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Conformation of Membrane Fusion-Active 20-Residue Peptides with or without Lipid Bilayers. Implication of α-Helix Formation for Membrane Fusion

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Received December 28, 1989; Revised Manuscript Received February 15, 1990

ABSTRACT: Fusion of small unilamellar vesicles of egg phosphatidylcholine can be triggered with synthetic 20-residue peptides. Taking the N-terminal amino acid sequence of HA-2 polypeptide of influenza virus as a guideline, we designed and synthesized several peptides having amphiphilic structures. Among the peptides so far studied, those active to induce membrane fusion took an α-helical conformation in the presence of phospholipid bilayers, while a peptide which was unable to induce membrane fusion was in a β-structure. Mixing of a pair of positively and negatively charged peptides, which had a complementary arrangement of electric charges to each other, resulted in α-helix formation at neutral pH, the condition of forming a randomly coiled conformation for each peptide. We concluded that α-helix formation was one of the necessary conditions to trigger a process of membrane fusion, at least in the present set of peptides. Characteristic features of these amphiphilic peptides are also described.

Hemagglutinin (HA)[1] of influenza virus is a glycoprotein responsible for the virus' ability to catalyze fusion of the membranes of the virus and a targeting cell (White et al., 1983). In vitro studies showed the HA-induced liposome fusion was triggered at a pH as low as 5.5 (Maeda & Ohnishi, 1980), the pH of the mildly acidic environment in endocytic vacuoles where in vivo fusion takes place to release the viral genetic material into the host cell cytoplasm. HA protein is made of two components, HA-1 and -2, and an inspection of the amino acid sequence of the protein revealed a hydrophobic region at the N-terminus of HA-2, which suggested the region as an interaction site with lipid membranes. We synthesized an eicosapeptide having the same amino acid sequence (1–20) of HA-2 [virus strain A/PR/8/34(H-1)] and found that the peptide actually induced membrane fusion of small unilamellar vesicles of egg PC, with a similar dependence on pH (active below pH 6) as the virus-mediated fusion (Murata et al., 1987a). Also, a closely related peptide, which had the amino acid sequence of the N-terminal 20 residues of influenza HA-2 strain B/Lee/40, has been reported to induce fusion of PC vesicles (Lear & DeGrado, 1987).

The structure of lipid bilayers is not rigid, and we expect that rather than the amino acid sequence itself, secondary or higher structures trigger the process of membrane fusion. We have synthesized HA-2-related peptides (Figure 1), and pH-dependent liposome fusion activities have been found for peptides II–VI (Murata, personal communication). In this paper, we describe the conformations of our synthetic peptides in solution, with or without phospholipid bilayers, to elucidate a correlation between peptide structure and the peptide activity of membrane fusion.

[1] Abbreviations: Boc, *tert*-butyloxycarbonyl; CD, circular dichroism; Fmoc, fluorenylmethyloxycarbonyl; HA, hemagglutinin; IR, infrared; MES, 2-(*N*-morpholino)ethanesulfonic acid; PC, phosphatidylcholine.

Materials and Methods

Peptides. Peptides I–III were synthesized by a Boc methodology with [[(phenylacetamido)methyl]amino]polystyrene (prepared from Bio-Rad SX-1). Peptide coupling was carried out with an amino acid symmetric anhydride and monitored with the Kaiser test (Kaiser et al., 1970). Peptides were cleaved and deblocked with HF [a low–high procedure (Tam et al., 1983)] or with trifluoromethanesulfonic acid (Tam et al., 1986). Peptides III–VII were prepared by an Fmoc methodology (Eberle et al., 1986) using Ultrosyn A polyacrylamide–Kieselgel resin (Pharmacia–LKB) and Fmoc amino acid pentafluorophenyl esters (Kisfaludy & Schoen, 1983). Peptide cleavage from resin (1 g) was achieved by keeping the resin in a mixture of anisole (3 mL), 2-mercaptoethanol (1.5 mL), and trifluoroacetic acid (10 mL) at room temperature for 6 h. Each peptide was purified by a successive application of Sephadex G25 gel filtration, ion-exchange chromatography (DEAE-Toyopearl for acidic peptides, CM-Toyopearl for peptide VI), and reversed-phase HPLC [Cosmosil 300C4 for peptides I and II, elution with acetonitrile–2 mM phosphate (pH 7.1); Cosmosil C18-P or YMC A343-S5 C18 for the others, elution with acetonitrile–5 mM ammonium acetate]. The purity of the peptides was confirmed in every aspect of amino acid composition (Table I) and analytical HPLC. The samples of peptide III prepared by Boc and Fmoc methodology were not distinguished on HPLC.

Spectroscopy. CD spectra were obtained with a JASCO J-20 spectropolarimeter modified as to have a quartz stress-modulator, and the ellipticities are expressed as a mean residue weight basis, $[\theta]_{MRW}$, with units of degrees centimeter squared per decimole. Cells having 0.2–10-mm optical path lengths were used, depending on peptide concentrations; most measurements were carried out with a 0.2-mm cell. All the peptides, except VI, precipitated below about pH 5, CD mea-

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I. H-Gly Leu Phe Gly Ala Ile Ala Gly Phe Ile Glu Gly Gly Trp Thr Gly Met Ile Asp Gly-OH

