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Liposome-Induced Conformational Changes of an Epitopic Peptide and its Palmitoylated Derivative of Influenza Virus Hemagglutinin

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The conformation of synthetic $HA_{317-329}$-NH_2 representing the major B- and T-cell epitopic region of influenza virus hemagglutinin, its palmitoylated derivative ($HA_{317-329}$-Thr(Pal)-NH_2), and the intersubunit peptide ($HA_{317-341}$-NH_2) comprising also the fusion peptide, were studied in aqueous buffer and in the presence of neutral and negatively charged liposomes. The free peptide is unordered in aqueous solution, even in the presence of liposomes. However, grafting the palmitic acid or the fusion peptide onto the C-terminus of the peptide enables the hydrophilic $HA_{317-329}$ to adopt folded (turn) and β-strand structure on the surface of neutral and negatively charged liposomes, respectively. The results emphasize the importance of some kind of anchor for achieving a specific conformation of epitopic peptide $HA_{317-329}$-NH_2 on the surface of liposomes. © 1998 Academic Press

Specific immune responses against proteins and viruses can be achieved using synthetic antigenic peptides. However, free peptides are weakly immunogenic when used without carrier molecules [1,2]. Several alternatives have been proposed to improve the immunogenicity of epitopic peptide sequences. Among them, the covalent attachment of fatty acid chain has been shown to enhance the immunogenicity of unmodified peptides [3], and their ability to prime cytotoxic T lymphocytes [4-6]. Recently, many lipopeptides such as the HIV-1 V3(190-211) sequence modified by N^{ϵ}-palmitoyl-L-lysine [3,7] or tripalmitoyl-S-glyceryl-cysteine containing N-acylated peptide derivatives [8] were synthesized and investigated. The synthesis of N-acylated derivatives seems to be simpler compared to that of O-acylated ones, however, the latters are more biodegradable.

The general lack of cross-reactivity between synthetic peptides and native proteins or viruses is believed to be due to the conformational dissimilarity between the peptide and the corresponding sequence within the intact molecule [9]. Experiments with synthetic peptides of influenza virus hemagglutinin (HA) revealed that of a number of peptides, including those representing the major antigenic regions, only the 24 residues fragment 305-328 elicited antibodies which cross-reacted with the virus or intact HA [10]. In the infectious form of the virus HA is cleaved between residues 328 and 329 to form the heavy (HA1) and light (HA2) chains. It is the 305-328 residues sequence of the C-terminus of the HA1 chain which can be recognized by both B and T cells. The shortest peptide which was able to elicit the production of antibodies was a decapeptide corresponding to the 319-328 sequence, subtype H3 (but not subtype H1, H2 or H6) [11]. On the base of our earlier spectroscopic studies on 317-329 fragments from H1-H3 subtypes, we suppose that α-helicity does not play a determinant role in the recognition of T cell epitopic peptides [12].

It is reasonable to suppose that modification by fatty acid promotes the interaction of polypeptides with membranes, however, the effects of these hydrophobic lipid groups on the conformation of peptides have scarcely been studied [13]. This paper reports conformational studies on three synthetic peptides: the C-terminal 13 residues of the HA1 chain, $HA_{317-329}$-NH_2, its O-palmitoylated derivative, $HA_{317-329}$-Thr(Pal)-NH_2 and the intersubunit peptide, $HA_{317-341}$-NH_2. Circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopic measurements were performed in buffer and in the presence of liposomes to reveal the conformational consequences of covalent attachment of the palmitic acid or the fusion peptide to the 317-329 peptides.

MATERIALS AND METHODS

Peptide synthesis. $HA_{317-329}$-NH_2 (VTGLRNIPSIQSR-NH_2) and $HA_{317-341}$-NH_2 (VTGLRNIPSIQSRGLFGAIAGFIEG-NH_2) were synthesized by solid-phase technique using Boc chemistry [14]. Side-

