lipid environment. Although some reports indicate that the secondary structure of various model proteins and polypeptides can be affected by the interaction with phospholipids (4–9), data concerning the true integral membrane proteins are scarce and often controversial.

One of the techniques that can be used to study conformational properties of proteins is infrared spectroscopy. Vibrational bands of proteins, and particularly the amide I band, are sensitive to the nature of the secondary structure as this involves specific hydrogen bonding of the C=O and N–H groups (Refs. 10–12 and references therein). Yet, despite a great potential, the practical usefulness of the technique was severely limited by the overlapping of spectral components characteristic of various secondary structures. This impeded or even precluded reliable identification of individual components in the broad contours of amide bands. However, recently developed resolution enhancement methods, such as Fourier deconvolution (13, 14) and Fourier derivative spectroscopy (15, 16) have helped overcome this obstacle and infrared spectroscopy emerges now as a powerful tool to probe details of protein conformation in solution. The combination of resolution enhancement methods with band fitting procedures allows for quantitative assessment of various components of protein secondary structure, such as α-helices, β-structures, and turns (17, 18). The technique is of particular value in the structural studies of membrane-associated proteins; in contrast to some other optical methods (*e.g.* circular dichroism), the infrared analysis is essentially free of potential artifacts due to the light scattering on membrane fragments.

In the present report we present the results of a detailed infrared spectroscopic investigation of the secondary structure of lipophilin in a phospholipid environment. Lipophilin, known also as proteolipid apoprotein or Folch protein, is a major component of the human central nervous system myelin (19–21). It comprises more than 50% of the total myelin protein and seems to play a crucial structural role. Lipophilin is rich in hydrophobic amino acids (22) and behaves in every respect as a typical integral membrane protein (20, 21). When incorporated into lipid vesicles it was shown to exist as a spherical particle 52 Å in radius protruding outside the bilayer on both sides (23).

MATERIALS AND METHODS

The phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL) and 2-chloroethanol was purchased from Aldrich and was redistilled shortly before use. Lipophilin was prepared from human central nervous system myelin as described by Gagnon *et al.* (19). The protein was lyophilized and stored at −20 °C. The aqueous form of lipophilin was obtained by dialysis of a protein solution in 2-chloroethanol against distilled water (24). The protein was reconstituted with phospholipids (dimyristoylphosphatidylcholine or dimyristoylphosphatidylglycerol) by using two different procedures. (i) The protein and lipid, at the appropriate proportion, were co-dissolved in 2-chloroethanol and the organic solvent was then removed by freeze drying. Aqueous membrane suspensions were obtained by dispersing the dry sample in buffer at a temperature slightly above that of the lipid phase transition. (ii) An aqueous solution of lipophilin at about 2 mg/ml was added to the lipid powder and the recombinants were obtained by extensive vortexing of the mixture. The sample was then lyophilized and rehydrated by suspending in the desired buffer. The latter procedure was reported to be an efficient and nonperturbing method for membrane reconstitution (25). In all cases the buffer contained 50 mM Hepes[1] and 50 mM NaCl adjusted to pH 7 and was prepared in D_2O (MSD Isotopes, 99.8% purity). The lipid to protein weight ratio in the reconstituted samples was 2:1. The final content of deuterium oxide was 90% by weight.

Samples for infrared measurements were placed between calcium fluoride windows that were assembled into a demountable cell (Har-

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[1] The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMPC, dimyristoylphosphatidylcholine.

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rick Scientific, Ossining, NY). The thickness of the spacer was 0.012 mm. Fourier-transform infrared spectra were recorded using a Digilab FTS-15 instrument equipped with a HgCdTe detector. For each spectrum 500 interferograms were collected, co-added, apodized with a triangular function, and Fourier-transformed to give a resolution of 2 cm^{-1}. In order to eliminate spectral contributions of atmospheric water vapor, the instrument was continuously purged with dry nitrogen. The technique of Fourier deconvolution was used to resolve overlapping infrared bands (13, 14). Curve fitting was performed using standard procedures (26).

