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Incorporation of synthetic peptide helices in membranes of tetraether lipids from *Thermoplasma acidophilum*. A calorimetric study

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Four analogues of the membrane-modifying, α-helical polypeptide antibiotic alamethicin were synthesized. The α-helical deca-, undeca-, heptadeca-, and icosapeptides were mixed with the main tetraether lipid of the Archaebacterium *Thermoplasma acidophilum* (MPL), dipalmitoylphosphatidylcholine (DPPC) and dihexadecylmaltosylglycerol (DHMG) in various ratios and the modification of the lipid phase transition was determined by differential thermal analysis (DTA). The polypeptides form mixed phases with MPL and DPPC, however, not with DHMG. Heptadeca- and icosapeptide exert a much stronger reduction of enthalpy (ΔH) than deca- and undecapeptide and bind about 0.5 molecule of MPL (or one molecule of DPPC) per peptide molecule. ΔH of the DPPC pretransition is reduced by the deca- and the undecapeptides and completely disappears with heptadeca- and icosapeptides (at 0.2 mole of peptide / mole of lipid). The modulation of the melting point T_m by the incorporation of peptides is more pronounced with MPL than with DPPC, the heptadecapeptide exhibiting the strongest reduction (with MPL) and the strongest broadening of the transition peak (with DPPC). Helix length, amphiphilicity and charge of the polypeptides can be correlated with the observed modifications of the lipid phase transitions.

Introduction

Interactions between phospholipids and synthetic peptide analogues of amphiphilic helices [1] are of major interest as models for channel forming proteins or membrane bound receptors.

In order to elucidate membrane interactions of natural polypeptides such as alamethicin [2], melittin [3] and valinomycin [4], the effect of defined synthetic peptides on phase transitions of model lipids are reported in the present study. In particular, a synthetic icosapeptide of α-helical secondary structure, analogous to the voltage-dependent pore forming alamethicin, has been investigated. This icosapeptide (P20) was synthesized via deca- (P10), undeca-(P11) and heptadecapeptides (P17) of amino acid sequences containing the lipophilic alanine and α-aminoisobutyric acid residues, which are the main components of peptaibols such as alamethicin [5]. In lipophilic environments each of the four polypeptides has a pronounced α-helical core [6–10]. Three of the peptides are mainly hydrophobic, only the P20 carries a hydrophilic head group comprising the triply charged tripeptide Asn-Arg-Arg at the N-terminus which shows a reversed asymmetry in voltage-dependent pore formation compared to the C-terminally polar alamethicin [5,11,12]. The peptides P10 and P11 are not membrane spanning, but P20 is long enough to span the membrane and P17 is almost as long. Hence, their incorporation into various lipid systems was investigated.

The basic structure of the main phospholipid (MPL) from *Thermoplasma acidophilum* is a symmetric membrane spanning macrocycle. It consists of two saturated

Abbreviations: Aib, α-aminoisobutyric acid; Boc, *t*-butoxycarbonyl; ΔH, enthalpy change; DHMG, dihexadecylmaltosylglycerol; DPPC, dipalmitoylphosphatidylcholine; DTA, differential thermal analysis; Me, methyl.

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methylbranched C_{40} hydrocarbon (diphytanyl) chains linked to glycerol molecules at both ends via ether bonds. At one end, the residual glycerol hydroxyl is glycosylated, at the other end of the macrocycle the glycerol carries a phosphate which in turn is esterified to another glycerol.

Thus, the macrocycle of MPL does not constitute a membrane bilayer but a monolayer of about the dimension (4–7 nm) of common phospholipid bilayers [13,14]. For comparison, the bilayer forming model phospholipid DPPC and the artificial etherglycolipid dihexadecylmaltosylglycerol (DHMG) were chosen.

Methods and Materials

Chemicals

Organic solvents (Resi quality) were purchased from Baker Inc., Groβ-Gerau (F.R.G.). All other chemicals were of analytical grade, they were purchased from Merck, Darmstadt (F.R.G.) or from Sigma, Deisenhofen (F.R.G.).