II. H-Gly Leu Phe Gly Ala Ile Ala Asp Phe Ile Glu Gly Gly Trp Glu Gly Leu Ile Glu Gly-OH

III. H-Gly Leu Phe Glu Ala Ile Ala Glu Phe Ile Glu Gly Gly Trp Glu Gly Leu Ile Glu Gly-OH

IV. H-Gly Leu Leu Glu Ala Leu Ala Glu Leu Leu Glu Gly Gly Trp Glu Gly Leu Leu Glu Gly-OH

V. H-Gly Leu Phe Glu Ala Ile Ala Glu Phe Ile Glu Gly Gly Tyr Glu Gly Leu Ile Glu Gly-OH

VI. H-Gly Leu Phe Lys Ala Ile Ala Lys Phe Ile Lys Gly Gly Trp Lys Gly Leu Ile Lys Gly-OH

VII. H-Gly Leu Glu Phe Ala Ile Glu Ala Phe Ile Glu Gly Gly Trp Glu Gly Leu Ile Glu Gly-OH

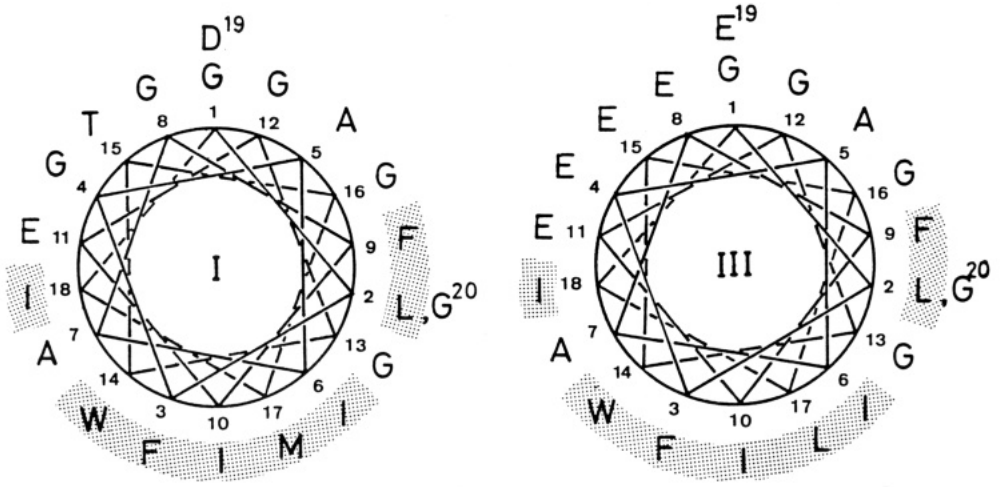

FIGURE 1: Amino acid sequence of peptides I–VII (top) and helical wheel representations of peptides I and III (bottom). Shaded residues are hydrophobic.

Table I: Amino Acid Composition of Peptides I–VII[a]

peptide	Asp	Thr	Glu	Gly	Ala	Met	Ile	Leu	Phe	Tyr	Lys
I	1.13	0.98	0.98	7.00	2.04	0.80	2.77	0.96	1.83		
II	0.95		2.91	6.00	2.08		2.76	1.92	1.85		
III			4.88	5.00	1.94		2.73	2.02	1.86		
IV			5.14	5.00	1.95			7.31			
V			4.86	5.00	2.00		2.84	2.12	2.00	0.99	
VI				5.00	2.04		2.83	2.07	1.89		4.80
VII			4.92	5.00	1.95		2.71	1.92	1.87		

[a] Hydrolysis was carried out in 6 M HCl at 110 or 120 °C for 24 h. Tryptophan was not determined.

surements were limited up to pH 5.2 or higher. Concentrations of peptides were determined by amino acid analysis.

IR spectra were recorded with a JASCO FT/IR-8000 at a resolution of 4 cm^{-1} for 1–3% solutions of peptides in buffered D_2O which were sandwiched between CaF_2 plates spaced at 25 μm. The peptide spectrum was obtained by subtraction of the solvent absorption from the solution spectrum, and the pD of the solution was assumed as the glass electrode meter reading +0.4. For some of the peptides, a sample in required solvent conditions was frozen at liquid nitrogen temperature and lyophilized, and the IR spectrum was obtained for a KBr tablet. Absorption component analyses were carried out, under an assumption of Lorentzian band shapes, with a fitting program installed in the instrument.

Phospholipids. Sonicated egg PC vesicles prepared as described previously (Murata et al., 1987a) were a generous gift of Dr. M. Murata, Kyoto University. CD spectral measurements of solutions containing phospholipids were carried out at peptide/lipid mole ratios of 1/15 to 1/100. Due to absorption flattening accompanying inevitable light scattering in the presence of lipid vesicles, the absolute values of ellipticity are somewhat ambiguous at the shorter wavelengths (Glaeser & Jap, 1985).

Gel Filtration. Analytical gel filtration experiments were carried out with Superose 6 or 12 (Pharmacia), Cellulofine GCL 90SF (Seikagaku Kogyo), or TSK G2000SW as gel matrices.

Results

Peptide I. Although peptides II–VII were readily soluble in water and studies were possible at a wide range of concentrations, peptide I, however, showed a limited solubility in aqueous media. Molecular associations of peptide I were extraordinary even for dilute solutions as was shown by its elution nearly at the void on TSK G2000SW gel chromatography. As no high-grade IR spectrum was obtained, arguments on the secondary structure of the peptide were confined to CD spectra, which were almost independent of pH or the presence of lipids (Figure 2). Although the presence of lipids did not give a significant change in the CD spectrum (peptide to lipid mole ratio more than 1 to 50), evidence of direct interaction of peptide I and lipid vesicles was obtained from gel chromatography with Superose 6, which had much higher exclusion limit than TSK G2000SW. The peptide was coeluted with lipids at the void volume of the column (0.3 M KCl, pH 6.80). There was observed only one ellipticity minimum in the 190–250-nm region; the spectra resembled that of β-structure [for assignments of secondary structures from CD bands, see Yang et al. (1986)]. However, the band was much wider than that of normal β-structure, suggesting

