Eur. J. Biochem. 242, 243–248 (1996)

Structural properties of the putative fusion peptide of hepatitis B virus upon interaction with phospholipids

Circular dichroism and Fourier-transform infrared spectroscopy studies

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(Received 17 July/13 September 1996) – EJB 96 1070/3

A peptide corresponding to the N-terminal sequence of the S protein from hepatitis B virus (Met-Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-Leu-Leu-Val-Leu-Gln) has been previously shown to interact with phospholipids and promote vesicle aggregation, phospholipid mixing, and liposome leakage, as well as erythrocyte lysis [Rodríguez-Crespo, I., Núñez, E., Gómez-Gutiérrez, J., Yélamos, B., Albar, J. P., Peterson, D. L. & Gavilanes, F. (1995) *J. Gen. Virol. 76*, 301–308]. The conformation of this putative fusion peptide has been studied, both at low and high peptide concentrations, by means of circular dichroism and Fourier-transform infrared spectroscopy, respectively. When the peptide is dissolved in trifluoroethanol, a significant population of α-helical structure is found in spite of the proline residue at position 11. In contrast, this hydrophobic oligopeptide has a high tendency to form large β-sheet aggregates in aqueous buffers. Most of these aggregates can be eliminated by centrifugation. The peptide remaining in the supernatant adopts a non-ordered conformation. The aggregates can be dissociated by the anionic detergent sodium cholate, but the peptide still maintains an extended conformation. In the presence of acidic phospholipid vesicles, the putative fusion peptide adopts a highly stable β-sheet conformation. Thus, unlike the fusion peptides of other viruses, an extended conformation seems to be the preferred structure when interacting with phospholipids. Such a conformation should be responsible for its membrane destabilization properties.

Keywords: hepatitis B virus; fusion peptide.

One of the initial infective steps for an enveloped virus is the destabilization of the target cell membrane performed by the viral envelope proteins, which results in lipid intermixing between the viral and cellular bilayers. In general, these fusion proteins in the virus envelope possess a common motif referred to as the fusion peptide, which is a hydrophobic stretch located at the N-terminal region of one of the viral envelope glycoproteins [1]. The fusion peptide is able to interact with the target membrane and perform the subsequent destabilization step through either a pH-induced conformational change at the acidic endosomes or as a consequence of its exposure upon interaction with a virus receptor at the plasma membrane. Thus, these sequences are usually considered to be strong lipid interacting regions capable of altering lamellar structures of the bilayer and promoting non-lamellar transient phases necessary in the fusogenic process.

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Abbreviations. HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; FTIR, Fourier-transform infrared; CF_3CH_2OH, trifluoroethanol; Myr_2 Gro*P*Gro, dimyristoylglycerophosphoglycerol; PtdCho, phosphatidylcholine; PtdGro, phosphatidylglycerol; CCA, convex constraint analysis.

Hepatitis B virus (HBV), the prototype member of the hepadnavirus family, is a small DNA-containing virus that infects human hepatocytes eliciting an important liver pathology [2]. The translation of the S region of HBV DNA yields the envelope proteins which assemble as lipoprotein particles referred to as hepatitis B surface antigen (HBsAg) [2–4]. In the surface of the mature virions, three different proteins can be observed, all of them sharing the same C-terminal 226 amino acids. The large (L) protein consists of 389 (or 400, depending on the subtype) amino acids and possesses the preS1 + preS2 + S sequences of HBV. The medium (M) protein consists of 281 amino acids, with 55 N-terminal amino acids corresponding to the preS2 sequence. Finally, the small (S) protein consists of the S sequence of HBV and possesses 226 amino acids [3].

Sequence similarities between the N-terminal hydrophobic stretch of the S protein from HBV and various fusion peptides of retroviruses and paramyxoviruses have been previously reported [5]. Synthesis of a 16-amino-acid peptide allowed the study of its interaction with lipid vesicles and with red blood cells. This synthetic peptide was able to perform vesicle aggregation as well as to haemolyse erythrocytes in a pH-dependent manner [6]. A detailed study of the fusogenic properties of this peptide was performed by means of several fusion assays employing different fluorescent probes [7], which demonstrated the increase of its lipid-interacting properties at acidic pH values

and showed a clear preference for negatively charged phospholipids in its interaction.