RESULTS

Vibrational spectra of proteins consist of several characteristic bands in the mid-infrared region. The vibrational mode most useful for the analysis of protein secondary structure is the amide I band between approximately 1600 and 1700 cm^{-1}. In order to avoid uncertainties associated with the subtraction of a strong H_2O band around 1640 cm^{-1} (16), the lipophilin/phospholipid samples used for infrared measurements were suspended in D_2O buffer. The amide I region of the infrared spectrum at 20 °C of lipophilin reconstituted with dimyristoylphosphatidylcholine (DMPC) is shown in Fig. 1*A*. The spectrum shows a maximum at about 1652 cm^{-1} and an intense shoulder between 1625 and 1630 cm^{-1}. While these two spectral features already indicate the presence of both α-helical and β-type conformations (10, 12), further details are likely to be hidden by the partial overlapping of bands that are characteristic of various components of protein secondary structure (15–18, 27). Indeed, the Fourier-deconvolved spectrum shown in Fig. 1*B* reveals the presence of five components in the amide I region of the lipophilin/DMPC sample in D_2O buffer. The frequencies of the component bands identified in the resolution-enhanced spectrum can be used subsequently as input parameters for curve fitting of the original broad amide I band contour (without knowing the number and approximate positions of the components any curve-fitting analysis would be meaningless). The result of such a curve-fitting analysis of the spectral region of the amide I band of lipophilin in a DMPC environment at 20 °C is shown in Fig. 2. The frequencies of the best fitted component bands are given in Table I; these frequencies correspond closely to those identified in the resolution-enhanced spectrum. Also listed in Table I are the fractional areas (as integrated intensities) of the individual components.

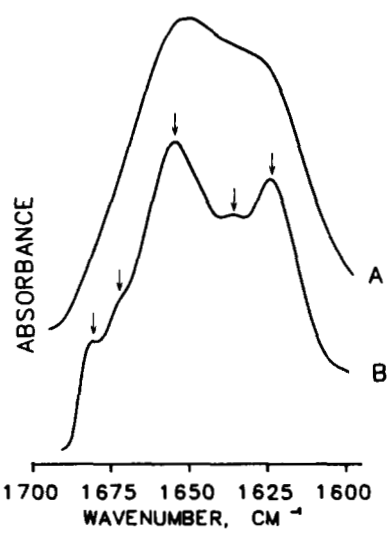

FIG. 1. **The amide I region of the infrared spectrum at 20 °C of lipophilin reconstituted with dimyristoylphosphatidylcholine.** *A*, original absorbance spectrum; *B*, same spectrum after band narrowing by Fourier deconvolution using a 18 cm^{-1} half-width Lorentzian line and resolution enhancement factor (a *K* value) of 2.2 (see Refs. 13 and 14).

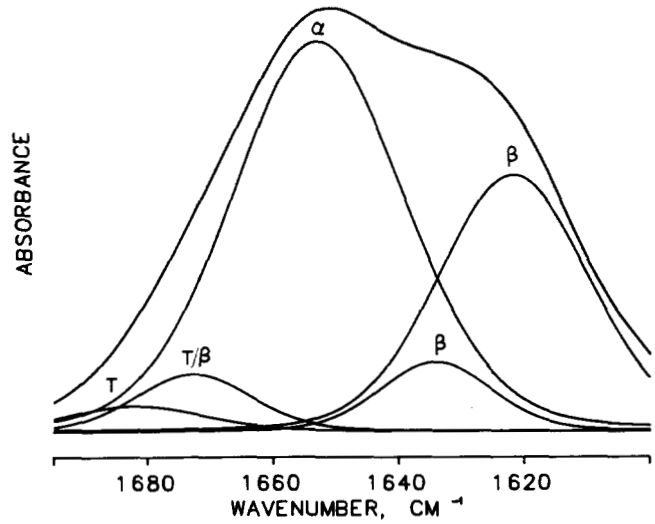

FIG. 2. **The amide I band contour with the best fitted individual component bands for lipophilin in a dimyristoylphosphatidylcholine environment at 20 °C.** The symbols α, β, and *T* stand for α-helices, β-structures, and turns, respectively.

