Association Characteristics of Amphiphilic α-Helices Connected by Flexible Links

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(Received October 2, 1990)

Amphiphilic helical peptides interlinked with sequences bearing a flexible mobility were designed and synthesized. The sequences containing glycine and/or cystine were adopted for the linkage parts. The secondary structure of the peptides was investigated by circular dichroism(CD) and the super-secondary structure was estimated. The CD spectra and their pH dependence show that the peptides have almost the same α-helix contents as designed and that the helices associate to be "coiled-coil" at neutral pH. The apparent helix contents increase at low pH and some of helices are subject to denaturation at high pH. The denaturation of α-helix bundles by guanidine hydrochloride is contemplated to be three-state transition. It is proposed that the peptides construct α-helix bundles with both loose and tight contacts between helices. Two kinds of folded structure constructed with 2 or more helices are also suggested.

The construction of artificial proteins is a very attractive field. Several new designs[1-5] of proteins have been presented in the last several years.

In nature there exist "coiled-coil"[6] and "4-helix bundle"[7] structures which are formed by 2 and 4 α-helices, respectively. Amphiphilicity of cylindrical helices is characteristic of these structures. The contact faces of associated helices are rich in hydrophobic residues and the opposite faces are often hydrophilic. Nishino et al.[8] investigated the association of amphiphilic helices by means of a size exclusion chromatography (SEC). They tested the effect of hydrophobic and hydrophilic amino acids in helices on the association. DeGrado et al.[9,10] revealed an association free energy and the number of association by measuring the concentration dependence of CD intensity. Mutter et al.[11] proposed a new design of peptides and the structure was analyzed with CD and SEC. In these studies the linked helices were found to form more stable aggregates than single helices.

In this work, we designed and synthesized several amphiphilic helical peptides interlinked with flexible connections. The important characteristic of our designed peptides is flexible linkage of amphiphilic helices. The flexible links are expected to play an important role in arranging helices in a stable packing. Moreover, the peptides may be useful as "induced fitting" host molecules. The formation of a bundle structure by amphiphilic helices is already known, however, the folding course and the influence of temperature and pH on a folded structure are not investigated well. In order to make clear the super-secondary structure as well as the folding behavior of linked α-helices the CD spectra were analyzed in detail.

Experimental

Materials. *t*-Butoxycarbonyl-protected amino acids and 4-(bromomethyl)phenylacetic acid were purchased from Kokusan Chemical Works and used without further purification. The other reagents were trifluoroacetic acid (TFA), dicyclohexylcarbodiimide (DCC), ninhydrin, diethyl phosphorocyanidate (DEPC), triethylamine (Et_3N), cesium carbonate (Cs_2CO_3), trifluoromethanesulfonic acid (TFMSA), dichloromethane, *N,N*-dimethylformamide (DMF) distilled over CaH_2, and dimethyl sulfoxide (DMSO), which were purchased from Wako Pure Chemical. The solvents were dried over molecular sieve 4A.

Peptide Synthesis and Purification. The peptides P2, P3, P4x, P4w and P6, of which the sequences are shown in Fig. 1 with one letter abbreviations,[12] were synthesized manually by a solid-phase method. The initial Boc-protected amino acid was anchored onto copoly(styrene-1%-divinylbenzene) (Bio-beads S-X1, Bio-Rad) intermediated with 4-(hydroxymethyl)phenylacetamidomethyl group by the method of Mitchell et al.[13] The second and the third residues from C-terminus were introduced in one time with DEPC-Et_3N[14] in order to be free from 2,5-piperazinedione formation.[15] The subsequent residues were coupled stepwise. The side-chain-protecting groups used were Glu(OBzl), Lys(ClZ), and Cys(Acm)[16] (Bzl, benzyl; ClZ, 2-chlorobenzyloxycarbonyl; Acm, acetamidomethyl). The double couplings were carried out for each step by means of DEPC-Et_3N and symmetrical carboxylic anhydride methods.[17] Finally the peptide was deprotected and cleaved from the resin by TFMSA-Me_2S-*m*-cresol system[18] at 0 °C for 28h. For the peptides P4x, P4w, and P6, Cys(Acm) was deprotected and oxidized to be cystine with 0.01 M (1 M=1 mol dm^{-3}) iodine in water-acetic acid (1 : 4) at 20 °C for 1 h.