We have studied the conformation of this N-terminal hydrophobic stretch by means of circular dichroism (CD) and Fourier-transform infrared (FTIR) spectroscopies, both in the presence and absence of phospholipid vesicles, to determine the structural features mediating its binding to phospholipid vesicles and its ability to perform membrane destabilization.

EXPERIMENTAL PROCEDURES

Materials. Deuterium oxide (D_2O, 99.9% by atom) was purchased from Sigma. Dimyristoylglycerophosphoglycerol ($Myr_2GroPGro$), egg phosphatidylglycerol (PtdGro) and egg phosphatidylcholine (PtdCho) were from Avanti Polar Lipids. PtdCho and PtdGro had the same fatty acid composition. All other reagents were obtained from Merck and Sigma. All solvents were HPLC grade.

Methods. *Peptide synthesis.* A sixteen-amino-acid peptide covering the N-terminal region of the S protein of HBsAg (Met-Glu-Asn-Ile-Thr-Ser-Gly-Phe-Leu-Gly-Pro-Leu-Leu-Val-Leu-Gln) was synthesized as the C-terminal amide on an automated multiple peptide synthesizer (AMS 422, Abimed) using a solid-phase procedure and standard Fmoc chemistry in 25 μmol base as previously described [7]. The peptide was purified by reverse-phase HPLC on a C_{18} column. Purity and composition of the peptide were confirmed by reverse-phase HPLC and by amino acid analysis [7]. The peptide to be used in the FTIR spectroscopy measurements was lyophilized at least three times in 10 mM HCl to remove any residual trifluoroacetic acid used both *in peptide synthesis* and in the HPLC mobile phase [8] (the trifluoroacetate ion has a strong infrared absorbance at 1670 cm^{-1}, which interferes with the characterization of the amide I band [9]).

Circular dichroism. Aliquots of peptide from a stock solution at 10 mg/ml in trifluoroethanol (CF_3CH_2OH) were dried under a stream of nitrogen. These peptide films were dissolved in CF_3CH_2OH and CF_3CH_2OH/water mixtures or resuspended in medium buffer (100 mM NaCl, 5 mM Tris, 5 mM citrate, 5 mM Mes, 1 mM EDTA) at the appropriate pH at a concentration of 120–140 μg/ml. The spectrum of the peptide in monomeric or small multimeric form in aqueous solution was recorded after elimination of large peptide aggregates by centrifugation at 15900 *g* in a Beckman Microfuge E for 15 min. The effect of phospholipids and sodium cholate was determined by adding increasing amounts of these compounds to peptide aliquots. The mixtures were allowed to react for 1 h at 37 °C. When lipids were added, the samples were subjected to probe sonication in an ice bath. The clear sonicated solutions were then analyzed by CD.

CD spectra were recorded on a Jovin-Yvon Mark III dichrograph at 25 °C and with a 1-mm pathlength cell at a scanning speed of 0.5 nm/s. A minimum of three spectra were accumulated for each sample. The peptide concentration was determined by amino acid analysis on a Beckman 6300 automatic amino acid analyzer.

Deconvolution of the CD data was performed according to the convex constraint analysis (CCA) method [10]. This method relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without refering to spectra from model systems. This analysis provides evidence that the CD spectrum of a protein contains independent information of at least four different additive secondary structures [10].

FTIR spectroscopy. Lyophilized aliquots of peptide were hydrated at concentrations in the range 1–10 mg/ml in D_2O buffers to avoid the interference of H_2O infrared absorbance (1645 cm^{-1}) on the protein amide I band [11]. Lyophilized lipids hydrated in the same D_2O buffer were either mixed immediately with the peptide or submitted to sonication in a water bath for 30 min before or after the addition of the peptide. Use of such alternative procedures to prepare the mixtures produces no differences in the observed spectral features of the samples.