1,2-Dihexadecyl-3-*O*-β-D-maltosyl-*sn*-glycerol (DHMG) was synthesized and kindly provided by Dr. L. Six, Regensburg (F.R.G.).

Synthesis of the polypeptides

Four α-helical polypeptides P10, P11, P17 and P20 were synthesized which possess the C-terminal lipophilic decapeptidemethyl ester segment (Ala-Aib-Ala-Aib-Ala)$_2$-OMe in common. In order to impose a pronounced amphiphilicity all four peptides were synthesized with a free, positively charged N-terminus. The decapeptide H-(Ala-Aib-Ala-Aib-Ala)$_2$-OMe · HCl (P10) was prepared from Boc-Ala-Aib-Ala-Aib-Ala-OMe [8] by coupling the Boc-protected pentapeptide acid to the pentapeptidemethyl ester, chromatographic purification of the resulting protected decapeptide and splitting off the Boc group with HCl/acetic acid as described in detail elsewhere [8]. The analytically uniform product was elongated by Boc-Gln-OH and the resulting undecapeptide ester was N-terminally deprotected to yield H-Gln-(Ala-Aib-Ala-Aib-Ala)$_2$-OMe (P11).

P11 was elongated by fragment condensation with Boc-Ala-Leu-Ile-Leu-Leu-Ala-OH to obtain the fully protected heptadecapeptide which was chromatographed and N-terminally deprotected to yield H-Ala-Leu-Ile-Leu-Leu-Ala-Gln-(Ala-Aib-Ala-Aib-Ala)$_2$-OMe (P17).

Finally, Boc-Asn-Arg(NO$_2$)-Arg(NO$_2$)-OH was coupled to P17 in order to obtain the fully protected 20-peptide, which was hydrogenated and N-terminally deprotected to yield the icosapeptide ester H-Asn-Arg-Arg-Ala-Leu-Ile-Leu-Leu-Ala-Gln-(Ala-Aib-Ala-Aib-Ala)$_2$-OMe · HCl (P20).

The peptides were found to be uniform in thin-layer chromatography. Amino acid and enantiomer analysis showed the correct values and ^{13}C-NMR spectroscopy proved the identity of the compounds. Circular dichroism and NMR measurements in alcoholic solvents revealed a dominating α-helical conformation for all four peptides. α-Helices are also found for a number of structurally related Aib containing polypeptides of this type [5–10] including alamethicin [2,15].

Details of the syntheses and analyses of the four peptides are given elsewhere [16].

The sequence Asn-Arg-Arg-Ala-Leu-Ile-Leu-Leu-Ala-Gln C-terminally elongated by (Ala-Aib-Ala-Aib-Ala)$_2$-OMe has been chosen for three reasons. (i) It is part of an epitope of the interferon HuIFN-α(Ly), (ii) it has high α-helical propensity and strong amphiphilicity, (iii) its C-terminal part possesses a conformationally restricted α-helix stabilized by four α-aminoisobutyryl residues [6–10].

P20 was designed for voltage-current experiments in black lipid bilayers because of its polarity inverse to that of the voltage-dependent pore forming alamethicin which has a hydrophilic C-terminus. Most interesting P20 exhibited the expected asymmetry effects in current/voltage characteristics indicating an opposite polarity in the channel forming mechanisms [11]. However, the step-wise non-integral substructure in membrane current fluctuations typical of the alamethicin single pore [12] could not be resolved, because P20 is lacking the pore state stabilizing principle of alamethicin.

Growth of Thermoplasma acidophilum and isolation of the main phospholipid (MPL)

Thermoplasma acidophilum was obtained from the Max-Planck-Institute for Biochemistry, Munich (F.R.G.) and semicontinuously grown in a modified Freundt-medium at 59°C and pH 2 under moderate aeration using a Biostat S fermenter, Braun AG, Melsungen (F.R.G.). Cells were harvested in the late-exponential growth phase, washed, concentrated, centrifuged, and lyophylized [17,18].