Each crude peptide was desalted by gel filtration with Sephadex G-15 using 0.1 M acetic acid. The crude yields of the peptides P2, P3, P4x, P4w, and P6 were 24, 17, 29, 30, and 17%, respectively. The collected peptide solution was then applied to a column of CM-Sephadex C-25(1.7 cm by 70 cm) and eluted with a linear gradient 0.01 to 0.60 M ammonium acetate, pH 6.5, using a total volume of 1000 ml (Fig. 2). Two peaks were obtained for peptide P2, however they were found to be the same composition by means of amino acid analysis. The later peak seems to correspond to a dimer. Reversed-phase HPLC's with columns of octadecyl or 2-cyanoethyl silica gel were attempted but resulted in strong adsorption to column packings. The adsorption also occurred in the chromatography with CM-Toyopearl

(P2) AGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG

(P3) GELKKLLEELKKLLEGKPGGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG

(P4x) GELKKLLEELKKLLEECKGKPGGLKKLEELLKKLEELLG
GELKKLLEELKKLLEECKGKPGGLKKLEELLKKLEELLG

(P4w) CGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG
CGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG

(P6) CGGELKKLLEELKKLLEGKPGGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG
CGGELKKLLEELKKLLEGKPGGELKKLLEELKKLLEEAKGKPGGLKKLEELLKKLEELLG

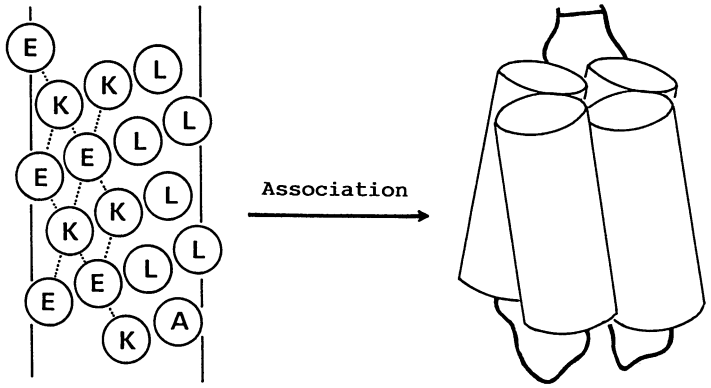

Fig. 1. Primary structures of the peptides represented with one letter abbreviations (upper) and drawing of a helical part to form bundle structure (lower). The peptides were named P2, P3, P4x, P4w, and P6 as shown in parentheses. The underlined sequences are α-helix parts predicted with the method of Chou and Fasman.[28] Cystine bonds are indicated with vertical line segments.

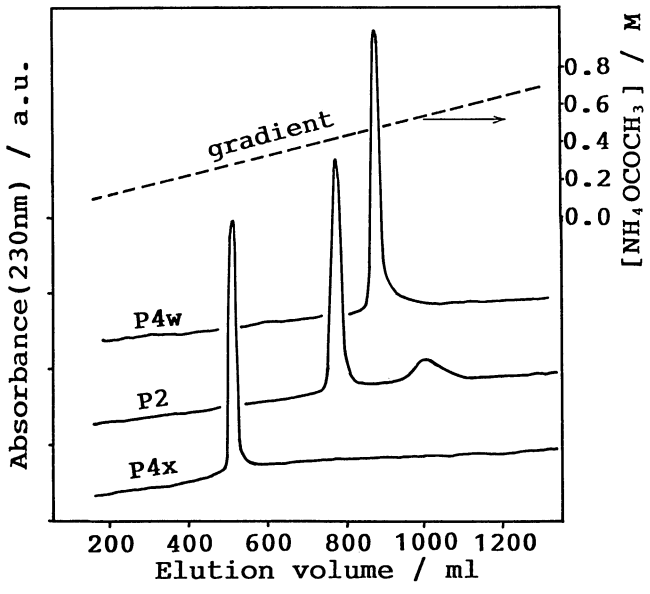

Fig. 2. Ion-exchange chromatography on CM-Sephadex C-25 with linear gradient.

650M and TSKgel G3000PW, and delayed elution was observed. Finally, the peptide was desalted by gel filtration and lyophilized to a fluffy powder.

The formation of a cystine linkage was confirmed by means of iodine titration[19] and quantitation of cysteine with di-(2-pyridyl) disulfide.[20] The amino acid compositions are listed in Table 1. The observed values have good agreements with the theoretical ones.

Table 1. Amino Acid Analyses of the Peptides[a]

Amino acid	P2	P3	P4x /2	P4w /2	P6 /2
Glu	8.91(9)	13.34(13)	8.97(9)	9.03(9)	13.38(13)
Gly	5.09(5)	7.35(8)	4.71(5)	4.82(5)	8.29(9)
Ala	1.75(2)	1.21(1)	(0)	1.00(1)	1.13(1)
Leu	13.26(13)	19.10(19)	13.31(13)	13.16(13)	19.20(19)
Lys	9.80(10)	14.32(15)	9.00(10)	9.33(10)	14.36(15)
Pro	0.93(1)	1.69(2)	0.86(1)	0.90(1)	1.81(2)

a) The values for peptides P4x, P4w, and P6 are related to their half-peptides. The values in parentheses are theoretical.