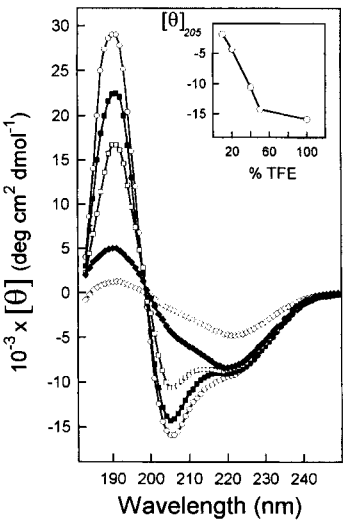

Fig. 1. CD spectra of the N-terminal peptide of the S protein from HBV in CF_3CH_2OH and CF_3CH_2OH/water mixtures. 100% CF_3CH_2OH (○), 50% CF_3CH_2OH (■), 40% CF_3CH_2OH (□), 20% CF_3CH_2OH (◆) and 10% CF_3CH_2OH (◇). In this figure, as well as in Figs 2, 4 and 5, the peptide concentration (70–80 μM) was determined by amino acid analysis.

Infrared measurements were performed in a Nicolet 520 instrument equipped with a DTGS detector. Samples were placed into a thermostatically controlled dismountable cell with CaF_2 windows and maintained at room temperature for 1 h to ensure that the isotopic H-D amide proton exchange reached equilibrium, as judged by a constant minimal absorbance at the residual amide II band in the peptide's infrared spectrum. The pathlength used was 50 μm and the sample chamber was continuously purged with dry air. A minimum of 250 scans/sample was taken, averaged, apodized with a Happ-Genzel function and Fourier transformed to give a nominal resolution of 2 cm^{-1}. Self-deconvolution was performed using a Lorenztian bandwidth of 18 cm^{-1} and a resolution enhancement factor of 1.8.

RESULTS

The CD spectra of the N-terminal peptide of the S protein from HBV in CF_3CH_2OH and CF_3CH_2OH/water mixtures at low peptide concentration (70–80 μM) are shown in Fig. 1. In pure CF_3CH_2OH, the peptide has a spectrum with two minima at 205 nm and 222 nm and a positive band at 190 nm, which is indicative of the adoption of a significant amount of α-helical conformation [12]. Deconvolution of this spectrum by the CCA method [10] renders 23% α-helix, 16% β-sheet, 15% β-turn and 46% random structures. Hence, though the peptide possesses a proline residue at position 11, the preceding glycine residue allows the possibility of an α-helix to occur. At CF_3CH_2OH concentrations lower than 50%, the shape of the spectrum changed considerably, increasing the ellipiticity at 205 nm and exhibiting a minimum at around 220 nm which is characteristic of extended structures [13]. Thus, increasing amounts of water induced the disappearance of α-helical structures and a progressive increase

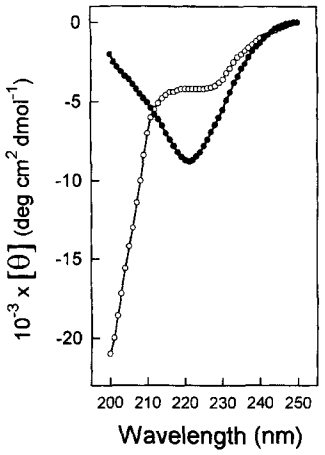

Fig. 2. CD spectrum of the N-terminal peptide in aqueous solution. Aliquots of peptide were resuspended in medium buffer at the appropriate pH. The spectrum (●) was recorded after incubation for 30 min at 37 °C. This sample was centrifuged in a Beckman Microfuge E for 15 min and the CD spectrum of the supernatant was immediately recorded (○). Identical results were obtained at both pH 7.0 and pH 5.0.

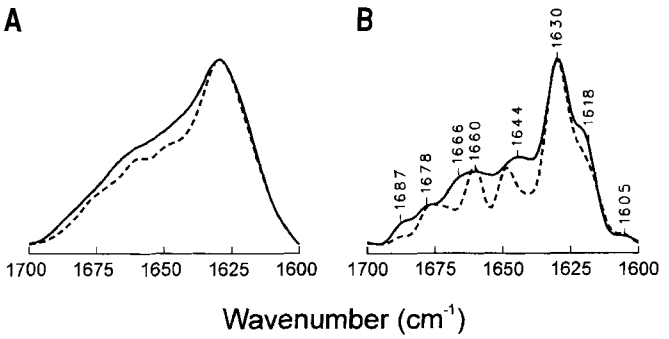

Fig. 3. Infrared amide I band region of the original (A) and deconvolved (B) spectra of the N-terminal peptide. The peptide, 5.8 mM, was hydrated in D_2O medium buffer at pH 7.0 (——) and pH 5.0 (– – –). In this figure, as well as in Fig. 6, the spectra of the buffers alone were subtracted from those of the peptide-containing samples.

in β-sheet conformation, probably due to peptide-peptide interactions. The isodichroic point at 199 nm would be indicative of both helical and extended conformations.