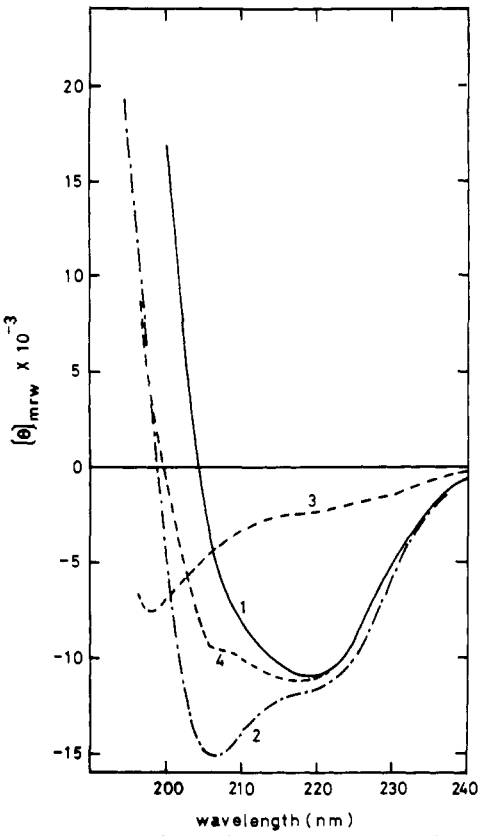

FIGURE 2: CD spectra of peptides I and II. 1, peptide I (10^{-5} M) + egg PC (10^{-3} M) in 0.1 M KCl–10 mM MES, pH 5.8 (the spectrum at pH 5.26 was superimposable). 2, peptide I (10^{-4} M) in 50% trifluoroethanol (10 mM phosphate, pH 7.4). 3, peptide II (5×10^{-4} M) in 0.1 M KCl–10 mM phosphate, pH 5.49. 4, peptide II (5×10^{-4} M) at pH 5.03.

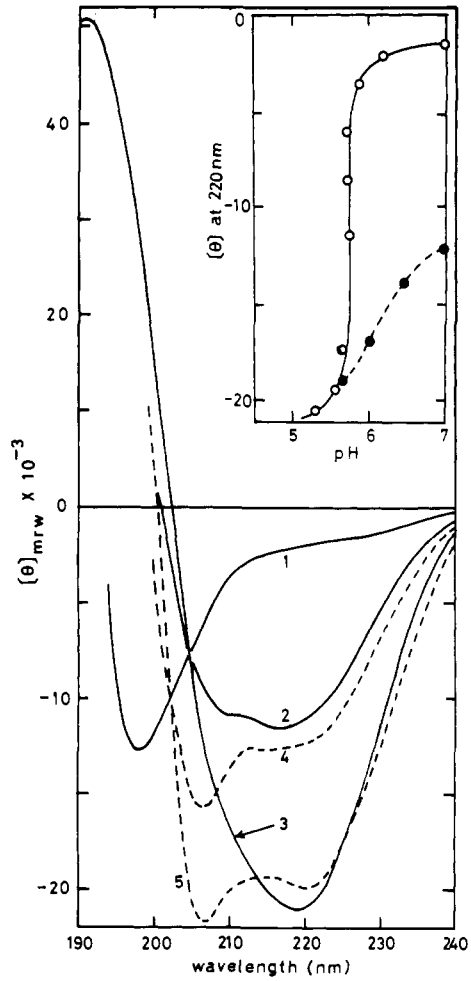

FIGURE 3: CD spectra of peptide III (7.34×10^{-4} M, without lipids for 1, 2, and 3; 3.67×10^{-4} M, with 5 mM egg PC for 4 and 5, in 0.1 M KCl–10 mM MES). 1, pH 7.0. 2, pH 5.75. 3, pH 5.28. 4, pH 7.0. 5, pH 5.6. The inset shows the relationship of $[\theta]_{mrw}$ at 220 nm and pH at the conditions described above; open circles and solid line for without lipids, closed circles and broken line for the presence of lipids.

that α-helix or distorted helix is also present.

Peptide II. The CD spectrum (Figure 2) is dependent on the concentration of the peptide. When the concentration of peptide II was as high as 5×10^{-4} M, the spectrum changed its shape drastically upon a decrease of the pH of the solution. Dilution of a pH 5 solution, which showed an α-helix-like spectrum, changed the spectrum to a random-coil type. The phenomenon was completely consistent with the result of gel filtration (data not shown) and suggested that self-aggregation of the peptide in solution stabilized an ordered structure(s).

Peptides III and V. Peptide V gave a similar dependence of the CD spectrum on pH change as that found for peptide III. An extensive study was performed with peptide III because the compound showed a CD spectrum closely resembling that of peptide I which had the HA-2 sequence. A pH titration of peptide III in water was accompanied with a CD spectral change, suggesting a transition from a randomly coiled sttate to an ordered structure(s) as shown in Figure 3. A severe dependence of the CD spectra on the concentration of peptide III and on the time elapsed after a solution was prepared was also noticed; when the concentration of III increased, a much greater amount of ordered structure was obtained. An apparent isoelliptic point was observed at 204.5 nm in Figure 3, and it may suggest that the spectral change represents a two-state transition, namely, a transition between two unique states, one at high pH and one at low pH. Therefore, we first considered the possibility of a distorted helix as a unique structure under the low-pH conditions; IR spectra of the amide I region, however, clearly revealed a peak at 1620 cm^{-1} which was unambiguously assigned to a β-structure (Krimm & Bandekar, 1986). A typical spectrum is shown in Figure 4 for a solution of III (1.5×10^{-2} M) at pD 6.6. Although CD spectral measurement of such a concentrated solution was limited to wavelengths longer than 225 nm, the CD spectrum from 225 to 250 nm could be overlapped with the spectrum of III at pH 5.75 in Figure 3, showing that the ordered structures were present under the conditions at which the IR spectrum was measured. IR spectra did not distinguish between α-helix and random coil for 1645 cm^{-1} absorption of III (Krimm & Bandekar, 1986). However, as the CD spectra showed that the α-helix should be also present, we concluded that the presence of an apparent isodichroic point was accidental and that peptide III had both α-helix and β-structure as its ordered structure.

Ordered structure formation of III was also dependent on salt concentration (Figure 5). In this case, the ordered structure formed at neutral pH with increasing salt concentration was α-helix. Under acidic conditions (a CD spectrum at pH 5.7 is shown in Figure 5 as an example), however, CD spectra at high salt concentrations showed a similar contribution from β-structure as observed at low-salt concentrations. When egg PC was present, peptide III gave CD spectra dominated by α-helix (Figure 3). About a 1.5-fold increase of ellipticity in the 205–230-nm region was observed when the pH was changed from 7 to 5.6.