Measurement. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer. Quantities of peptides were determined from amino acid analyses after hydrolysis[21] in 10 M HCl-TFA mixture (2 : 1, v/v) for 50 min at 165 °C. The total of the molar ratios of accurately measurable amino acids was used to calculate the peptide concentrations.

CD spectra from 195—205 nm to 260 nm were recorded using a JASCO J600 spectropolarimeter with 10 mm path length cells at a controlled temperature. Peptide concentrations were 6.0×10^{-4} M (for residue) for the measurements

of temperature and pH dependence. The denaturation with guanidine hydrochloride (GuHCl) was measured for 1.2×10^{-3} M (for residue) solution in a 1 mm cell. Mean residue molar ellipticity([θ]) were reproducible within 3%. Sodium phosphate buffer (0.015 M) containing 0.15 M NaCl was used in the pH range from 2.00 to 11.00 where the pH values were adjusted with HCl or NaOH. For the solutions with pH 1.30 and pH 11.50, HCl-NaCl and NaOH-NaCl buffer were used, respectively. GuHCl solutions were prepared with stock buffer solutions of 0.015 M 3-morpholino-1-propanesulfonic acid with and without 8.0 M GuHCl. Each solution of 0—8.0 M GuHCl was adjusted to pH 7.00.

Results and Discussion

Design of Peptides. The peptides were designed to fold in helix-bundle structures (Fig. 1). The peptides P2, P3, P4x, P4w, and P6 contain 2, 3, 4, 4, and 6 helical parts, respectively. P4x and P4w are respectively cross-linked at different positions with disulfide bonds.

The principles for constructing the helical parts are proper choice of residues which tend to form α-helix and arrangement of hydrophobic and hydrophilic residues on opposite sides of a helix cylinder. The hydrophilic residues, Glu and Lys, were arranged to form a salt-bridge between positions i and $i+3$ or $i+4$ which is favored in nature.[22] The sequences of α-helical region are aligned in the order of Lys-Lys-Leu-Glu-Glu-Leu-Leu or Glu-Glu-Leu-Lys-Lys-Leu-Leu. Each helical part contains 14—17 residues and is possible to form a helix winding 4—5 times.

A loop with 5 residues or a cystine bond were used as the linkage part connecting two α-helical parts. The former is -Gly-Lys-Pro-Gly-Gly- and the latter is -Cys-Lys-, Cys-Gly- or Cys-Gly-Gly- coupled by disulfide bond. Glycine residue was chosen because it tends to form turn structures and has flexible mobility compared with α-substituted amino acid. Proline was chosen because of its specific tendency to terminate α-helix chain.[23] These residues widely occur in loop regions. As a loop region seemed to be exposed to water, lysine was added to the sequence as a hydrophilic residue.

The possible conformations of these peptides were examined with computer graphics, which showed us that the helices could associate and form a bundle structure with only a small distortion energy at a loop region. The stable association of the helices may be possible because their linkage parts have high flexibility.

CD Spectra of Peptides. The CD spectra of the peptides are shown in Fig. 3. A typical pattern of α-helix was observed for each peptide. In this condition the peptides seem to undergo little denaturation as shown later (Fig. 5). Generally, three classes, i.e. α-helix, β-sheet, and remainder, are most often used for secondary structures. However, it was pointed out that more splitted classes[24] of β-sheet are necessary

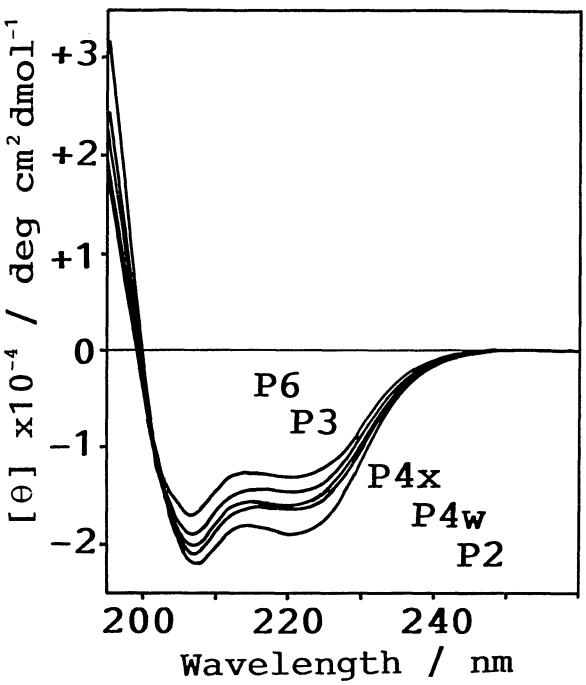

Fig. 3. CD spectra of the peptides in sodium phosphate buffer, pH 7.00, at 20 °C.