Dilution of a concentrated solution of the fusion peptide, dissolved in CF_3CH_2OH, into aqueous buffers leads to the progressive appearance of large aggregates. The CD spectrum of such aggregates is shown in Fig. 2. This is characteristic of β-structure, with a minimum at 221 nm. The low peptide concentration employed precludes the effect of light scattering on both the shape and magnitude of the spectrum. Moreover, this spectrum did not significantly change upon decreasing the pathlength of the cuvette used for recording it. When these aggregates are eliminated by centrifugation, the supernatant has the typical CD spectrum of nonordered structures (Fig. 2). Amino acid analysis of this supernatant indicated that about 15% of the total peptide could be recovered after centrifugation of the sample. Hence, though the peptide is able to adopt α-helical structure in CF_3CH_2OH and when it is in a monomeric or small multimeric form in aqueous solution it is in a nonordered structure, it has a high tendency to aggregate probably through interchain hydrogen bonding giving rise to a β-sheet. Identical results were obtained at either pH 7.0 or pH 5.0.

FTIR analysis performed at high peptide concentrations (0.6–5.8 mM) in D_2O aqueous buffers both at neutral and acidic

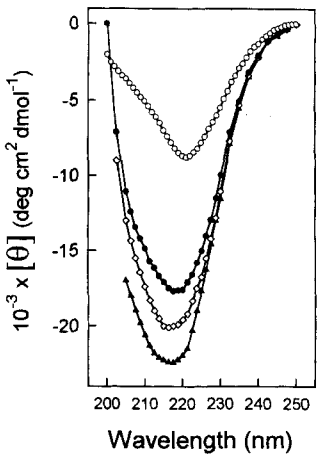

Fig. 4. Effect of sodium cholate on the CD spectrum of the N-terminal peptide. 0 mM cholate (○), 5 mM cholate (●), 10 mM cholate (◇) and 20 mM cholate (▲). The peptide was resuspended in medium buffer at pH 7.0 and increasing amounts of sodium cholate were added.

pH values indicate that the peptide mainly adopts a β-sheet structure with a maximum absorbance at 1630 cm^{-1} (Fig. 3A), thus confirming that the peptide possesses a high tendency to adopt an extended conformation, probably by hydrogen bonding multiple peptide monomers. Deconvolution of the spectrum (Fig. 3B) shows that the amide I region exhibited other maxima that can be assigned to side chains (1605 cm^{-1}), low frequency β-sheets or aggregated strands (1618 cm^{-1}), non-ordered structures (1644 cm^{-1}), α-helix (1660 cm^{-1} and 1666 cm^{-1}) and β-turn or high-frequency component of the antiparallel β-structure (1678 cm^{-1} and 1687 cm^{-1}) [14–17]. Only minor differences were observed between pH 7.0 and pH 5.0, and these were related to the higher proportion of extended conformation at pH 5.0 as well as to the slightly different proportion and spectral shifts of the minor bands.

The presence of the anionic detergent sodium cholate increases the solubility of the fusion peptide in aqueous buffers. The effect of this membrane mimicking detergent, at concentrations in the range 5–20 mM, on the CD spectrum is shown in Fig. 4. Both below and above the critical micellar concentration (14 mM) the spectrum changed considerably, decreasing the ellipticity values and shifting the minimum from 221 nm to 217 nm. Hence, the hydrophobic environment provided by the detergent modifies to a certain degree the extended structure observed for the peptide aggregates. The displacement (4 nm) toward lower wavelengths of the ellipticity minimum observed upon interaction with the detergent may be due to a change in the polarity in the local environment of the adopted β-structure. Similar environment-dependent shifts have been observed in the CD spectra of other proteins upon interaction with lipids [18, 19]. Additionally, FTIR studies indicated that the β-sheet is the main conformation both in the presence and absence of sodium cholate. The presence of the detergent produces a slight shift in the position of the β-sheet band to higher frequencies, which is consistent with the disruption of peptide aggregates during the detergent solubilization process (data not shown).