Lipids were extracted from freeze-dried cells as described by Langworthy et al. [17]. MPL was isolated and purified chromatographcally over silic acid columns using chloroform-methanol gradients [18]. Controls were carried out by thin-layer chromatography using various solvent systems [19].

Differential thermoanalysis (DTA)

DTA was performed by means of a Mettler TA 3000/DSC 30 instrument equipped with a liquid nitrogen cooling device. Small quantities of dissolved lipid ($\leqq 5$ μl) were added repetitively into DTA pans, dried under a stream of nitrogen gas and kept under vacuum for 16 h. About 1 mg DPPC or DHMG or 3–5 mg

MPL were applied per pan. The lipid mass was determined gravimetrically. Dissolved peptide was added in similar manner, and its quantity again being determined gravimetrically. 20 μl of buffer solution (0.38 M sodium cacodylate/HCl, pH 7) was added and the pans were sealed. Maximal incorporation of peptides into the lipid phase was achieved by repeated freeze-thawing cycles. Peptide to lipid ratios refer to the total amount of sample, although little is known about the partition between aqueous and lipid phases. Nevertheless, the hydrophobic character of the peptides dominates and thus our estimations were considered a reasonable approximation.

In case of MPL the peptide-lipid mixture contained 50% of the cryoprotectant ethyleneglycol.

Heating scans were accomplished as indicated, at 0.017 K/s (DPPC, DHMG) and 0.06 K/s (MPL). Further details have been published elsewhere [20].

Results

Fig. 1 shows the heating curves of MPL in the presence of various ratios of the polypeptides P10, P11, P17 and P20. It is obvious that the phase transitions become broadened and quenched. The enthalpy change ΔH and the temperature of maximal heat flow were determined and plotted as functions of the molar ratios of peptide over lipid ($C_{peptide}/C_{lipid}$) (Figs. 2, 3). ΔH is

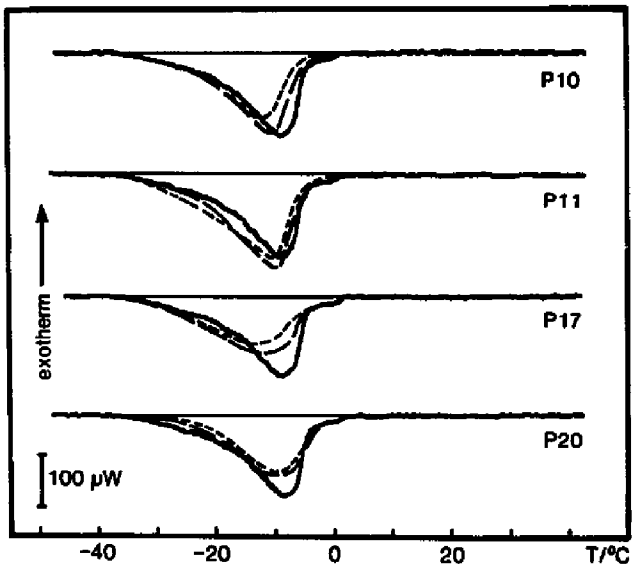

Fig. 1. DTA heating scan of hydrated samples of MPL and mixed samples of MPL plus the peptides P10, P11, P17, and P20. Heating rate $dT/dt = 0.06$ K/s. MPL (———); MPL + P10 (– – –, $C_{pep}/C_{lip} = 0.07$; ---, $C_{pep}/C_{lip} = 1.08$); MPL + P11 (– – –, $C_{pep}/C_{lip} = 0.51$; ---, $C_{pep}/C_{lip} = 0.88$); MPL + P17 (– – –, $C_{pep}/C_{lip} = 0.32$; ---, $C_{pep}/C_{lip} = 0.55$); MPL + P20 (– – –, $C_{pep}/C_{lip} = 0.18$; ---, $C_{pep}/C_{lip} = 0.42$).