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chain protecting groups were as follows: Tos (Arg), Bzl (Thr, Ser), cHex (Glu). The peptide chains were elongated on an MBHA resin (0.73 mmoles/g). Couplings were performed with N,N′-dicyclohexylcarbo-diimide (DCC), with the exception of Asn, Gln and Arg which were incorporated as their OBt-esters. As for *the synthesis of the palmitoylated peptide,* $HA_{317-329}$-Thr(Pal)-NH_2 a side-chain unprotected threonine derivative (Boc-Thr-OH) was first attached to the resin and then its hydroxyl function was acylated with palmitoyl chloride in presence of pyridine on the solid phase. After repeated acylation, the peptide chain was elongated further. The completed peptide resins were treated with liquid HF/dimethyl sulphide/p-cresol/p-thiocresol (88:6:4:2, v/v), at −5°C, 45 min. HF was removed and the resulted free peptides were solubilized in 30% aqueous acetic acid, filtered and lyophilized. The crude peptides were purified by semi-preparative reverse phase HPLC on a C-18 column (16 × 250 mm). The peptides were eluted with a linear gradient of aqueous acetonitrile 24% → 80% MeCN, 0.1% trifluoroacetic acid (TFA) in 60 min, 4 ml/min flow rate. The appropriate fractions were pooled and lyophilized. The purified peptides were characterized by amino acid analysis, HPLC and mass spectrometry using a Finnigan TSQ 7000 tandem quadrupole electrospray mass spectrometer. Peptide purity was above 97% (HPLC and capillary zone electrophoresis).

Preparation of phospholipid liposomes. For neutral liposomes 10 mg dipalmitoyl phosphatidylcholine (DPPC) and for negatively charged liposomes 9 mg DPPC+1 mg dipalmitoyl phosphatidic acid (DPPA) were dissolved in 1 ml chloroform and dried by a stream of N_2 gas for 1 h. The dry lipid was hydrated in 3 ml of 5 mM Hepes buffer (pH 7.4) with repeated vortex mixing at 50°C for 20 min. The suspension was sonicated at 50°C for 45 min (until a clear solution was obtained) using a MSE Type 150W ultrasonic disrupter (21 Kc/sec, 10 μ amplitude). The oxygen was expelled by bubbling N_2 through the suspension before sonication. After sonication the suspension was centrifuged for 30 min at 4000 g to remove the titanium dust originated from the probe. For the CD measurements the suspension was diluted with Hepes buffer to a lipid concentration of 1.5 mM. Prior to measurements the peptide-liposome samples were incubated at 25°C for 30 min.

Circular dichroism. CD spectra were monitored using a Jobin-Yvon Mark VI dichrograph. Measurements were carried out at 25°C using an optical cell with a path length of 0.02 or 0.1 cm. Ellipticity is expressed as mean residue ellipticity, $[\Theta]_{MR}$, in units of deg.cm^2.dmole^{-1}.

Infrared spectroscopy. Fourier transform infrared measurements were performed in D_2O (Aldrich, 99.9%) at room temperature on a Bruker IFS-55 FTIR spectrometer at a resolution of 2 cm^{-1} using a 0.05 cm cell with CaF_2 windows. The amide I region of the spectra was decomposed to individual bands by the Levenberg-Marquardt nonlinear curve-fitting method using weighted sums of Lorentz and Gauss functions. The choice of the starting parameters was assisted by Fourier self-deconvolution (FSD) [15]. Since the peptides were eluted with a solvent mixture containing TFA, all peptides were present as trifluoroacetate salts. Thus, in the FTIR spectra the component at 1673 cm^{-1} can be correlated with the ν_{as} (COO^-) vibration of trifluoroacetate [16].

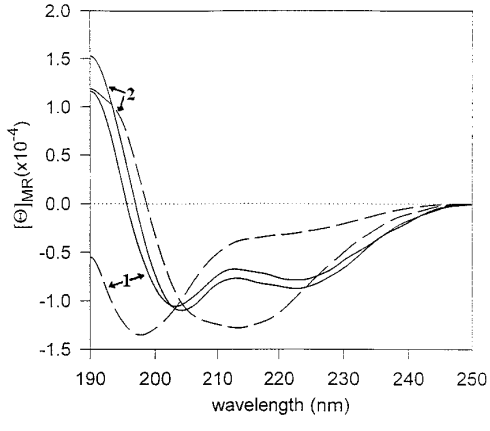

FIG. 1. CD spectra of $HA_{317-329}$ (1) and $HA_{317-329}$-Thr(Pal)-NH_2 (2) in TFE (—) and in 5 mM Hepes buffer, pH 7.4 (---). Peptide concentration: 30 μM.