Peptide IV. In the absence of egg PC, α-helix was dominant for this peptide, and the presence of phospholipids increased the content of α-helix 1.3–1.5 times (Figure 6).

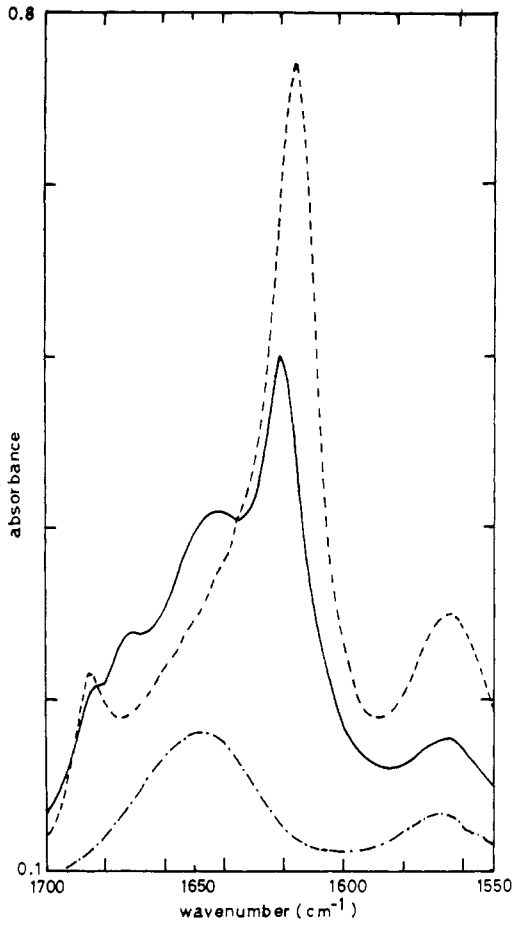

FIGURE 4: IR spectra. Solid line, III (1.7×10^{-2} M) in 0.1 M NaCl–5 mM MES/D_2O, pD 6.6. Broken line, VII (2×10^{-2} M) in the same media as above, pD 7.5. Chain line, solid precipitates from a mixture of III and VI solutions, measured as a KBr disk.

Peptide VI. Peptide VI, a lysine analogue of peptide III, behaved similarly in spectroscopic aspects; the only difference was that the changes took place as expected at basic pH (Figure 7). High concentrations of salt (Figure 7) or the presence of egg PC (Figure 8) also assisted α-helix formation at neutral pH. Peptides III and VI had an unordered conformation at neutral pH, but upon mixing their solutions, an ordered structure(s) was (were) formed (Figure 9). The complex formed with III and VI was in a highly aggregated state and readily precipitated unless it was much diluted; therefore, the observed CD spectrum for 0.017 mM III + VI (Figure 9) might be distorted at shorter wavelengths due to a turbid solution. Nonetheless, the spectrum displays two minima characteristic of α-helix. The IR spectrum (KBr) of a lyophilized sample of the precipitated complex showed an absorption at 1647 cm^{-1} and not around 1620–1630 cm^{-1} (Figure 4), the spectrum of the amide I region being almost identical with that of a solution of III in 50% C_2H_5OD at pD 7.0, the conditions that favored α-helix formation.

Peptide VII. This peptide which was designed to adopt a β-structure was shown to actually have the desired conformation in every aspect of CD and IR spectra. A CD spectral change accompanied with pH titration of VII is shown in Figure 10, the existence of an isoelliptic point at 210 nm suggested the transition was two state, namely, from an unordered state at a pH higher than 7.0 to a β-structure at pH lower than 6.5, when the concentration of VII was 1 mM. The conformation of the peptide was dependent on the peptide concentration (Figure 11). β-Structure in solution was unequivocally identified by a strong IR absorption at 1620 cm^{-1}

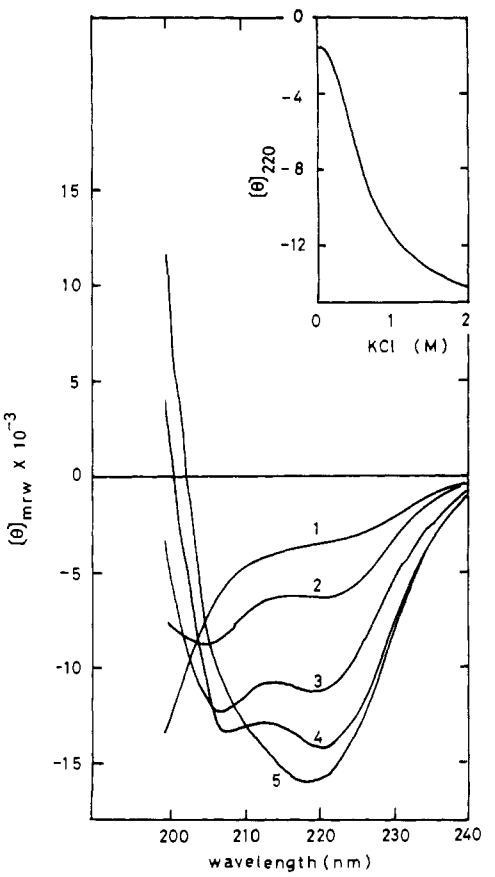

FIGURE 5: CD spectra of III (8.7×10^{-4} M) at different concentrations of KCl (in parentheses). Spectra 1–4 were at pH 7.0, spectrum 5 at pH 5.7: 1 (0.25 M); 2 (0.5 M); 3 (1.0 M); 4 (2.0 M); 5 (1.0). The inset shows the change of $[\theta]$ at 220 nm with KCl concentration.