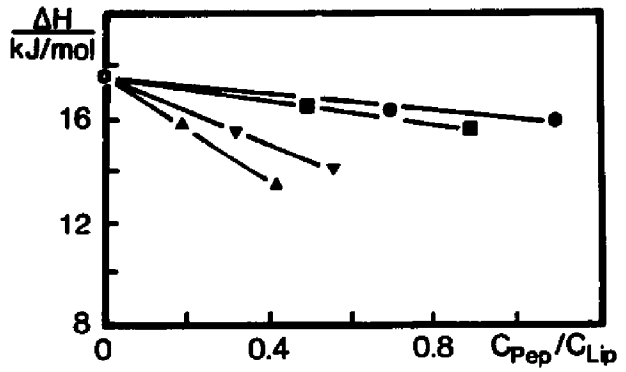

Fig. 2. Enthalpy change ΔH of the thermotropic transition of hydrated samples of MPL (○) and of mixtures with the peptides P10 (●), P11 (■), P17 (▼) or P20 (▲). ΔH is given per mol lipid.

linearly reduced with peptide concentration, and its slope increases with the length of the peptides (Fig. 2).

T_m is generally shifted to lower temperatures (Fig. 3), the effect of P17 being much stronger than that of P10, P11 and P20.

The results for MPL were compared to those for DHMG and DPPC, however, it must be considered that 1 mole of MPL is equivalent to two moles of DPPC or DHMG. The heating curve of DHMG (Fig. 4) shows a sharp main transition at 56.8°C and a small preceding endotherm around 55.5°C. The latter has also been observed by others (Six, personal communications); its origin, however, is not known (possibly reorganization or hydration of headgroups). The addition of P20 leads to a significant reduction of the transition at 55.5°C and to an increase in the low temperature tail (51–55°C), however, T_m, $\Delta T_{1/2}$ and ΔH remain virtually unchanged. The effect of the other peptides on the phase transition of DHMG was even smaller ($dT_m \cdot C_{lip}/dC_{pep} \leqq 0.36$ K, $d\Delta T_{1/2} \cdot C_{lip}/dC_{pep} \leqq 0.9$ K, $d\Delta H \cdot C_{lip}/\Delta H \cdot dC_{pep} \leqq 0.09$).

Judging from the marginal modulation of the thermodynamic properties of the lipid phase transition the

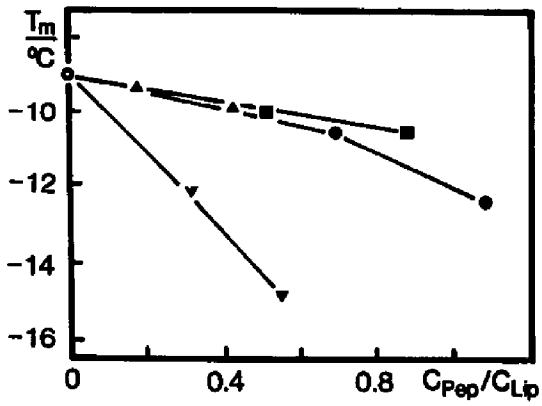

Fig. 3. Temperature of maximal heatflow (T_m) of the thermotropic transition of MPL (○) and of mixed samples with the peptides P10 (●), P11 (■), P17 (▼) and P20 (▲).

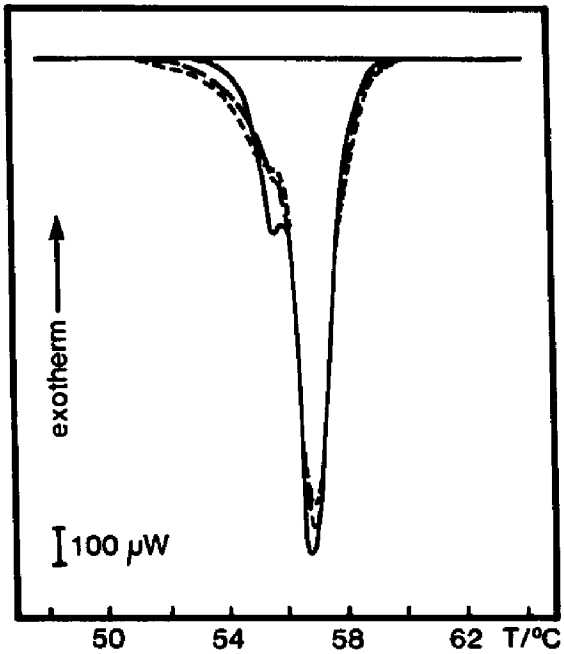

Fig. 4. DTA heating scans of hydrated samples of DHMG and mixed samples with the peptide P20. Heating rate $dT/dt = 0.017$ K/s. DHMG (———); DHMG + P20 (– – –, $C_{pep}/C_{lip} = 0.16$; ---, $C_{pep}/C_{lip} = 0.20$).

