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The (i, $i+4$) Phe–His Interaction Studied in an Alanine-based α-Helix

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Two models have been considered for the helix-stabilizing Phe–His$^+$ interaction in C-peptide: (1) the H-bond model in which His$^+$ acts as an H-bond donor and the aromatic ring of Phe acts as an acceptor, and (2) a helix dipole model, in which Phe constrains His so that there is a stronger interaction between His$^+$ and the helix dipole. To decide between these models, we compared the effect on helix stability of the Phe–His interaction near the middle *versus* close to the C terminus of an alanine-based peptide. The nature of the interaction is the same at either position, in agreement with the H-bond model. The results show further that a weak helix-stabilizing Phe–His interaction can be detected when His is uncharged. Replacement of Phe by the saturated analog Cha (β-cyclohexylalanine) gives no interaction, as predicted by the H-bond model.

Keywords: Phe–His interaction; Cyclohexylalanine; α-helix stability; amino–aromatic interaction

1. Introduction

The interaction of Phe8 with His12 accounts in part for the unexpected stability of the C-peptide helix (the N-terminal 13 residues of RNase A) (Dadlez *et al.*, 1988; Shoemaker *et al.*, 1990). It produces a distinctive pH dependence of the helix content, causing a large drop in helix content as the pH is raised above 5·3. Since its discovery, the mechanism by which the Phe–His interaction stabilizes the helix has been of interest. It seems likely that the "helix macrodipole" is involved in this interaction since the histidine is near the C terminus of the peptide and the C-peptide helix is most stable when the histidine is positively charged (His$^+$) (Shoemaker *et al.*, 1990). In the crystal structure of RNase A, the aromatic rings of His and Phe are very close together in an edge-to-face arrangement. This suggests that the Phe–His interaction is an aromatic–aromatic interaction in which helix stability is enhanced by the interaction of the slightly negative aromatic carbon atoms in the face of the Phe ring with the slightly positive aromatic hydrogen atoms on the edge of the His ring (Burley & Petsko, 1985). An amino–aromatic interaction (Burley & Petsko, 1986; Levitt & Perutz, 1988) is also consistent with the spatial arrangement of the Phe and His side-chains in the crystal structure of RNase A. In this model, an amino or amide hydrogen atom interacts with the negative charge density of the Phe ring. This type of interaction is expected to be stronger at low pH when the His side-chain is charged; the charge resides on both imidazolium nitrogen atoms, making them more electronegative (Wilbur & Allerhand, 1977). Because the amino–aromatic interaction has been compared by analogy to H-bond formation (Levitt & Perutz, 1988), we refer to it as the H-bond model. The interaction of Phe and His$^+$ could combine features of both the aromatic–aromatic and amino–aromatic models, and might be expected to yield a particularly strong interaction. An alternative model is a helix dipole model in which Phe forces His$^+$ closer to the C terminus of the peptide by steric constraint, thus causing an increase in the strength of the interaction of His$^+$ with the helix macrodipole. At present, the mechanism by which the Phe–His interaction contributes to helix stability is not known.

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The Phe–His interaction in C-peptide is characterized by a large drop in helix content when His^+ is titrated to His^0, about three times larger than can be accounted for by the His^+-dipole interaction observed in the absence of Phe8. The interaction is not screened significantly in 3 M-NaCl, unlike the His^+-dipole interaction. Residue swap experiments in which Ala11 and His12 are switched (giving F8H11A12) suggest that Phe interacts with His^+ and not with His^0, and only when Phe and His are spaced $(i, i+4)$ (Shoemaker *et al.*, 1990). Nuclear magnetic resonance studies show that the Phe8 side-chain protons have pH-dependent chemical shifts with a pK_a consistent with the titration of His12. This indicates that the two side-chain rings are close together in space, and is evidence for a direct side-chain–side-chain interaction (Shoemaker *et al.*, 1990; Dadlez *et al.*, 1988).

Studies of the Phe–His interaction in C-peptide are limited by the complex sequence of this peptide. Experimental design is difficult since there are limited options for moving or replacing residues without making interpretation of the results impossible. In addition, C-peptide is not a good model α-helix, since it has a kink near Thr3 (Osterhout *et al.*, 1989).

Side-chain interactions can be studied with few complicating effects in simple alanine-based helices. These peptides have been used to study Glu–Lys ion pairs (Marqusee & Baldwin, 1987) and to demonstrate the high helix propensity of alanine (Marqusee *et al.*, 1989). The effect of placing a helix-destabilizing residue (Gly) at various positions in the helix has been determined (Chakrabartty *et al.*, 1991). Here we use an AAKAA repeating-sequence peptide to investigate the helix-stabilizing interaction of Phe and His. Circular dichroism (CD†) is used to measure the changes in helix content caused by placing the Phe–His pair either in the center of the peptide or at the C-terminal end. To determine the role of the His^+-dipole interaction in the pH dependence of the helix content, we replaced Phe with Ala to make peptides with only His at the same position.