To ascertain the peptide structure when interacting with phospholipids, we performed the structural characterization of the peptide at increasing $Myr_2GroPGro$ concentrations. The N-terminal peptide induces aggregation of acidic phospholipids [7], which prevents CD analysis. Nonetheless, if the Myr_2GroP-Gro-peptide mixtures are subjected to probe sonication, the suspensions become completely transparent and CD analysis can be performed. Fig. 5 shows the results obtained at pH 7.0; similar

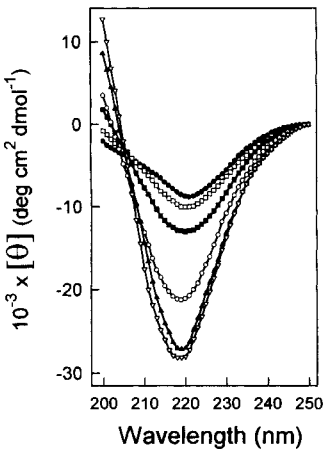

Fig. 5. CD spectra of the N-terminal peptide in the presence of Myr_2-Gro*P*Gro. The peptide was resuspended in medium buffer at pH 7.0 and increasing amounts of Myr_2Gro*P*Gro were added. After incubation for 1 h at 37 °C, the samples were subjected to probe sonication. The lipid/peptide molar ratios are as follows: (□), 1.5:1; (■), 3:1; (○), 5:1; (▲), 7:1 and (▽), 10:1. The upper spectrum (●) corresponds to the peptide in the absence of lipids.

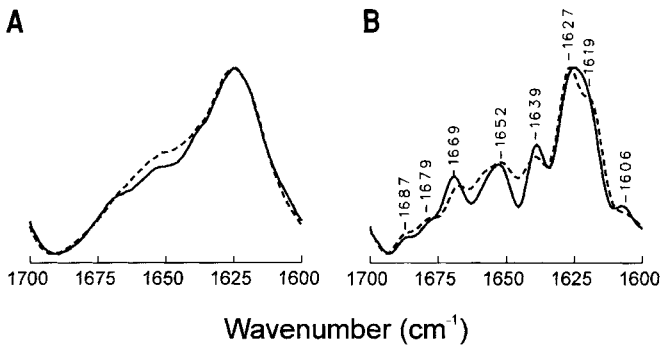

Fig. 6. Infrared amide I band region of the original (A) and deconvolved (B) spectra of the N-terminal peptide in the presence of phospholipids. The peptide, 5.8 mM, was hydrated in D_2O medium buffer at pH 7.0 and incubated with PtdGro (——) and PtdCho (– – –) at a lipid/peptide molar ratio of 5:1.

results were obtained at pH 5.0. Comparable results to those obtained with sodium cholate were observed. The progressive interaction of the peptide with the lipid bilayer leads to a substantial decrease in the ellipticity at 220 nm. Also, a significant displacement (from 221 nm to 218 nm) of the ellipiticity minimum toward lower wavelengths was observed. Lipid/peptide molar ratios higher than 10:1 did not modify the spectrum any further. Whereas it is difficult to accurately assess the relative percentage of secondary structure in small peptides by CD, it seems reasonable to conclude by comparison with the spectra of different β-proteins [13] that the peptide is predominantly adopting an extended conformation. The CCA method performed on the spectrum of the peptide interacting with the highest amount of Myr_2Gro*P*Gro rendered almost a 90% β-sheet conformation. However, due to the variable ellipticity contribution of β-turns [20], the tentative presence of a certain amount of β-turns cannot be completely excluded from the CD analysis.

We also attempted CD characterization of the peptide interacting with PtdCho vesicles, but the resulting lipid-peptide suspensions were turbid even after exhaustive sonication, which prevented quantitative analysis. Nevertheless, the spectra still exhibited the characteristic β-sheet minimum centered at around 220 nm (data not shown).