TABLE I

Positions (in cm^{-1}) and fractional areas (in percentage) of the amide I bands of lipophilin under different conditions

Lipophilin/ DMPC at 20 °C	Lipophilin/ DMPC at 82 °C	Lipophilin/ DMPG[a] at 20 °C	Lipophilin in 2-chloroethanol at 20 °C
1622 (30%)	1626 (45%)	1621 (24%)	1623 (19%)
1634 (6%)	1643 (6%)	1635 (12%)	1635 (12%)
1654 (54%)	1655 (27%)	1654 (55%)	1654 (63%)
1673 (7%)	1672 (19%)	1672 (5%)	1676 (6%)
1683 (3%)	1689 (3%)	1680 (4%)	1685 (<1%)

[a] DMPG, dimyristoylphosphatidylglycerol.

The most prominent feature in the amide I region of the infrared spectrum of the lipophilin-DMPC complex in D_2O buffer is the band at 1654 cm^{-1} (Fig. 2 and Table I). This component, which accounts for 54% of the total area of the amide I band, can be assigned unambiguously to protein segments in the α-helical conformation (10, 11, 17). Fourier deconvolution reveals that the shoulder seen in the original spectrum of Fig. 1*A* consists of two bands: a major component at 1622 cm^{-1} and a smaller one at 1634 cm^{-1}. The frequencies of both bands are highly characteristic of β-structures (17, 18). In fact, the presence of more than one "β-component" in the spectral region between 1620 and 1640 cm^{-1} has been observed for a number of other proteins (17).[2] These components most likely represent β-type segments with a slightly different pattern of hydrogen bonding. Although tempting, the assignment of the different "β-bands" to specific classes of β-strand (*i.e.* parallel and antiparallel) is not straightforward and, at least at present, would be purely speculative. It should also be emphasized that while the lipophilin/lipid samples were prepared as dispersions in D_2O buffer, the infrared spectra argue against any H→D exchange of the amide groups in α-helical and β-strand structures. Therefore the bands considered above represent protonated and not deuterated amide groups. This is indicated by the virtually identical positions of the infrared bands representing α-helices and β-strands in the lipophilin spectra measured in D_2O

[2] W. K. Surewicz, M. A. Moscarello, and H. H. Mantsch, unpublished data.

and in the fully protonated 2-chloroethanol (see below and Table I). Furthermore, no low frequency shift of the respective infrared bands (as expected for deuterium-exchanged amide groups) could be detected when the lipophilin spectra recorded immediately after sample dispersion in D_2O buffer were compared with those measured after 24 h. The above observations are not surprising as even in the water-soluble globular proteins the ordered structures such as α-helices and β-strands are fairly resistant to hydrogen/deuterium exchange (Ref. 28).[2] Deuteration of the amide groups of helical and β-strand structures should be particularly difficult in the case of the highly hydrophobic lipophilin embedded in a lipid bilayer matrix.

The assignment of the two minor components at 1673 and 1682 cm^{-1} (Fig. 2) is less certain as both bands due to turns and coupled high frequency vibrations of β-segments may contribute to the spectral region between 1670 and 1690 cm^{-1} (10, 17, 29). The frequencies of bands due to these different structures may in certain cases be very close or even coincide. In previous analyses of infrared spectra of water-soluble proteins, turns have been assigned to bands around 1665 cm^{-1} as well as to frequencies above 1680 cm^{-1} (17, 18). A usually weak band around 1675 cm^{-1}, on the other hand, has been attributed to in-phase vibrations of β-segments in antiparallel conformation (17). In the infrared spectrum of lipophilin the "turn band" around 1665 cm^{-1} is, however, missing. In this case we are inclined to attribute both the band at 1682 cm^{-1} as well as the band at 1673 cm^{-1} (or at least the major part of it) to turns. The assignment of the latter band as a β-component would result in an unrealistically low estimate of the content of turns. Models of the organization of lipophilin in the lipid bilayer have assumed folding of the protein into five membrane domains (22, 30, 31). Several of these have been isolated (30) and the structure of the C-terminal domain has been characterized (32). Obviously, a considerable amount of turns is required to separate the transmembrane segments.