in order to obtain an improved accuracy in the analysis of CD spectra. In addition, the CD spectra of poly(amino acid)'s forming β-sheet were related to no single reference spectrum.[25] From these results, the CD analysis of β-sheet seems to be somewhat vague. The remainder structure is also vague because it has no defined structures. On the other hand, α-helix contents appears to be determinable more quantitatively than the other structures.

Chen et al.[26] introduced an empirical chain length dependent factor for a helix and obtained a good agreement with theoretical CD intensity. In Chen's equation, $[\theta]_H=[\theta]_H^\infty\cdot(1-k/n)$, mean residue ellipticity of a helix is a function of chain length, n. The parameters, $[\theta]_H^\infty$ and k, are listed in the literature.[26] According to this equation, the number, n, was calculated on the assumption that $[\theta]_{222}$ for nonhelical residues are from 1000 to 5000 deg cm^2 dmol^{-1} which are the values reported for remainder structures.[27] This leads to the results that the numbers n are smaller by about 3 to 5 per helix than the numbers predicted with the method of Chou and Fasman.[28] The helix contents with this method, which is named here "method-A", are almost consistent with the values obtained with CONTIN program[29] as listed in Table 2. The stabilization of helices with hydrophobic interaction between helices is so significant that it is difficult to interpret these reduced helix contents.

Then, we applied another method which is named "method-B" to determine helix contents. As reference data the method-B adopts the [θ] values of tropomyosin and its model peptide[30] which form "coiled-

Table 2. The Helix Contents Calculated with Various Methods

Peptide	Designed[a]	CONTIN[b]	Method-A[c]	Method-B[c]
	%	%	%	%
P2	78	60	63	82
P3	78	48	54	69
P4x	74	50	56	73
P4w	78	56	57	74
P6	75	38	49	63

a) The values were calculated with the prediction method of Chou and Fasman.[28]. b) The program version 2DP(1984) of CONTIN[29] was used. c) The helix contents were determined with the method described in the text on the assumption that $[\theta]$ values for nonhelical structures were 3000 deg cm^2 dmol^{-1} at 222 nm.

coil", while the method-A and CONTIN are based on the data of familiar globular proteins. The reference $[\theta]_H^\circ$ values for method-B are −1.52, −2.45, −2.57, −2.46, −2.54, −2.67, −2.77, −2.68, −2.11, −1.38, −0.93, −0.56, −0.29, and -0.09×10^4 deg cm^2 dmol^{-1} at every 3 nm from 204 to 243 nm. In a similar manner as above, the differences between the number n and the designed number are estimated to be about −1 to 2 per helix. Thus, the helix contents with method-B are almost consistent with the designed helix contents as listed in Table 2.

Consequently, it may be concluded that the helices of our peptides are coiled-coil structure rather than "general" α-helix found in globular proteins. The peptides containing more than two helices, P3—P6, would have certain helix bundle structures constructed with coiled-coil structural units. These structures are discussed precisely in following sections. The coiled-coil structure has small distortion in comparison with "general" α-helix. This seems to cause the difference in $[\theta]_H^\circ$ values between coiled-coil and "general" α-helix.

In order to estimate the secondary structures of nonhelical parts the calculated spectra for helical regions were subtracted from the observed spectra. The subtracted spectra are converted to mean residue ellipticity and shown in Fig. 4. The spectra have large negative CD peaks at less than 205 nm and resemble the spectra of random-coiled poly(amino acid)'s which have negative peaks with the intensity about −5.0 to -2.5×10^4 deg cm^2 dmol^{-1} at 196—202 nm.[31] As the β-sheet shows positive $[\theta]$ value at this wavelength region, the nonhelical parts of our peptides are considered to have not a β-sheet but a remainder structure which is specific for our designed sequences.