FTIR spectra of the N-terminal peptide interacting with PtdGro and PtdCho vesicles are depicted in Fig. 6. The spectra shown are those obtained at pH 7.0 and at a lipid/peptide molar ratio of 5:1. Similar results were obtained at pH 5.0. In the presence of either neutral or anionic phospholipids, a high intensity band centered at around 1627 cm^{-1} was observed. It is known that spectral components in the 1610–1629-cm^{-1} range are characteristic of strongly hydrogen-bonded β-structures [21, 22]. Other minor differences occurred in the 1640–1690 cm^{-1} region where minor spectral components were observed. Hence, compared to the structure adopted in solution, only slight conformational changes seem to occur when interacting with phospholipids. The characteristic low-frequency band of extended structures is the predominant spectral component in all cases although a higher contribution of possible turn components is observed in the presence of phospholipids. In this regard, there are glycine and serine residues in the peptide sequence that are known to be capable of inducing turn structures. Identical results to those shown were obtained when the phospholipid vesicles were sonicated. Moreover, increasing the lipid/peptide molar ratio up to 20:1 did not significantly alter the shape of the spectrum. The β components observed in the presence of anionic phospholipids were very stable as indicated by the fact that the amide I band did not significantly change when the samples were submitted to thermal denaturation up to 70 °C (data not shown).

Since divalent cations have been previously proposed to determine the secondary structure of the HIV-1 fusion peptide when interacting with lipid vesicles [23], we also tested the effect of the presence of Ca^{2+} in the peptide structure either in aqueous buffers or when interacting with phospholipid vesicles. Neither the FTIR nor the CD tecniques were able to detect any significant change in the peptide secondary structure either in solution or when interacting with PtdGro vesicles up to a final Ca^{2+} concentration of 5 mM (data not shown).

DISCUSSION

Although fusion induced by N-terminal peptides does not necessarily reflect the fusogenic activities of viral fusion proteins, studies on the membrane destabilization effects caused by these peptides might help to understand the mechanism of lipid-protein interactions performed by viral glycoproteins in the initial infective steps. We have undertaken the structural characterization of a 16-amino-acid peptide corresponding to the N-terminal sequence of the S protein from HBV, which has been previously shown to associate and destabilize membranes [6, 7]. In CF_3CH_2OH, this peptide is able to adopt a significant population of α-helical structure. However, this was not unexpected from the known tendency of CF_3CH_2OH to induce helical structure on a variety of other peptides or proteins. As an example of this, a synthetic peptide from actin, known to be partially folded as a β-strand in the three-dimensional protein structure is α-helical in CF_3CH_2OH [24].

In aqueous buffers, both at pH 5.0 and pH 7.0, CD and FTIR indicate that the putative fusion peptide from HBV adopts a non-ordered conformation when in monomeric or small multimeric form, but with a high intrinsic tendency to form large β-sheet aggregates, which should be formed by monomer-monomer hydrogen bonding. Also, the latter conformation is maintained when these large aggregates are progressively dissociated by increasing amounts of sodium cholate. Moreover, addition of acidic phospholipid vesicles allows the peptide to adopt a highly

stable β-sheet conformation, which suggests that this might correspond to the active conformation of the peptide when interacting with lipids.

In the CD analysis, the low ellipticity values observed are remarkable when the peptide is in the presence of phosphatidylserine vesicles or sodium cholate. The shape of the CD spectrum is comparable to that of extended structures. However, the low ellipticity values make it difficult to adjust the spectrum by comparison to known β-proteins. All β-proteins, like immunoglobulins, concanavalin or pepsinogen have ellipticity values at 215–220 nm of −8000 deg cm^2 $dmol^{-1}$ [13], although synthetic peptides that adopt β-sheet structure have values close to −20000 deg cm^2 $dmol^{-1}$ [25, 26]. A stronger hydrogen bond network or the Cotton effect of the phenylalanine side chain at position 11 could be responsible for the observed ellipticity values. The contributions of aromatic amino acids side chains to the far-ultraviolet CD spectrum have been observed in proteins although their exact contributions to the CD spectrum remain unclear [27].

The lipid vesicle destabilization induced by the putative fusion peptide from HBV increases at acidic pH [7]. When comparing the conformational features of the peptide at pH 7.0 and pH 5.0, a slight pH-dependent conformational change is observed. Since phosphatidylserine has no chemical groups whose titration could modify the interaction in the pH range studied, we presume that the partial protonation of the side chain of glutamic acid at position 2 might be responsible for the structural modification induced by the pH change. However, this glutamic acid residue is not conserved among hepadnaviruses [2]. Such lack of conservation would indicate a greater relative importance of the hydrophobic residues, particularly the tripeptide Phe-Leu-Gly, in eliciting the fusogenic properties.