The secondary structure of lipophilin reconstituted with DMPC was also studied as a function of temperature. While infrared spectra of the protein in the temperature range between 20 and 35 °C are indistinguishable, further increase in temperature results in changes in the amide I band contour (Fig. 3). The intensity ratio of the peak at 1652 cm^{-1} to that at 1628 cm^{-1} decreases as the temperature is increased. This suggests that the content of β-structure increases at the expense of α-helices. The temperature-induced spectral changes are not rapid and progress gradually over a wide temperature range. Fourier deconvolution of the infrared spectrum measured at 82 °C (the highest temperature applied in this study) indicates the presence of five component bands in the amide I region (Fig. 4). These components are, however, not identical with those identified in the spectrum at or below 30 °C. The bands representing the β-structure at 20 °C (at 1622 and 1634 cm^{-1}) are replaced in the spectrum at 82 °C by a single band centered at 1626 cm^{-1}. Moreover, in addition to the α-helix band at 1655 cm^{-1}, a new component at 1643 cm^{-1} can be distinguished in the high temperature spectrum. This minor band is characteristic of deuterated amide groups of non-ordered protein fragments (10, 17). It most likely represents helical fragments which at high temperature underwent the transition to non-ordered structures and thus became accessible to deuterium exchange. Curve-fitting analysis (Table I and Table II) indicates that at 82 °C the α-helical content of lipophilin drops to 27%. The estimate of the content of β-structure at 82 °C is subject to considerable uncertainty due to the relatively large area occupied by the component at 1672 cm^{-1}. As discussed above, the assignment of the band at this

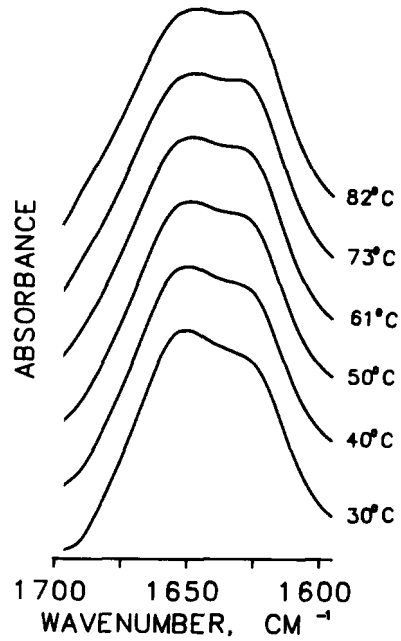

FIG. 3. **Temperature dependence of the infrared spectrum in the amide I region of lipophilin reconstituted with dimyristoylphosphatidylcholine.** Spectra were recorded in a heating cycle.

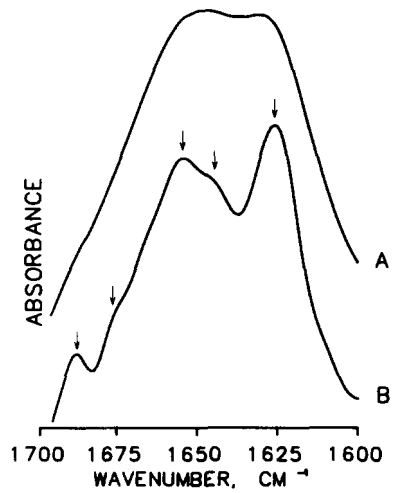

FIG. 4. **The amide I region of the infrared spectrum at 82 °C of lipophilin reconstituted with dimyristoylphosphatidylcholine.** *A*, original absorbance spectrum; *B*, same spectrum after band narrowing by Fourier deconvolution using parameters given in the legend to Fig. 1.

TABLE II

Total estimate of the content of α-helix and β-conformation in lipophilin under different conditions

Sample	α-Helix	β-Structure[a]
	%	
Lipophilin/DMPC, 20 °C	54	36
Lipophilin/DMPC, 82 °C	27	45
Lipophilin/DMPG,[b] 20 °C	55	36
Lipophilin in 2-chloroethanol, 20 °C	63	31

[a] The content of β-structure is based on low frequency β-components only (see text) and may be somewhat underestimated, particularly in the case of the lipophilin/DMPC sample at 82 °C.

[b] DMPG, dimyristoylphosphatidylglycerol.