Thermal Transition of the Peptides. The temperature dependence of $[\theta]_{222}$ is shown in Fig. 5. Each peptide undergoes gradual thermal transition. The slope of transition curves is not steep compared with CM-tropomyosin but similar to that of Hodges' peptide.[30] The helices of the peptides are not fully denatured even at 90 °C. The peptide P2 is the most stable and its transition curve is different from the others. It is suggested that the peptide with two amphiphilic helices make a stable folded structure while more than two helices results in somewhat different folded structure containing a helix associated by weaker interac-

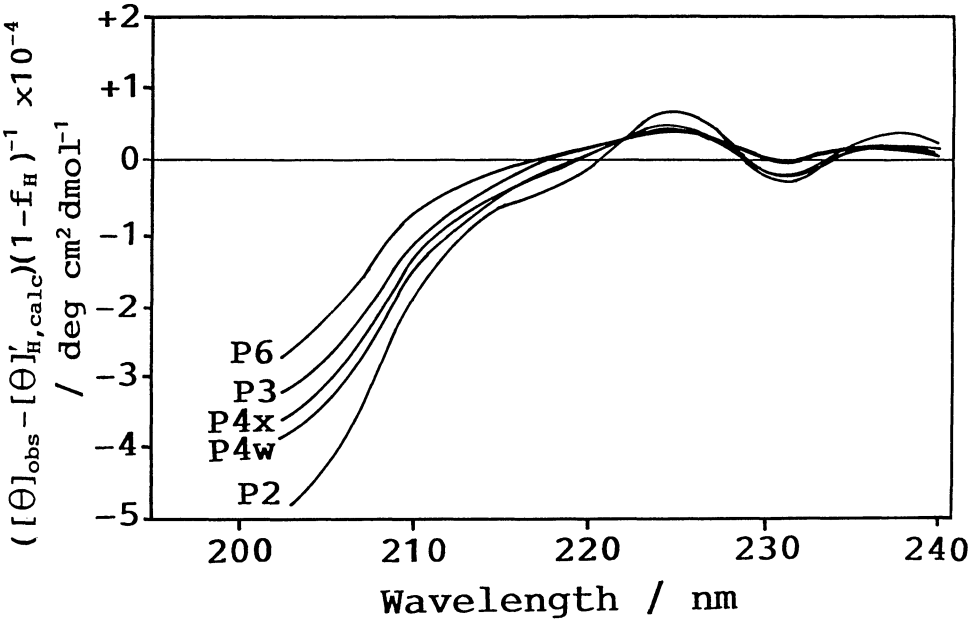

Fig. 4. Remained CD spectra after subtracting those of α-helical parts. $[\theta]_{obs}$, $[\theta]'_{H,calc}$ are mean residue ellipticities which are observed and calculated for helical parts, respectively and f_H is a mole fraction of helical residue.

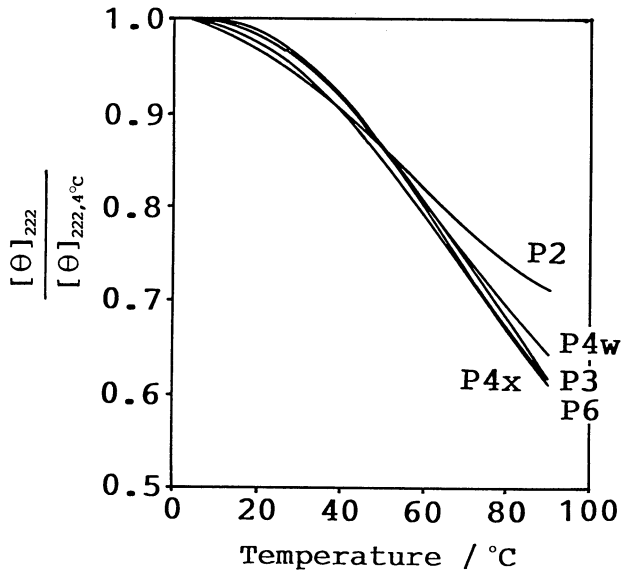

Fig. 5. Temperature dependence of mean residue ellipticity at 222 nm in phosphate buffer, pH 7.00. The values of $[\theta]_{222}$ are normalized at 4 °C.