As indicated by sucrose density centrifugation, at a phospholipid/peptide molar ratio of 5:1 almost 50% of the peptide is bound to the phospholipids [6]. Nevertheless, only slight conformational changes are observed upon interaction with phospholipids, both at low and high peptide concentrations and molar ratios even higher than 5:1. The absence of large conformational changes is not due to limited conformational flexibility of the peptide since it has been shown to be α-helical in organic solvents. Several proteins have been reported not to alter significantly their secondary structure upon interaction with acidic phospholipids. For instance, snake venom cardiotoxins or the pore-forming domain of colicin A display small, although significant, changes upon interaction with $Myr_2GroPGro$ vesicles as monitored by FTIR spectroscopy [28, 29]. Similarly, CD studies have shown that a synthetic leucine-rich repeat peptide from *Drosophila* chaoptin adopts a partial β structure in aqueous solution and is also predominantly a β structure in the presence of phospholipids [30].

Most fusion peptides studied to date adopt an α-helical conformation when interacting with lipids. For instance, this is the case of the fusion peptide of the influenza virus hemagglutinin, which is predominantly α-helical when interacting with lipids, as shown both by CD [31] and FTIR spectroscopy [32]. FTIR spectroscopy studies have also shown that although wild-type simian immunodeficiency virus fusion peptide and other derivatives are unable to adopt α-helical conformations in the absence of lipids, the addition of increasing amounts of lipid vesicles results in a corresponding increase in the amount of α-helix [33]. Interestingly, the same authors have observed that a variant of this fusion peptide with a high proportion of β-turns is unable to promote efficient vesicle leakage and fusion [33]. However, there is increasing evidence for the existence of peptides adopting extended structures when interacting with lipid bilayers. As an example, the human immunodeficiency virus fusion peptide adopts an α-helical structure at low peptide/lipid ratios, but is also able to bind to the lipid vesicles as a β-sheet at higher peptide/lipid ratios [34]. This peptide adopts mostly an extended antiparallel β-structure under conditions supporting fusion [23]. CD analysis of the fusion peptide of measles virus also confirms the conclusion that it adopts a 73% β-sheet structure when interacting with liposomes [35].

Moreover, several cases have been described in which toxins or other pore-forming proteins lyse cells through a barrel-stave mechanism in which a β-barrel is adopted by means of homooligomerization and formation of a pore that allows a passive flux of ions and small molecules across the bilayer [36]. The non-viral putative fusion peptide of PH-30, a protein active in sperm-egg fusion, has recently been determined to exist in an essentially unordered conformation in solution, but binding to membranes results in a conformational transition to a β-sheet structure [37]. CD studies on synthetic signal peptides also indicate that the β-conformation is a common structural feature in highly hydrophobic environments [38]. Consequently, lipid destabilization and fusion may also be performed by alternative structures other than α-helices.

How the N-terminal peptide of HBV is folded in the mature virions and how it becomes exposed during the viral fusion step are not known at present. Previous studies have shown that treatment of HBsAg particles with leucine amino-peptidase produces only trace amounts of amino acids, which indicates that the N-terminal sequence of the S protein is not exposed in these particles, or alternatively, its exposure is not enough for the enzyme to react with [39]. However, the membrane non-permeant amino-group-specific reagent trinitrobenzenesulfonic acid reacts with the N-terminal methionine group, since no phenylthiohydantoin-methionine release is observed upon Edman degradation of the HBsAg-treated protein [39]. Hence, it is presumed that the hydrophobic N-terminal sequence of the S protein is almost completely buried in the viral membrane, probably interacting with the viral lipids. This might imply that a structural change is necessary to allow the interaction of this peptide with the target membrane during the fusion process. In apparent support of this possibility, it has been recently described that in cultures of HepG2 cells inoculated with natural serum-derived HBV virions digested with V8 protease, HBV DNA can be detected intracellularly in a time-dependent manner following infection [40]. Since V8 protease cleaves next to the putative fusion peptide sequence, it can be argued that proteolysis-dependent exposure of a fusion domain within the envelope protein of HBV is necessary during natural infection. However, further studies are necessary to assess this point definitely.

The authors would like to acknowledge Dr J. G. Gavilanes for critical reading of the manuscript. This work has been partly supported by grants from the *Dirección General de Investigación Científica y Técnica* (Spain) (PB93-093 and PM95-0108) and from the National Institutes of Health (USA) (AI 15955).

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