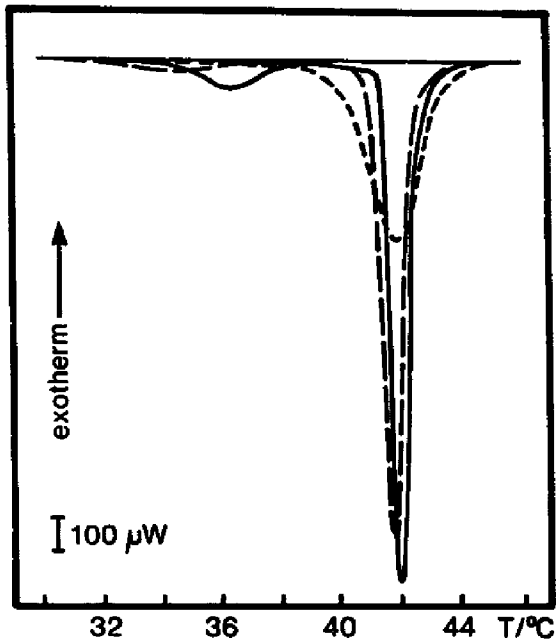

Fig. 6. DTA heating scan of hydrated samples of DPPC and mixtures with the peptide P17. Heating rate $dT/dt = 0.017$ K/s. DPPC (———); DPPC + P17 (– – –, $C_{pep}/C_{lip} = 0.24$; ---, $C_{pep}/C_{lip} = 0.51$).

incorporation of the polypeptides into DHMG membranes is low.

DPPC exhibits a marked main transition at 41.8°C preceded by a pretransition around 35°C (Fig. 5). By addition of peptide (Figs. 5–7) T_m stayed almost constant ($dT_m \cdot C_{lip}/C_{pep} \leqq 0.9$ K) (Fig. 8), however, the peak width of the maximal transition was broadened (Fig. 9) increasing with peptide length. ΔH is quenched

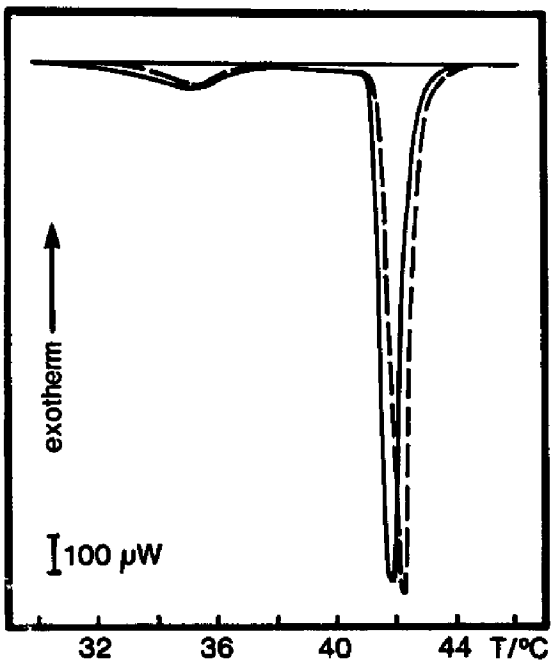

Fig. 5. DTA heating scan of hydrated samples of DPPC and mixtures with the peptide P10. Heating rate $dT/dt = 0.017$ K/s. DPPC(———); DPPC + P10 (– – –, $C_{pep}/C_{lip} = 0.28$).