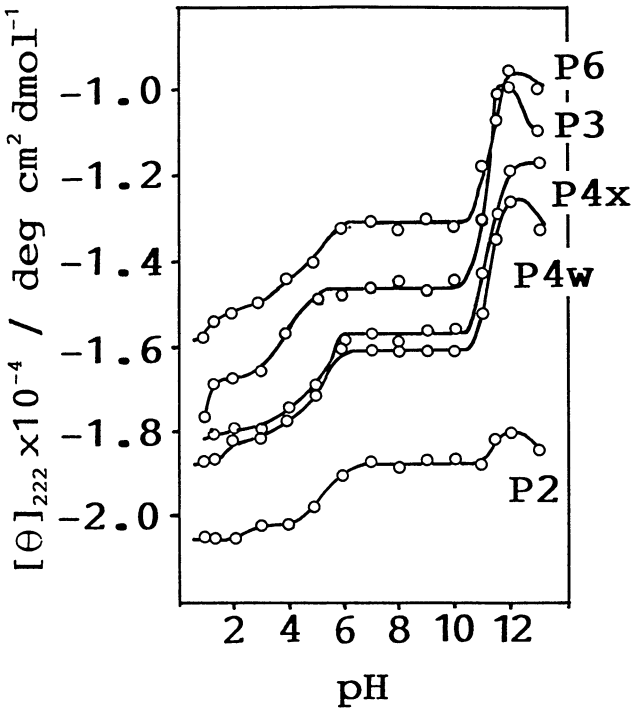

Fig. 6. The pH dependence of mean residue ellipticity at 222 nm.

tion. The disulfide bond does not appear to make a major contribution to the stability.

pH Dependence. The pH dependence of $[\theta]_{222}$ are shown in Fig. 6. For each peptide the intensity at 222 nm is constant at pH 6 to 11. These five peptides have a common tendency that the negative intensity increases in acidic conditions and decreases in basic conditions. The pH regions of the both transitions are consistent with pK_a of Glu and Lys side chains. At pH 12 the spectra are characteristic pattern of α-helix. Though only Glu side chains dissociate in this basic conditions, the denaturation of helices is restricted to some extent. Some denaturation may be attributed to weakened amphiphilicity because the Glu residues exist only at every two turns of helix.

In contrast to the basic conditions, the acidic ones cause a negative shift of $[\theta]_{222}$. The intensity increases by about 20%. If this is due to the increase of a helix content, the linkage region of the peptide will be shortened and become difficult to make a helix bundle. Therefore, it is reasonable to consider that the helix contents are constant but the type of helix changes from coiled-coil to otherwise "general" helix found in globular proteins. This change seems to result in the increase of the intensity of $[\theta]_H^\circ$ as discussed in the previous section.

As mentioned by Eisenberg et al.,[32] apolar carbon atoms of hydrophilic residues such as Lys and Glu have significant contributions to the stability of the folded structure of proteins. So, even if hydrophilicity is reduced in acidic or basic solutions, where either Lys or Glu is ionized, the helices of these peptides may not be necessarily denatured. The experimental results suggest that the helices of these peptides are susceptible to denaturation only at basic pH. As Lys has a long methylene side chain, non-protonated Lys at basic pH play a role as more hydrophobic residue

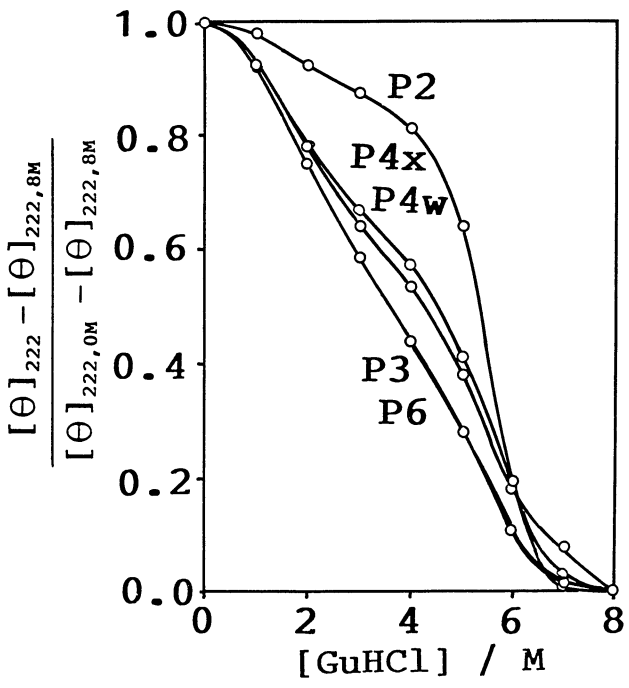

Fig. 7. Guanidine denaturation curves. The values of $[\theta]_{222}$ are normalized to be 1.0 in 0 M GuHCl and 0.0 in 8 M GuHCl. The ellipticities of these peptides in 8 M GuHCl were about 500—900 deg cm^2 dmol^{-1}.

than Glu at acidic pH, which is exposed to water and make α-helix unstable. Insufficient amphiphilicity would cause partial denaturation of helices in basic solutions. Thus, amphiphilicity is important in stabilizing α-helices.