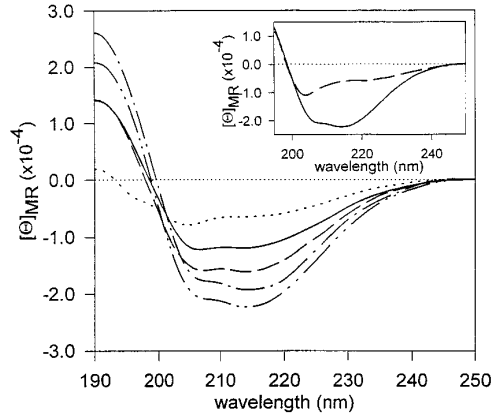

FIG. 2. CD spectra of $HA_{317-329}$-Thr(Pal)-NH_2 at different concentrations in 5 mM Hepes, pH 7.4. 15 μM (···), 30 μM (—), 60 μM (---), 120 μM (-·-), 240 μM (-···-). *Inset:* CD spectra of $HA_{317-329}$-Thr(Pal)-NH_2 (240 μM) in 5 mM Hepes, pH 7.4 without (—) and with 0.2% (w/v) Triton (---).

RESULTS AND DISCUSSION

Figure 1 presents the CD spectra of $HA_{317-329}$-NH_2 and $HA_{317-329}$-Thr(Pal)-NH_2 in trifluoroethanol (TFE) and in 5 mM Hepes buffer (pH 7.4). The low intensity, blue shifted helical (class C) spectrum of the free peptide in TFE can be correlated with the stabilization of certain subtypes of β-turns [17]. The intensive β-turn acceptor band at 1638 cm^{-1} in the FTIR spectrum also supports the presence of this conformational element [12]. Introducing the palmitoyl moiety into the C-terminus does not result in any significant spectral change in TFE, however, the two peptides show different CD spectra in buffer. In buffer solution the free peptide is present in a predominantly unordered or semiextended (PPII) conformation [18], while the broad negative band in the CD spectrum of $HA_{317-329}$-Thr(Pal)-NH_2 is indicative of a mixture of β-sheet and turn conformers. The increased population of ordered conformations upon introducing palmitoyl group may account for the hydrophobic environment provided by the acyl chain. This may be of significance in the recognition events keeping in mind, that the most structured peptides in solution often correspond to the best immunogens [19].

Characteristic differences in the concentration dependence of the CD spectra of the free and lipoderivative of $HA_{317-329}$ were observed in Hepes buffer. While