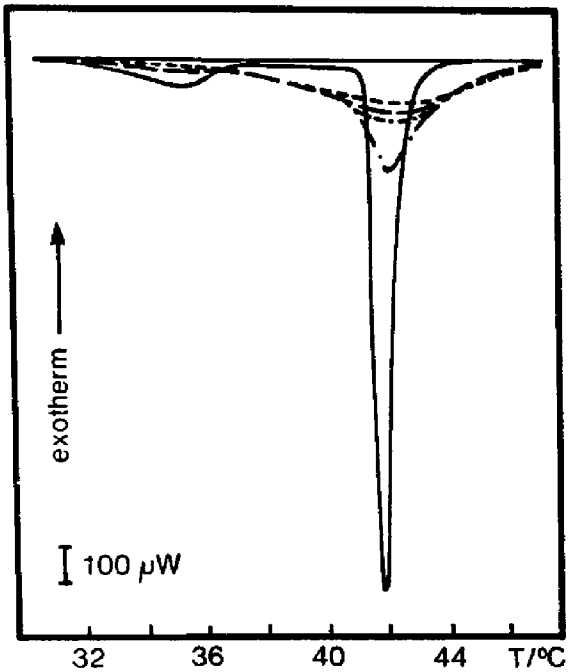

Fig. 7. DTA heating scan of hydrated samples of DPPC and mixtures with the peptide P17. Heating rate $dT/dt = 0.017$ K/s. DPPC (———); DPPC + P17, metastable phase (– – –, $C_{pep}/C_{lip} = 0.24$; ---, $C_{pep}/C_{lip} = 0.51$); stable phase (– · – · –, $C_{pep}/C_{lip} = 0.24$; · · · · ·, $C_{pep}/C_{lip} = 0.51$).

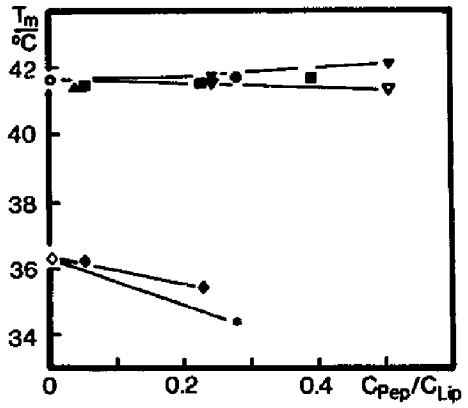

Fig. 8. Temperature of maximal heatflow (T_m) of the thermotropic pretransition of hydrated samples of DPPC (◇) and mixtures with P10 (★) and P11 (◆) and of the main transition of DPPC (○) and mixtures with P10 (●), P11 (■), P17 (from stable (▽) and metastable (▼) solid-analogue phase) and P20 (▲). Heating rate dT/dt = 0.017 K/s.

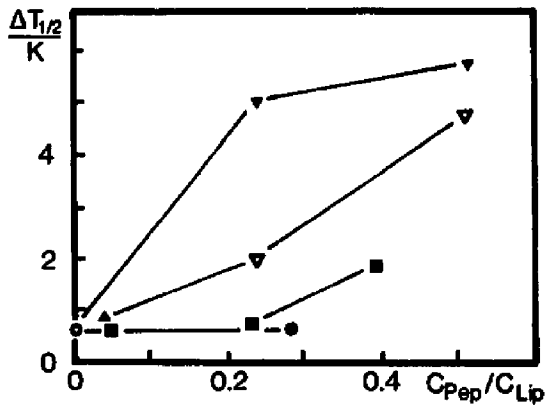

Fig. 10. Half-height width of the thermotropic main transition of hydrated samples of DPFC (○) and mixtures with P10 (●), P11 (■), P17 (of stable (▽) and metastable (▼) solid-analogue phase) and P20 (▲). Heating rate dT/dt = 0.017 K/s.

by all peptides (Figs. 5–7) in a similar way as it was seen with MPL.

The transition temperature and enthalpy changes ΔH of the pretransition is reduced with P10 and low concentrations of P11 and it completely disappears with P17 and P20 and higher concentrations ($C_{pep}/C_{lip} \leqq 0.4$) of P11.