(Figure 4). Direct measurement of the CD spectrum of the solution for the IR spectrum was impossible, but 0.2-fold dilution (concentration of VII = 3 mM) of the IR sample (pD 7.5) with D_2O afforded a CD spectrum with $[\theta]_{218} = 6000$, which was nearly identical with the one at pH 6.4 in Figure 10.

Unlike the other peptides, the β-structure of VII was preserved after the addition of egg PC (Figure 11). Also, a dependence of the CD spectra on the peptide concentration was observed in the presence of lipids.

Structure of I–VII in 50% Trifluoroethanol. The structure of the peptides in media containing organic solvent was studied. All of the peptides showed spectra characteristic of α-helix (for examples, Figures 2, 8, and 11), which were similar to each other in shape and degree of ellipticity. Only one dominant absorption around 1645 cm^{-1} was observed in IR spectra, and the position of this absorption was consistent with that of α-helix (Krimm & Bandekar, 1986).

Discussion

A 20-residue peptide having a part of the N-terminal amino acid sequence of influenza HA-2 has been shown to mimic membrane fusion induced by the virus protein (Murata et al., 1987a). Structurally related peptides II–VI, which were designed to represent some of the features carried by the protein-based peptide I, were also able to mediate membrane fusion in a pH-dependent manner [peptides II–V were active below pH 6, while peptide VI was active above pH 8 (Murata, personal communication)]. We considered that the amphiphilicity of these peptide α-helices (if formed, see Figure 1) might be responsible for the pH-dependent fusogenic activity.

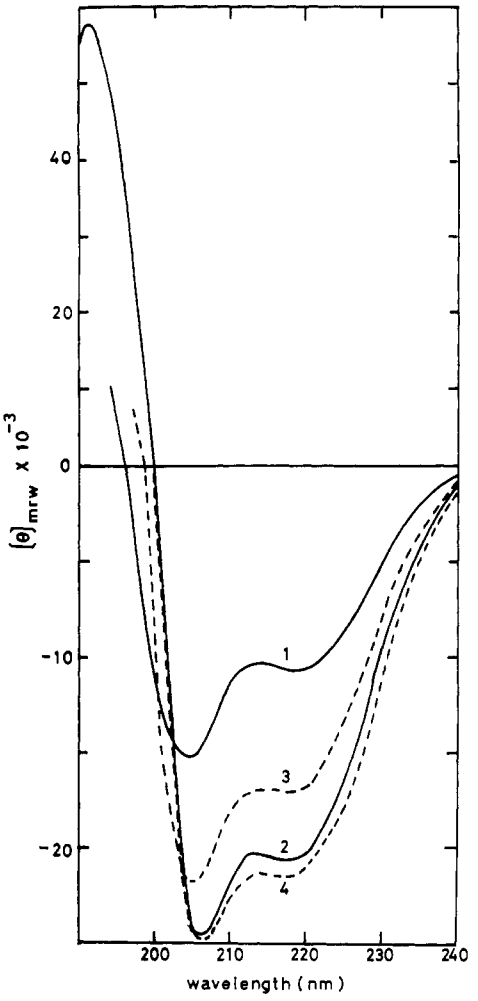

FIGURE 6: CD spectra of peptide IV. 1 (pH 7.26) and 2 (pH 4.95), 9.25×10^{-4} M of IV in 0.1 M KCl–5 mM phosphate or MES. 3 (pH 7.02) and 4 (pH 5.37), 1.98×10^{-4} M IV in the presence of 4 mM egg PC.

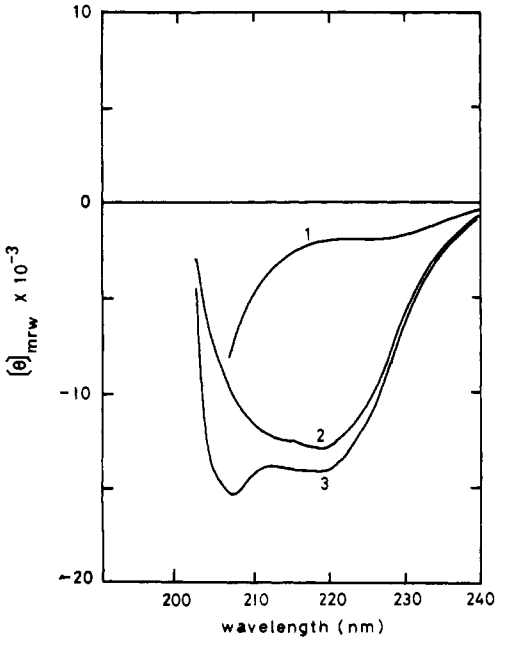

FIGURE 7: CD spectra of peptide VI [1.4×10^{-3}, buffered with 5 mM MES and 5 mM tris(hydroxymethyl)aminomethane]. 1, pH 5, 0.1 M KCl; 2, pH 8.8, 0.1 M KCl; 3, pH 5, 1.0 M KCl.

Distribution of hydrophobic side chains of an α-helix on a half-side of the lateral surface of the helix and hydrophilic

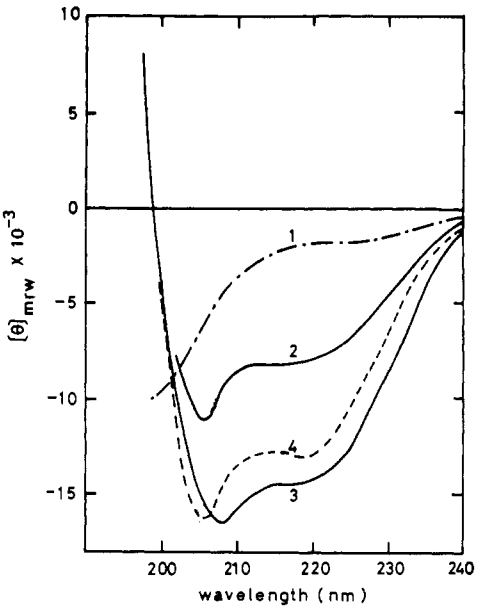

FIGURE 8: CD spectra of peptide VI at pH 6.9, 0.1 M KCl. Concentration of VI in parentheses. 1 (8.5×10^{-4} M); 2 (4.5×10^{-5} M) + egg PC (5×10^{-4} M); 3 (4.2×10^{-4} M) + egg PC (5 mM); 4 (4.2×10^{-4} M) in 50% trifluoroethanol.