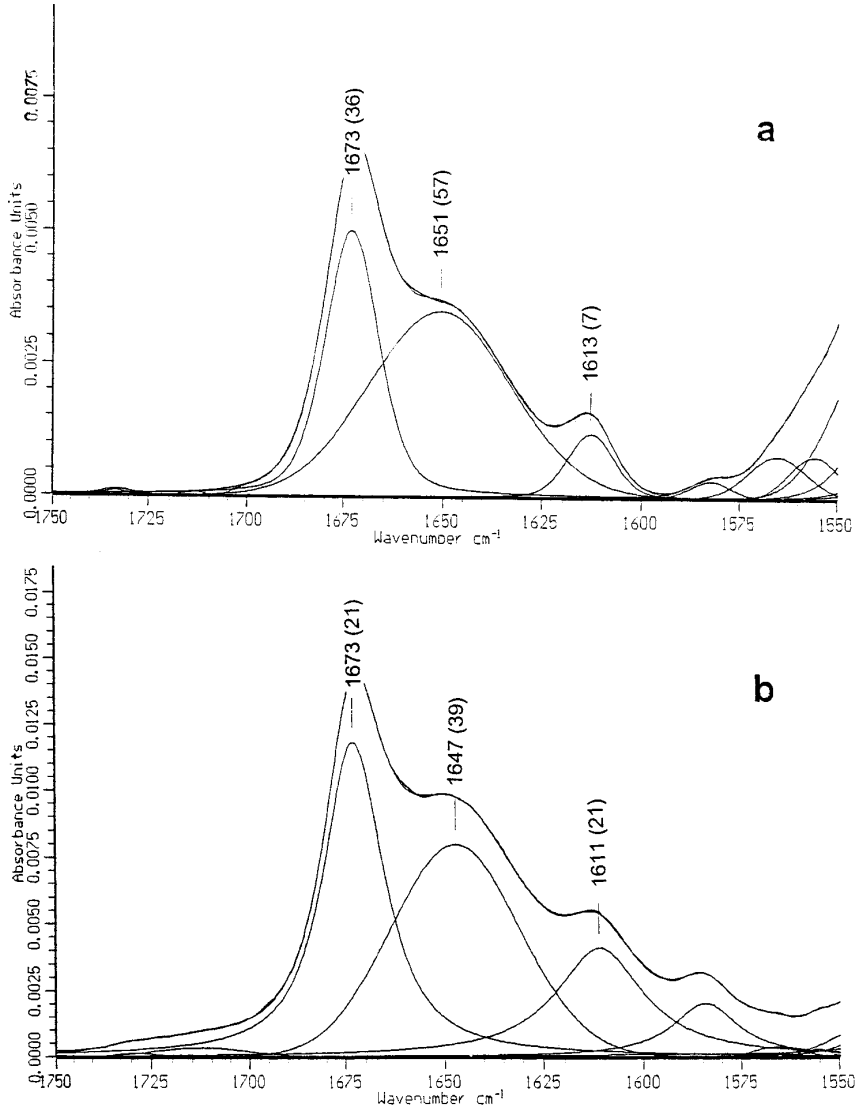

FIG. 3. FTIR spectra of $H_{317\text{-}329}$-Thr(Pal)-NH_2 in D_2O at 120 μM (a) and 240 μM (b) peptide concentrations. In parentheses: relative intensities in percentages.

the CD spectrum of the free peptide was practically independent of the concentration in the range of 15-240 μM (data not shown), a definite spectral change was monitored in the case of $HA_{317\text{-}329}$-Thr(Pal)-NH_2 (Fig. 2). At the highest concentration (240 μM), the lipopeptide was not fully dissolved (cloudy solution) and the spectrum was suggestive of a mixture of β-sheet (negative band at 215 nm), and folded conformations (shoulder at $\sim$205 nm). On dilution, the spectral contribution of the β-sheet gradually decreases and at 15 μM the spectrum shows enrichment of turn structures, though a significant amount of unordered conformation is also present (low positive band intensity). The FTIR measurements, performed in D_2O at 120 and 240 μM concentrations, support the results of CD spectroscopy. As Fig. 3 shows, two main amide I bands appear: one at 1611 and 1613 cm^{-1} - corresponding to a mixture of β-sheet and β-aggregation - and a stronger component at 1647 and 1651 cm^{-1} which can be correlated with a mixture of helical (or loop) and aperiodic secondary structures [15]. There is a difference, however, in the relative intensity (number in parenthesis in Fig. 3) of the two components. The spectrum of the more concentrated sample suggests higher relative amount of β-sheet conformation or aggregates.

Addition of the solubilizing agent Triton X-100 (0.2% w/v) to the Hepes solution of $HA_{317\text{-}329}$-Thr(Pal)-NH_2 at the highest concentration (240 μM), a CD spectrum similar to that of the most diluted (15 μM) sample was obtained (inset in Fig. 2). Apparently, at higher concentrations the lipopeptide forms micelles which are held together by hydrophobic interactions between palmi-

toyl chains. It is unlikely that molecular association is initiated by the peptide moieties. However, on the surface of the lipopeptide-micelles part of the peptide moieties gets in close vicinity to each other which leads to the formation of strong H-bonds between neighbouring β-strands adopting β-sheet conformation.

The secondary structure of the free, the palmitoylated and fusion peptide-coupled $HA_{317-329}$-NH_2 was compared in the presence of neutral and negatively charged liposomes at [lipid]/[peptide] ratio of 50. As Figure 4*a* shows, the CD spectra of the *free* peptide in Hepes buffer and in the presence of *neutral liposomes* are nearly identical: the peptide is disordered or semi-extended irrespective of the presence or absence of liposomes. Interaction between the hydrophylic peptide and the hydrophylic surface of liposomes is not excluded but due to the limited residence time the experimental presentation is difficult. Although the electrostatic attraction between the positively charged peptide and the *negatively charged* surface of liposomes resulted in a weak spectral change in the 210-240 nm range, apparently the induced conformational change was weak in the absence of some kind of anchor.

On the contrary, the covalently coupled palmitoyl chain greatly modifies the conformation of $HA_{317-329}$ in the presence of both neutral and negatively charged liposomes. The 205 nm band in the CD spectrum of the *lipopeptide* in the presence of neutral liposomes suggests enrichment of the population of the folded conformers (Fig. 4b). A similar observation was made by Macquaire *et al.* [20] when the conformational change induced by phospholipidic interface of model lipopeptide constructions was studied by NMR technique. In the presence of negatively charged liposomes, the shape of the CD spectrum (band at 215 nm) is suggestive of the dominance β-sheet conformation. The palmitoyl moiety has no chiral contribution in the spectral range investigated, hence the CD spectra reflect exclusively the change of the peptide conformation. Apparently, the palmitoyl moiety is inserted into the lipophilic interior of the liposomes and the peptide on the surface of the liposome adopts an ordered conformation. In the presence of the *neutral* liposomes the adoption of turn structures is most likely while *negatively charged* liposomes induce an extended conformation. Possibly, the electrostatic interaction between the peptide which contains basic amino acids and the acidic phospholipid promotes the extension of the peptide backbone and the stabilization of β-strand or β-sheet structure. Recently Nidome *et al.* reported that peptides with short acyl chain have a fairly strong perturbation activity on DPPC membrane while the peptides with long chain acyl groups (miristoyl or palmitoyl) showed weak membrane-perturbation (peptide was stably anchored to liposomes) and antibacterial activities [21].