DPPC plus P17 seem to form two different mixed phases: a stable phase present in the first heating curve after prolonged storage at 4°C ($t \geqq 20$ h) and a metastable solid-analogue state present in subsequent freezing and thawing cycles (Fig. 7) with an increased melting temperature (Fig. 8) and width (Fig. 9) and a reduced ΔH (Fig. 10) compared with the stable phase. Thus, the solubility of P17 in the stable solid-analogue phase of DPPC seems to be reduced, yet the three-fold broadening of $\Delta T_{1/2}$ (Fig. 9) and the reduction of ΔH (Fig. 10) indicate that still a significant fraction of the peptide remains dissolved in the lipid solid-analogue phase. This reorganization could be (i) an exclusion of peptide from the lipid domains and its accumulation at the domain borders or (ii) a submersion of the hydrophobic peptide completely into the hydrophobic bilayer under increased lipid headgroup interactions and reduced lipid headgroup spacing in the solid-analogue phase. The time constant for the recrystallization of the solid-analogue phase is in the order of several hours.

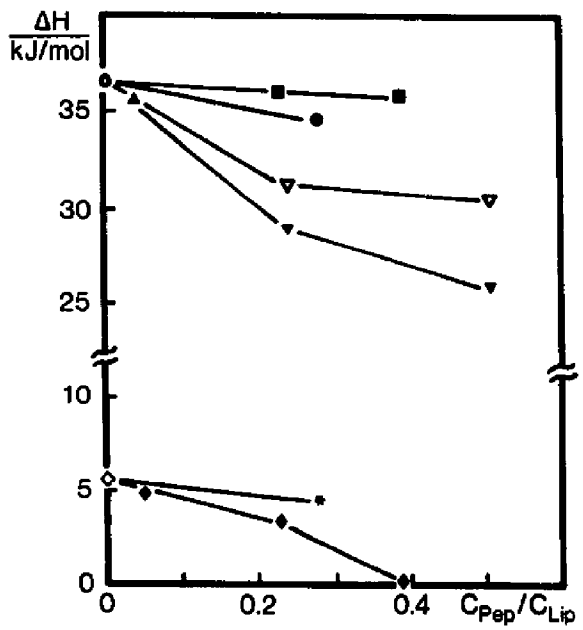

Fig. 9. Enthalpy changes (ΔH) of the pretransition of hydrated samples of DPPC (◇) and mixtures with P10 (★) and P11 (◆) and of the main transition of DPPC (○) and mixtures with P10 (●), P11 (■), P17 (of stable (▽) and metastable (▼) solid-analogue phase) and P20 (▲). Heating rate dT/dt = 0.017 K/s.

Discussion

In biological membranes, the protein content ranges from 25 up to 75% (w/w), depending on their origin and functions, e.g. inner mitochondrial versus nervous cell membranes. Thus the interactions between proteins and lipids are complex and interrelations between the different membrane constituents must be studied in model systems or even by means of theoretical membrane models. Hence, the distinct interaction of single membrane proteins as defined 'impurities' can be investigated on the cooperativity of lipid phase transitions, and on the number of lipid molecules immobilized per protein. Perturbation of the hydrophobic bilayer by proteins as well as their miscibility during thermotropic phase transitions can be evaluated.

Papahadjopoulos et al. [21] developed a classification of protein–lipid interactions on grounds of the effect on the transition temperature T_m and enthalpy ΔH.

Group 1 shows an increase of ΔH and concomitant increase in (or no effect on) T_m. These proteins or peptides predominantly interact electrostatically with polar lipid headgroups at the membrane surface and do not penetrate into the hydrocarbon layer resulting in a stabilization of the membrane. Examples are polylysine and ribonuclease [21–24].

Group 2 proteins cause a strong reduction of ΔH and T_m. These proteins or peptides are bound at the membrane surface by ionic interaction with the polar lipid headgroups, partially penetrate into and deform the hydrocarbon region, reducing the lipid cooperativity and thus destabilizing and fluidizing the membrane, as proposed for cytochrome *c* [21,25,26].