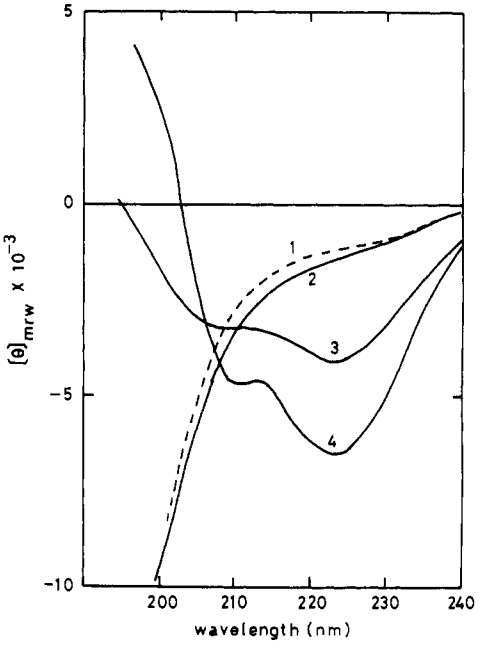

FIGURE 9: CD spectra of peptide III + VI. 1, III (9×10^{-4} M), 0.1 M KCl, pH 7.0. 2, VI (8.5×10^{-4} M), 0.1 M KCl, pH 6.9. 3, III (3.4×10^{-6} M) + VI (3.4×10^{-6} M), 1 mM piperidinomethanesulfonic acid, pH 7.0. 4, III (1.7×10^{-5} M) + VI (1.7×10^{-5} M), pH 6.4.

groups on the other side makes a peptide–phospholipid vesicle interaction in water possible. The present study on peptides I–VII supports this aspect. Peptides I–VI, which were active fusogens for liposomes, all took the α-helical conformation, at least partially for I and almost exclusively for II–VI, upon binding with phospholipids. On the contrary, peptide VII, which was inactive, assumed β-structure when it was adsorbed on lipid bilayers.

Peptide Designs. Peptides I–VI were designed according to a principle of amphiphilicity. Briefly, peptide I has the same sequence found at the N-terminal region (residues 1–20) of influenza virus HA-2 [strain A/PR/8/34 (Winter et al., 1981)], II and III have the modified sequences of I to make the peptides more soluble in aqueous media, and IV is an

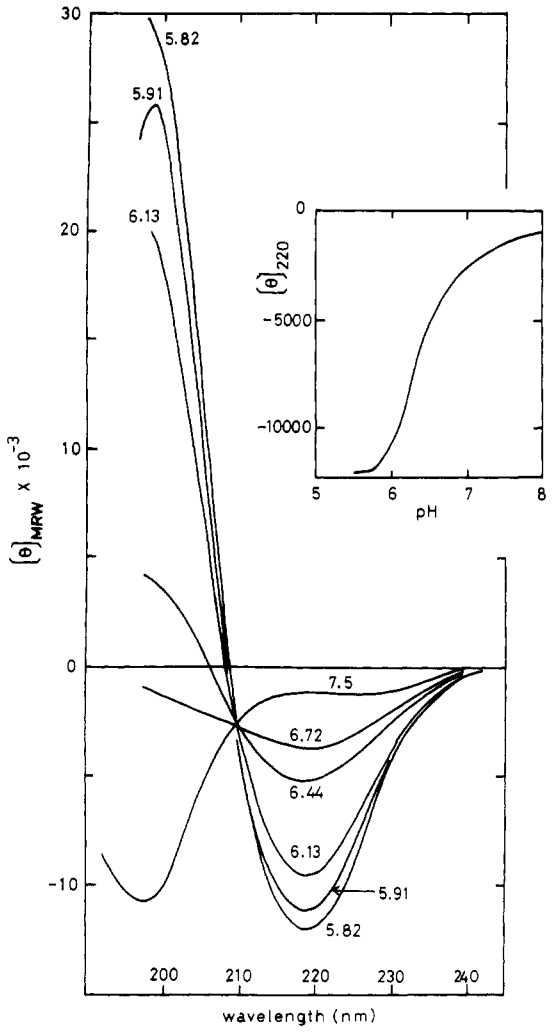

FIGURE 10: pH titration of peptide VII (10^{-3} M, 0.2 M KCl–5 mM MES). The numbers shown in the figure are the pH of the solutions; the inset shows a plot of $[\theta]$ at 220 nm vs pH.

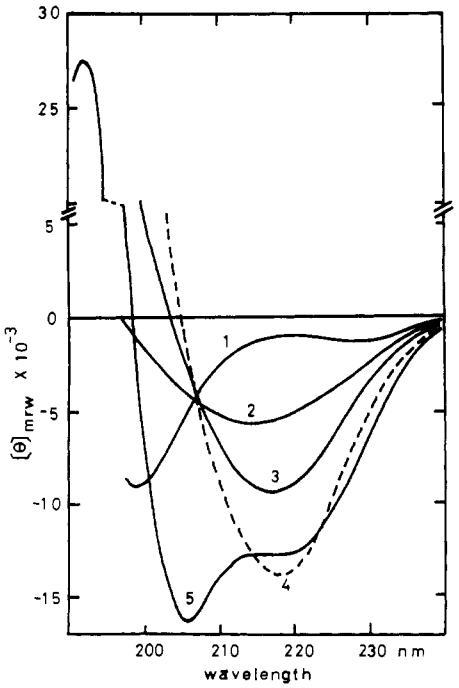

FIGURE 11: CD spectra of VII at pH 6 (0.1 M KCl–5 mM MES). Concentration of VII is in parentheses. 1 (10^{-6} M); 2 (5×10^{-5} M); 3 (7×10^{-4} M); 4 (4×10^{-4} M) + 5 mM egg PC; 5 (4×10^{-4} M) in 50% trifluoroethanol.

analogue of III, in which all of the hydrophobic residues except Trp are substituted by Leu. VI is a basic peptide, a Lys counterpart of III, and V has the same sequence as III except Tyr instead of Trp. In peptides II–VI, Gly and Ala residues were reserved as a part of the hydrophilic face of assumed amphiphilic α-helices. Peptide VII was designed to assume β-structure, also amphiphilic in nature. All the peptides except V reserve Trp at position 14, which will serve as a fluorescent reporter group for its environment.