Denaturation with Guanidine Hydrochloride. The helix structures were denatured with the increase of GuHCl concentration and $[\theta]_{222}$ values became about 1000 deg cm^2 dmol^{-1} in 8 M GuHCl. The relative changes of $[\theta]_{222}$ are shown in Fig. 7. The denaturation of P2 is a very steep transition in comparison with those of the other peptides. The midpoint of denaturation for P2 was shifted to a little higher GuHCl concentration when the peptide concentration was increased. This means that intermolecular association occurs in addition to intramolecular association of helices.

Each denaturation curve shows the fine structure that the first transition occurs at 2—3 M of GuHCl and the second at 5—6 M. For the peptide P2 the second transition is a major one, however for the other peptides almost the half of helices is subject to the first transition. It is reported that the peptide containing single amphiphilic α-helical strand takes α-helix form in associating with each other but that it can not form α-helix when isolated in a dilute solution.[9] Therefore, in our conditions no isolated α-helices exist. As all helices are not destroyed in the first denaturation step, the association of helices would exist even up to the second denaturation. So, both transitions relate to changes in association forms. These two types of transition can be explained by two types of interaction constructing a bundle structure. One of these interactions is relatively weak attractive force and the combined helices are susceptible to denaturation.

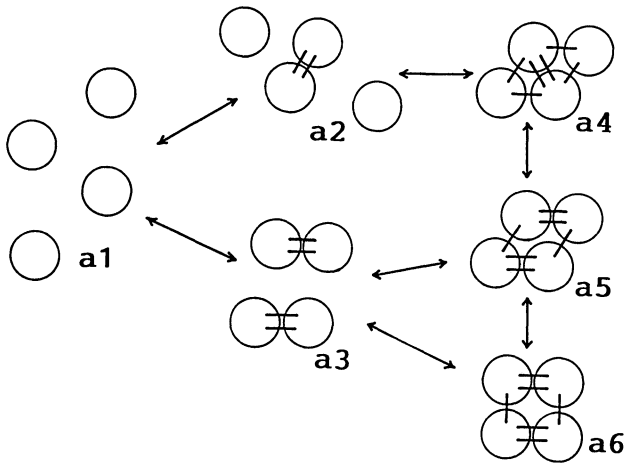

Fig. 8. Probable folded structures and defolding course. An example of 4-helix bundle is illustrated. Each circle represents α-helix as an axial view. Loose and tight contacts are represented with single (-) and double (=) line segments, respectively.

Another interaction brings about close contact of helices like coiled-coil structures.

Defolding Model of Linked Helices. In summarizing the results of denaturation experiments we can assume preliminary model for defolding as drawn in Fig. 8. It is considered that the association of helices is constructed with both loose and tight contacts. Most of P2 molecules take a folded structure with a tight contact, whereas some helices of the other peptides make a loose contact.

The experimental results of GuHCl denaturation are interpreted as follows. As described in the previous section, P2 takes an associated form such as $(P2)_2$. Most of P2 molecules take structures of **a5** or **a6** and turn to **a3** through the first denaturation point at 2—3 M GuHCl. In this course the helices are not subjected to deformation. On the other hand, not a minor molecules of P4x and P4w seem to take structures of **a4**. Through the first denaturation point they turn to **a2** and two helices among four undergo a denaturation, so that the total helix content decreases. The second denaturation point at about 6 M GuHCl is assumed to be related to a destruction of tight contact of helices like **a3** which results in entire transition to random coils. The denaturation of P3 and P6 is explained similarly to P4x and P4w but the number of associated helices could not be determined by only these experiments. It is interesting that the fractions corresponding to the first transition of P3 and P6 are a little larger than those of P4x and P4w. P3 and P6 may have more complicated association forms with loose contact.

The denaturation in basic solution can be explained by a similar consideration. As shown in Fig. 6, $[\theta]_{222}$ of P2 does not change largely in comparison with the others in a condition from neutral to basic. This course is assumed to be a denaturation of a loose contact. As most of P2 is related to a tight contact, the change of $[\theta]_{222}$ seems to result in a small change.

Richmond[33] gave the geometrical data for a packing of helices, that is, the inter-axial distance between helices is 1.05 nm and the outermost surface of leucine residue is 0.74 nm apart from a helix axis. These data lead to a consideration that on the average almost one residue contained in a winding pitch of α-helix contributes to hydrophobic interaction between two helices. As our helices contain -Leu- and -Leu-Leu- alternately in winding pitches, one of serial Leu residues may participate in a loose contact of helices.