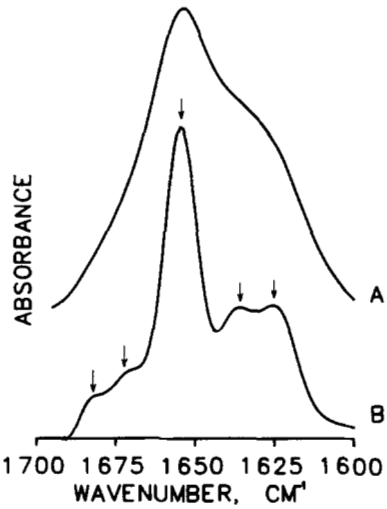

FIG. 5. **Infrared spectrum in the amide I region of lipophilin in 2-chloroethanol at 20 °C.** *A*, original absorbance spectrum; *B*, same spectrum after band narrowing by Fourier deconvolution using parameters given in the legend to Fig. 1. The weak absorbance in this region of 2-chloroethanol was subtracted using the solvent band at 1920 cm^{-1} as a reference peak.

frequency is not unambiguous as it may contain contributions from both turns and protein segments in a β-conformation. Despite this uncertainty, even when taken alone, the intense band at 1626 cm^{-1} already reflects the increased content of β-structure in the thermally denaturated protein.

As described under "Materials and Methods," lipophilin was reconstituted with DMPC by using two different methods. The infrared spectra in the amide I region of these two preparations are virtually identical. Very similar spectra were also obtained after protein reconstitution with an acidic phospholipid, dimyristoylphosphatidylglycerol, indicating that the secondary structure of lipophilin in a membrane environment is not affected significantly by the nature of the lipid headgroup (Table I and Table II).

The aqueous form of lipophilin is stable only in dilute solutions and the sensitivity requirements of our current Fourier-transform infrared instrumentation have precluded reliable studies of the secondary structure of lipophilin as an aqueous solution. High quality spectra, however, could be obtained for lipophilin dissolved in 2-chloroethanol (Fig. 5*A*). The amide I band contour of lipophilin in this solvent consists of a major peak at 1653 cm^{-1} and a distinct shoulder around 1630 cm^{-1}, just like in the case of lipophilin/phospholipid samples. Band narrowing by Fourier deconvolution (Fig. 5*B*) reveals that this shoulder consists of two components, the frequencies of which are almost identical to those observed in the spectra of lipophilin/phospholipid samples (Table I). However, compared to lipophilin in a phospholipid environment, the quantitative estimate obtained for the secondary structure of the protein in 2-chloroethanol is richer in α-helix and slightly poorer in β-type structures (Table II).

DISCUSSION

The present infrared spectroscopic study shows that the main component of the secondary structure of lipophilin in a phospholipid environment is the α-helix. Qualitatively, this is in accord with the results of a previous circular dichroism (CD) experiment (24). However, quantitative estimates of the protein secondary structure obtained from the analysis of infrared and CD data are considerably different. Estimates based on circular dichroism give a higher content of α-helix (75% *versus* 54% obtained in this study) and a significantly lower content of β-structure (8–15% *versus* the present estimate of at least 36%). The discrepancies between the two methods appear to be even more serious when the conformation of lipophilin in 2-chloroethanol is considered. According to the CD data the secondary structure of lipophilin in this solvent consists of 95% α-helix, with no β-strands, whereas the present estimate indicates that a substantial amount of β-structure is present also upon dissolution of lipophilin in the aliphatic alcohol (Table II).