Peptides I–V. These peptides, except I, took an unordered conformation in aqueous solutions at neutral pH. When the pH of the solution was lowered, an ordered structure(s) appeared, and the content was maximum at the lowest pH where the peptides remained in solution and spectral measurements were possible. These acidic peptides, and ordered structure formation with decreasing pH should be correlated with neutralization of carboxyl groups. Helix–coil transitions between pH 5 and 6 have been well established for poly(L-glutamic acid) (Wada, 1960) and glutamic acid–leucine copolymers (Nylund & Miller, 1965). Also, since the content of ordered structures was dependent on peptide concentrations as the more concentrated solutions formed the more ordered structures, the observed ordered structure formation must be a function of molecular association. Molecular associations stabilized ordered structures.

Except for the case of peptide IV, for which α-helical conformation was assigned rather unequivocally, the ordered structures of the other peptides at low pH were of a complex nature. For example, an IR spectrum of III showed two dominant amide I absorptions at 1620 and 1645 cm^{-1} with an intensity ratio 1:1.3, respectively. The amide I band at 1620 cm^{-1} was readily assigned to β-structure, but we could not discriminate the contributions from the α-helix and unordered conformation to the 1645 cm^{-1} band because both structures should have nearly the same band positions (Krimm & Bandekar, 1986). As described under Results, the conformational state of peptide III at the conditions in which the IR spectrum was measured corresponded to a CD spectrum at pH 5.75 in Figure 3, and a calculation of CD spectral components based on a set of reference spectra [we used a set of spectra 1, 5, and 7 of Stone et al. (1985)] gave 21% α-helix, 45% β-structure, and 34% random coil for a pH 5.75 CD spectrum or, in other words, a ratio of β-structure to a sum of α-helix and random coil of 1.24, a value nearly consistent with that obtained by IR spectroscopy. From this spectral evidence, we concluded that peptides I–III and V formed ordered structures comprising of α-helix and β-structure in solution at low pH. At present, we do not know whether these two ordered structures coexist within a molecule or the system is an ensemble of α-helical molecules and those having β-structure. It should be noted that an analysis based on protein structure data such as Chou–Fasman's procedure (Chou & Fasman, 1974) gave nearly identical potentials of α-helix and β-structure formation for these peptides [$\langle P_\alpha \rangle$ = 0.94 (peptide I) to 1.11 (peptide III); $\langle P_\beta \rangle$ = 1.04 (peptide I) to 0.90 (peptide III)]. However, one must be cautious for the application of statistics of protein structure to short peptides, for instance, polyglutamic acids having a polymerization number about 10 form β-structure almost exclusively at low pH (Rinaudo & Domard, 1976), while a glutamate residue is a strong β breaker in the treatment of Chou and Fasman (1974) and the α-helix is an exclusive conformation of high molecular weight poly(glutamic acids) at low pH. Peptide IV prefered α-helix in a wide range of pHs even without phospholipids, reflecting a high α-helix-forming potential of leucine residues.

Molecular association favored formation of ordered conformations of the peptides. Preliminary studies for peptides III, IV, and V with gel filtration techniques suggested the size of aggregates was fairly unique (monodisperse) and an apparent molecular weight of around 10000. We are not yet able to evaluate definitely the self-association constants of the peptides, and a more detailed study is now in progress. Unordered structure observed at neutral pH in solution might be due to repulsive negative charges on glutamic acid side chains. Shielding of the electric charges by adding salt induced a progressive formation of α-helix. A closely related example has been reported (Subbarao et al., 1987). It is interesting to note that the potential of the peptides to form an α-helix was higher when the pH was high or, in other words, when glutamic residues dissociated almost completely. At pH lower than 6.0, a significant amount of β-structure persisted, and the effect of added salt was small, probably due to a diminished amount of electric charges on the peptide at low pH (Figure 5).

α-Helix was the exclusive ordered conformation of peptides III–V if phospholipids were included in the solution. Induction of ordered secondary structure has been observed in many instances (Kaiser & Kezdy, 1983; Surewicz et al., 1987; Rizzo et al., 1987). α-Helix may be prefered when a peptide has similar potentials for α-helix and β-structure (Wu et al., 1982). Positive evidence of complexation of the peptides with egg PC vesicles was given by gel filtration; the peptides were coeluted with the vesicles at the column void.

Peptide VI + III. If peptide VI formed an α-helix at neutral pH, the positively charged lysyl side chains are distributed on the hydrophilic side of the helix surface in the same fashion as the negatively charged glutamyl side chains of peptide III, and a complementation of VI and III helices stabilized by an electrostatic interaction between oppositely charged groups might be possible. Actually, we observed helix formation when III and VI were mixed together at neutral pH. We think that this mode of complementation of peptides is the cause of the triggered membrane fusion when the vesicles complexed with peptide III and those with peptide VI were mixed at neutral pH, while vesicles complexed with III or VI alone do not give effective fusion at that pH (Murata, personal communication). Reflecting the electrostatic nature of the interaction between two peptides, high concentrations of added neutral salts prohibited helix formation.