The CD spectra of $HA_{317-341}$-NH_2 (epitopic peptide coupled with the fusion peptide) are illustrated in Fig. 4*c*. In buffer, the peptide is mostly unordered although the relatively intense negative band at longer wavelengths (210-240 nm) indicates the contribution of structured forms. In the presence of *neutral* liposomes the peptide exhibits a distinctly helical CD spectrum, while with *negatively charged* liposomes the spectrum

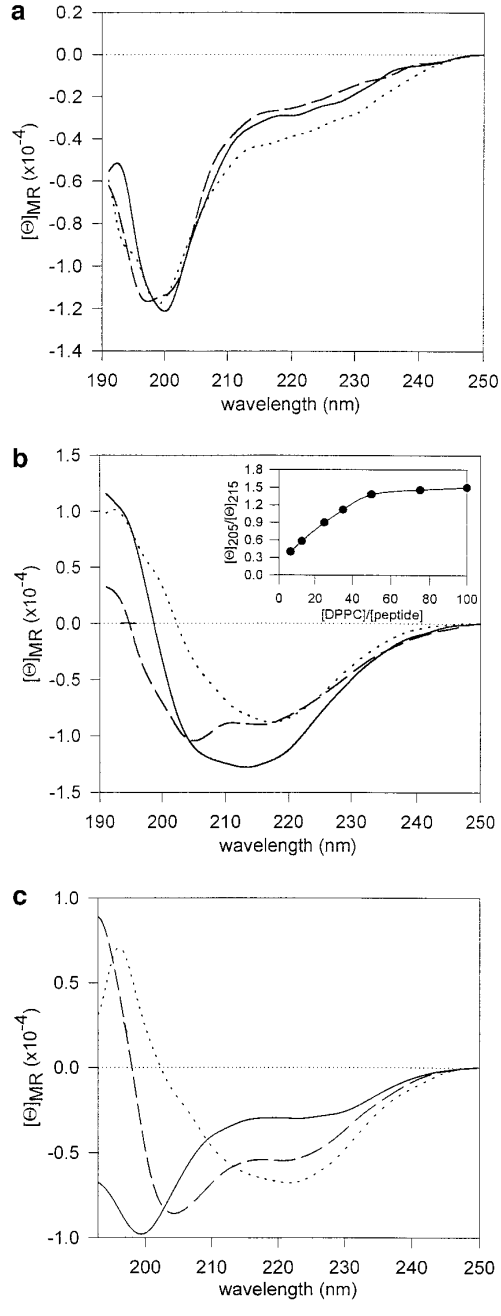

FIG. 4. CD spectra of $HA_{317-329}$ (a), $HA_{317-329}$-Thr(Pal)-NH_2 (b) and $HA_{317-341}$ (c) in 5 mM Hepes buffer, pH 7.4 (—), in the presence of neutral DPPC (---) and negatively charged, DPPC:DPPA (0.9:0.1) liposomes (· · ·). Peptides were dissolved at a concentration of 30 μM in 5 mM Hepes buffer containing 1.5 mM liposomes. *Inset* shows the ellipticity ratios measured at 205 and 215 nm in the function of [DPPC]/[peptide] ratios.

is characteristic of β-sheet conformation. It is worth noting that under the latter conditions the negative band is much broader than that of normal β-structure, suggesting that some α-helix and/or nascent helix derived from the fusion part is also present. Fusion peptides were shown to bind to lipid membranes with a transition of their secondary structure into α-helix [22].

CONCLUSION

This paper reports a definite shift towards ordered secondary structures (turn and sheet) of a B and T cell epitopic peptide $HA_{317\text{-}329}\text{-}NH_2$ of influenza virus hemagglutinin in solution when covelently coupled to palmitic acid. The relative amount of ordered structures was further increased when at higher concentrations the *lipopeptide* formed micelles or more importantly in the presence of DPPC liposomes. Contrary to this, no conformational change of the *free* peptide was detected in the presence of liposomes. This work therefore emphasizes (i) the importance of a built-in acyl group to induce a definite structure of short antigenic peptides in solution and (ii) an increase of the population of ordered conformers by stably anchoring the peptide to the lipid membrane. This may be of significance for achieving the specific conformation necessary for antibody recognition.

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