It is interesting that our peptide P4w and peptide α4 designed by DeGrado et al.[1] show different GuHCl denaturation curves from each other nevertheless both are made of 4 helical parts. This means that the linkage parts play a significant role to array helices. The flexible mobility of our linkage parts results in two steps of denaturation. The design of linkage parts will be important for specific functions of artificial proteins.

References

1) W. F. DeGrado, Z. R. Wasserman, and J. D. Lear, *Science*, **243**, 622 (1989).
2) M. Mutter and S. Vuilleumier, *Angew. Chem., Int. Ed. Engl.*, **28**, 535 (1989).
3) F. R. Carbone and S. J. Leach, *Int. J. Pept. Protein Res.*, **26**, 225 (1985).
4) E. T. Kaiser and F. J. Kézdy, *Science*, **223**, 249 (1984).
5) R. Moser, S. Frey, K. Münger, T. Hehlgans, S. Klauser, H. Langen, E.-L. Winnacker, R. Mertz, and B. Gutte, *Protein Eng.*, **1**, 339 (1987).
6) F. H. C. Crick, *Acta Crystallogr.*, **6**, 689 (1953).
7) P. C. Weber and F. R. Salemme, *Nature*, **287**, 82 (1980).
8) N. Nishino, Y. Sugita, T. Yamamoto, and T. Fujimoto, *Rep. Asahi Glass Found. Ind. Technol.*, **48**, 149 (1986).
9) S. P. Ho and W. F. DeGrado, *J. Am. Chem. Soc.*, **109**, 6751 (1987).
10) L. Regan and W. F. DeGrado, *Science*, **241**, 976 (1988).
11) M. Mutter and G. Tuchscherer, *Makromol. Chem. Rapid Commun.*, **9**, 437 (1988).
12) IUPAC-IUB Comm. Biochem. Nomencl., *Arch. Biochem. Biophys.*, **125**, i—v (1968).
13) A. R. Mitchell, S. B. H. Kent, M. Engelhard, and R. B. Merrifield, *J. Org. Chem.*, **43**, 2845 (1978).
14) N. Ikota, T. Shioiri, S. Yamada, and S. Tachibana, *Chem. Pharm. Bull.*, **28**, 3347 (1980).
15) B. F. Gisin and R. B. Merrifield, *J. Am. Chem. Soc.*, **94**, 3102 (1972).
16) D. F. Veber, J. D. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **94**, 5456 (1972).
17) H. Morii and K. Ichimura, *Bull. Chem. Soc. Jpn.*, **62**, 2730 (1989).
18) J. P. Tam, W. F. Heath, and R. B. Merrifield, *J. Am. Chem. Soc.*, **108**, 5242 (1986).
19) M. J. F. Diez, D. T. Osuga, and R. E. Feenay, *Arch. Biochem. Biophys.*, **107**, 449 (1964).
20) D. R. Grassetti and J. F. Murray, *Arch. Biochem. Biophys.*, **119**, 41 (1967).
21) A. Tsugita and J.-J. Scheffler, *Proc. Jpn. Acad., Ser. B*, **58**, 1 (1982).
22) M. Sundaralingam, Y. C. Sekharudu, N. Yathindra, and V. Ravichandran, *Proteins*, **2**, 64 (1987).
23) P. J. Flory, "Statistical Mechanics of Chain Molecules," John Wiley & Sons, New York (1969), Chap. 7.
24) I. A. Bolotina, V. O. Chekhov, and V. Y. Lugauskas, *Int. J. Quantum Chem.*, **16**, 819 (1979).
25) P. K. Sarkar and P. Doty, *Proc. Natl. Acad. Sci. U.S.A.*, **55**, 981 (1966).
26) Y.-H. Chen, J. T. Yang, and K. H. Chau, *Biochemistry*, **13**, 3350 (1974).
27) K. Hamaguchi and H. Takesada, "Tanpakushitu No Senkousei," Gakkai Syuppan Center, Tokyo (1971), Chap. 3.
28) P. Y. Chou and G. D. Fasman, *Biochemistry*, **13**, 222 (1974).
29) S. W. Provencher and J. Glockner, *Biochemistry*, **20**, 33 (1981).
30) R. S. Hodges, A. K. Saund, P. C. S. Chong, S. A. St.-Oierre, and R. E. Reid, *J. Biol. Chem.*, **10**, 1214 (1981).
31) A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Am. Chem. Soc.*, **90**, 4736 (1968).
32) D. Eisenberg and A. D. McLachlan, *Nature*, **319**, 199 (1986).
33) T. J. Richmond and F. M. Richards, *J. Mol. Biol.*, **119**, 537 (1978).