Peptide VII. In the present study, peptide VII was the only peptide which did not cause membrane fusion above pH 4.5, and the only one to show β-conformation irrespective of the presence of phospholipids. The amino acid sequence predicted the β-structure to be amphiphilic; namely, charged hydrophilic glutamyl side chains could appear on one side of β-sheet and hydrophobic residues on the other side, thus enabling an effective interaction with lipid membranes. As gel filtration suggested, a direct interaction between phospholipids and the peptide actually occurred but did not lead to membrane fusion. Peptide VII has some potential of helix formation since the conformation in 50% trifluoroethanol is α-helix.

α-Helix and Lipid Bilayers. All the fusion-active peptides, so far studied in the present work took an α-helical conformation when they were bound to lipid membranes. Lear and DeGrado (1987) also reported that their peptide was α-helical in the presence of phospholipids. These results suggest that α-helix must be responsible for inducing lipid membrane fusion.

Peptides bind to lipid membranes with a transconformation of their secondary structure into α-helix, since α-helices once formed are stabilized by an interaction with lipid bilayers due to their amphiphilicity programmed in their amino acid sequences. When the pH of the solution is nearly neutral, the location of helices might be restricted to the surface of bilayers to avoid unfavorable interactions of the charged face of the helix and the interior of bilayers. In a complementary system such as peptides III and VI each bound separately to different vesicles, an electrostatic attraction between negatively and positively charged α-helices of III and VI, respectively, enhances and stabilizes the association of these two kinds of vesicles. Dependence of the fraction of α-helix (in the presence of phospholipids) on pH is only gradual (Figure 3), while that of membrane fusion activities of the peptides is remarkable at a narrow range of pH, between 6 and 5 (Murata et al., 1987a). Therefore, the fusion activity must be correlated with exhaustive neutralization of electric charges on the α-helices, or in other words, activity vs pH profiles are parallel to the titration curves of peptides, as observed for succinylated melittin (Murata et al., 1987b). Once electric charges were neutralized, the hydrophilic side of the helices can penetrate into the interior of bilayers, or a transposition of the helices (or in other words, perturbations of lipid bilayers) becomes possible. A perturbation of lipid bilayers accompanying such a translocation of helices may trigger the process of membrane fusion. The β-sheets of peptide VII formed on lipid bilayers are probably too stable in this configuration to cause a perturbation of bilayers.

The following points should be solved in the future: What kind of a transposition of α-helices occurs to initiate membrane fusion? Do α-helices exist separately or associated themselves within lipid bilayers? Furthermore, the peptides themselves, apart from their potential to trigger membrane fusion, should be studied for their ability to form association-induced secondary structure in aqueous solutions. Protein tertiary structure is formed upon interactions between parts of a polypeptide chain. Those interactions cause secondary structure formation of the chain; without them, those local parts remain in a different conformation (mostly random coil). We think a thermodynamic or quantitative study of a peptide self-assembly system accompanied with ordered structure formation, as in the present case, will afford a basis for understanding the tertiary structure of proteins (Ho & DeGrado, 1987).

Acknowledgments

We are pleased to acknowledge the encouragement and many stimulating discussions of Drs. S. Ohnishi and T. Ooi. We thank Dr. M. Murata for his generous gift of egg PC vesicles and communication of his experimental results and also F. Kaneuchi at JASCO Co. for assistance in measurements of IR spectra.

Registry No. I, 111982-68-4; II, 127258-59-7; III, 127258-60-0; IV, 127258-61-1; V, 127258-62-2; VI, 127258-63-3; VII, 127258-64-4.

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Role of Arginine 67 in the Stabilization of Chymotrypsin Inhibitor 2: Examination of Amide Proton Exchange Rates and Denaturation Thermodynamics of an Engineered Protein†

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Received September 26, 1989; Revised Manuscript Received March 19, 1990

ABSTRACT: We have examined the contribution to protein stability of an interaction involving a charged hydrogen bond from an arginyl side chain (Arg67) in the serine proteinase inhibitor chymotrypsin inhibitor 2 (CI-2), by replacing this side chain with an alanyl residue by protein engineering. Using nuclear magnetic resonance spectroscopy (NMR), we have examined the effect of this mutation on the hydrogen–deuterium exchange rates of several backbone amide protons in the native and engineered proteins at 50 °C. These exchange rates provide a localized probe at multiple discrete sites throughout the protein and from comparison of native and mutant exchange rates allow calculation of the difference in free energy of exchange ($\Delta\Delta G_{ex}$) resulting from the mutation. The results show that for the majority of amides observed this mutation results in $\Delta\Delta G_{ex}$ of ca. 1.7 kcal mol^{-1} over the whole CI-2 molecule. However, for two relatively exposed amide protons the exchange rates are found to be far less perturbed, implying that local unfolding mechanisms predominate for these protons. Direct measurement of the stability of both proteins to denaturation by guanidinum hydrochloride shows that the interaction contributes 1.4 kcal mol^{-1} to the stability of the molecule. This value is comparable to those obtained from the NMR exchange measurements and indicates that the exchange processes reflect the differences in stability between the native and mutant proteins. Our results show (i) at the temperature of these experiments, NH exchange is due to unfolding processes; (ii) the whole CI-2 molecule (over the region for which data are available) forms a single cooperative folding unit; and (iii) the mutation studied results in a global destabilization of the protein by ca. 1.5 kcal mol^{-1}, a value consistent with the loss of the hydrogen-bonded interaction.

The conformation and stability of a protein depend upon a myriad of weak noncovalent interactions between the various constituent amino acid residues in the molecule. Collectively, these interactions produce a molecule that has a defined three-dimensional structure. Two relevant questions concerning the way these forces are used in the structural organization of proteins are (i) what is the precise energetic contribution of various individual interactions to the stability of the overall structure and (ii) how is the contribution of a single interaction distributed over the whole protein? Is the stabilization that is conferred localized to the immediate area of the protein around the particular residue involved or is this stabilization delocalized over the whole molecule?

Mutations within a protein are well-known to cause alterations in the stability of the molecule. Many "temperature-

† This work is funded by the Protein Engineering Club of the S.E.R.C.
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0006-2960/90/0429-6264$02.50/0