The reasons for the discrepancies between the estimates of lipophilin secondary structure obtained from the analysis of circular dichroism and infrared data are not fully clear. The sources of potential error in these two approaches are different. The CD spectrum of protein is affected by interfering absorption and, particularly in a membrane environment, by light scattering (33). Quantitative analysis of circular dichroism spectra relies on the set of reference data (34). These data, obtained from the spectra of aqueous solutions of globular proteins of known three-dimensional structure, may be not fully applicable to membrane proteins in a hydrophobic environment. Estimates of the secondary structure based on CD spectra are also influenced by uncertainties in protein concentration. The approach used in this study seems to be free of most of the problems listed above. Particularly, the analysis based on infrared spectra does not depend on any transferred secondary structure data. On the other band, a potential source of uncertainty in the quantitative interpretation of infrared data arises from the ambiguity in the assignment of the component bands between 1670 and 1680 cm^{-1} (see "Results"). This problem concerns particularly those cases where the characteristic "turn" band around 1665 cm^{-1} is not observed in the infrared spectra. Under certain circumstances the above ambiguity may affect the accuracy of the estimate of a β-structure. It should, however, not affect the estimate of the α-helix content. Another problem with the analysis of infrared spectra arises from the unknown intrinsic absorptivities of the amide I vibrations of C=O groups in different conformational states. The present approach is based on the assumption that the various amide I bands have comparable absorptivities and, accordingly, that the total fractional areas of bands assigned to various components of the secondary structure represent the real content of these structures. The good correlation that was found for more than 20 proteins between the secondary structure estimates obtained from x-ray data and infrared analysis (17)[2] strongly suggests that this assumption provides a realistic approximation that will not lead to systematic errors.

Thus, although certain approximations are involved in the quantitative interpretation of infrared spectra, we believe that the present estimate of a secondary structure, and particularly the relatively high content of β-type conformations, represents a real structural property of lipophilin. In fact, the main spectral features characteristic of a β-structure are seen even without deconvolution, and the overall shape of the amide I band contour is similar to that of other proteins containing both α-helices and sizable amounts of β-type conformations. Taking into consideration the uncertainties due to the assignment of the high frequency bands, the data in Table II represent only the lower limit of our β-structure estimates. The presence of a relatively large amount of β-type conformation is also consistent with the theoretical predictions based on an analysis of the amino acid sequence in lipophilin (22).

Previous differential scanning calorimetry experiments (35) as well as the results from our measurements of the lipid C–H stretching vibrations (data not shown) demonstrate that

DMPC in the presence of lipophilin undergoes a gel to liquid crystalline transition at a temperature close to that of the pure lipid, *i.e.* around 24 °C. The apparent lack of any differences between the amide I bands of DMPC-reconstituted lipophilin at 20 and 35 °C strongly indicates that the secondary structure of the protein is not affected even by relatively drastic changes in the physical state of the lipid matrix. Although, generally, very little is known about the effect of lipid fluidity on the conformational properties of membrane proteins, the behavior of lipophilin seems to resemble that of another integral protein, Ca^{2+}-ATPase, the conformation of which was reported to be insensitive to the phase transition of the surrounding lipids (36).

In view of the above, the changes in the conformation of lipid-associated lipophilin observed at higher temperatures represent most likely the direct effect of heating on the protein and not a secondary response due to the increased mobility of lipid acyl chains. The present data indicate that the thermally induced alteration in the secondary structure of membrane reconstituted lipophilin is not a sharp event but that it rather takes place over a wide temperature range. One of the consequences of the increased temperature is the reduction of the content of α-helical conformation. The above conclusions are in accord with the temperature-induced gradual decrease in the ellipticity at 222 nm found in a previous CD study of lipophilin in phosphatidylcholine vesicles (24). Unfortunately, no quantitative interpretation of the high temperature circular dichroism spectra is available. Analysis of the infrared data suggests that the drop in helicity of lipophilin in a membrane environment at high temperature is accompanied not only by the appearance of non-ordered polypeptide segments, but also by a further significant increase in the amount of β-structures. Early experiments with aqueous forms of lipophilin suggested that some of these forms exhibit considerable conformational flexibility and that at elevated temperatures they undergo a transition from predominantly helical to a β-type conformation (37). The present data indicate that a similar type conformational transition may occur also in a membrane environment. Of particular relevance to this finding may be the recent report (38) suggesting that the thermal denaturation of Ca^{2+}-ATPase is also accompanied by an increase in the content of β-sheet structure.

In conclusion, the detailed analysis of the infrared spectra of lipophilin indicates that the protein in a lipid environment contains both α-helices as well as a considerable amount of β-structure. The secondary structure of the protein is not affected by the lipid-phase transition. Incubation of the lipophilin-DMPC complex at high temperatures results in a reduced content of α-helical conformation and an increase in the amount of β-structure.

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