Group 3 proteins express a reduction of ΔH linear with the protein/lipid ratio and a variable influence on T_m (e.g. no effect [21], or reduction of T_m [27]). They are embedded in the hydrophobic phase of the membrane or span the bilayer by hydrophobic forces and perturb the fatty acid chain packing as seen with valinomycin by a concomitant reduction of T_m [27] or with invariant T_m as published for, e.g. gramicidin A [26], glycophorin [28,29], glucagon [30], myelin-lipophilin [21,31]. From the modulation of the thermotropic lipid phase transition it is concluded that the four polypeptides form mixed phases with MPL and DPPC. In general, the intensity of reduction of ΔH increases with the length of the amino acid chains for P10, P11 and P20, and T_m is only slightly altered. Hence, these polypeptides should be accounted as 'group 3' analogues being embedded in the membrane without strong deterioration or deformation towards their environment. This interpretation is in line with the physicochemical structure of the polypeptides, which consist of a rigid barrel-shaped α-helix with a strong hydrophobicity [5,8].

P17 deviates from the above correlation with the polypeptide chain length, but exhibits a pronounced reduction of T_m (Fig. 3) indicating a stronger deformation of the membrane. For this polypeptide, however, a rigid hydrophobic α-helix is expected which almost spans the membrane, so that its low polarity at both ends may interfere with the charge density [32] and hydrophilicity of the polar lipid headgroups and by forming clusters in the membrane. The polar side chain of glutamine in position 11 may play an essential role in this behavior, because the Gln carboxamide groups can form intermolecular hydrogen bonds especially in hydrophobic environments. Thus, the lipophilic helical polypeptide may be readily inserted as a relatively stable oligomeric aggregate into the membrane. The shorter hydrophobic moiety from position 12 to 17, together with the terminal Asn, remains more or less associated with the polar region of the lipids generating perturbation similar to melittin, the main constituent of bee venome.

In P20 Gln has a central position, so that the molecule is more symmetrical. Both hydrophobic moieties may be sufficiently extended to keep Gln – in spite of its polarity – within the hydrophobic core of the membrane. This behavior is known, i.e., from the DCCD-binding proteolipid in the F_0 part of ATP-synthase complexes and it causes much less perturbation than the asymmetry of P17.

The metastability of the mixed P17-DPPC solid-analogue phase indicates another anomaly (Figs. 8–10) as alterations in lipid–protein interaction [33] may result in phase separation.

With MPL, such phenomenon was not observed, which may result from its repetitively branched structure in the hydrocarbon layer permitting an efficient incorporation of proteins or from its membrane spanning extension which does not favour a submersion of peptides and phase separation. However, it may also result from the short storage times below the phase transition temperature and/or the high concentration of cryoprotectant in the buffer due to the subzero phase transition temperature of MPL.

For DHMG, the question arises whether the minuteness of the effect of the peptides on the phase transition is determined by their low extent of incorporation or by their negligable influence on the thermotropic properties of DHMG. P20 has a pronounced polar tail of three positive charges, which is expected to facilitate an ionic fixation to and subsequent insertion of the molecule into the lipid membrane [34]. If inserted, it should interact with the lipid headgroups and modulate the thermotropic parameters of the phase transition as in the case of MPL and DPPC. Fig. 6, however, shows merely a marginal modulation of the heating scan, so that it is concluded that the strong intermolecular H-bond interaction between DHMG headgroups ($T_m = 57°C$ compared with $T_m = 41°C$ for DPPC [35]) prevent a significant incorporation of all four peptides into the lipid bilayer of DHMG.

In summary, the artificial peptides P10, P11, P17, and P20 are incorporated into lipid membranes of MPL and DPPC, but not into DHMG. In addition, P20, the sole synthetic peptide in this series with an extremely polar tail, deforms the structure of the bilayer (monolayer of MPL) to a lower extent than P17; P20 compares to the natural peptide alamethicin whereas P17 rather resembles melittin.

Acknowledgement

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