To determine whether the interaction requires an aromatic ring, which has an accessible region of negative charge in its center (Fig. 1), we replaced Phe with non-aromatic β-cyclohexylalanine (Cha) which does not have an accessible region of negative charge. To interpret the helix contents of the various peptides, we determined the relative helix propensities of Ala, Phe and Cha.

2. Materials and methods

Peptides were synthesized and purified as previously described (Padmanabhan & Baldwin, 1991). F-moc-β-cyclohexyl-L-alanine was obtained from Novabiochem and was activated by BOP (benzotriazolyl-tris (dimethylamino)phosphonium hexafluoro-phosphate) and HOBT (1-hydroxybenzotriazole). Peptide identity was checked by Fab mass spectrometry. Concentration of peptide stocks was determined by tyrosine absorbance at 275 nm, as described by Brandts & Kaplan (1973). All CD measurements were made using an AVIV 60DS spectropolarimeter at 0°C. CD samples contained 20 mM-peptide. Single wavelength measurements were done in 1 mM each of sodium citrate, sodium phosphate and sodium borate, with NaCl concentrations of either 10 mM or 1 M. The pH was adjusted using HCl or NaOH. CD spectra were measured in 100 mM-KF, 1 mM-K_2HPO_4, (pH 7).

pH titrations were fitted to the Henderson–Hasselbalch equation using 2 pK_a values, one pK_a for the His and 1 for the single Tyr, plus the 3 Lys residues, since their titrations are not resolved.

Molecular graphics analysis was performed using BIOGRAF version 3.0 (provided by Molecular Simulations, Inc.) on a Silicon Graphics Iris 4D/20.

3. Results

(a) *Peptide design*

The series of 17-residue peptides used in this study (Table 1) is based on a sequence designed by Marqusee *et al.* (1989). Each peptide contains three repeats of the sequence AAKAA, with one tyrosine residue added for concentration determination. The three lysine residues are added for solubilization in water. The mean residue ellipticity of the reference peptide is pH-independent below pH 8, but increases sharply above pH 8 due to titration of the tyrosine and three lysine residues (Fig. 2). The reference peptide has a high helix content because of the high helix propensity of Ala (Marqusee *et al.*, 1989), but introduction of Phe or His into the sequence drastically lowers the helix content because of the low helix propensities of these residues (Shoemaker *et al.*, 1990; Padmanabhan *et al.*, 1990; Table 2).

The peptide F12H16 is designed to have the Phe–His interaction in the same position and spacing as in C-peptide: the His is one residue back from the C terminus of the peptide and the Phe, His spacing is $(i, i+4)$. For comparison, we made a peptide with $(i, i+5)$ spacing of the Phe and His (F11H16), where direct side-chain interaction between Phe and His is unlikely. We moved the Phe from position 12 to 11 in order to avoid moving the

Table 1
Peptide sequences

AKRef	Ac-YAAKAAAAKAA AAKAA A-(NH_2)
F12H16	Ac-YAAKAAAAKAA **F**AKA**H**A-(NH_2)
F11H16	Ac-YAAKAAAAK**F** AAKA**H**A-(NH2)
H16	Ac-YAAKAAAAKAA AAKA**H**A-(NH2)
F7H11	Ac-YAAKAA**F**AKA**H**AAKAA A-(NH2)
F6H11	Ac-YAAKA**F**AAKA**H**AAKAA A-(NH2)
H11	Ac-YAAKAAAAKA**H**AAKAA A-(NH2)
F12	Ac-YAAKAAAAKAA **F**AKAA A-(NH2)
Cha 12	Ac-YAAKAAAAKAA **Cha** AKAA A-(NH2)
Cha12H16	Ac-YAAKAAAAKAA **Cha** AKA**H**A-(NH2)
Cha11H16	Ac-YAAKAAAAK**Cha** AAKA**H**A-(NH2)

† Abbreviations used: CD, circular dichroism; Cha, β-cyclohexylalanine; *s* (as in *s*-value), helix propagation parameter of helix-coil theory.

A, alanine; **F**, phenylalanine; **H**, histidine; **Cha**, β-cyclohexyl alanine; Ac-, indicates that the N terminus is acetylated; –(NH_2) indicates that the C terminus is amidated.

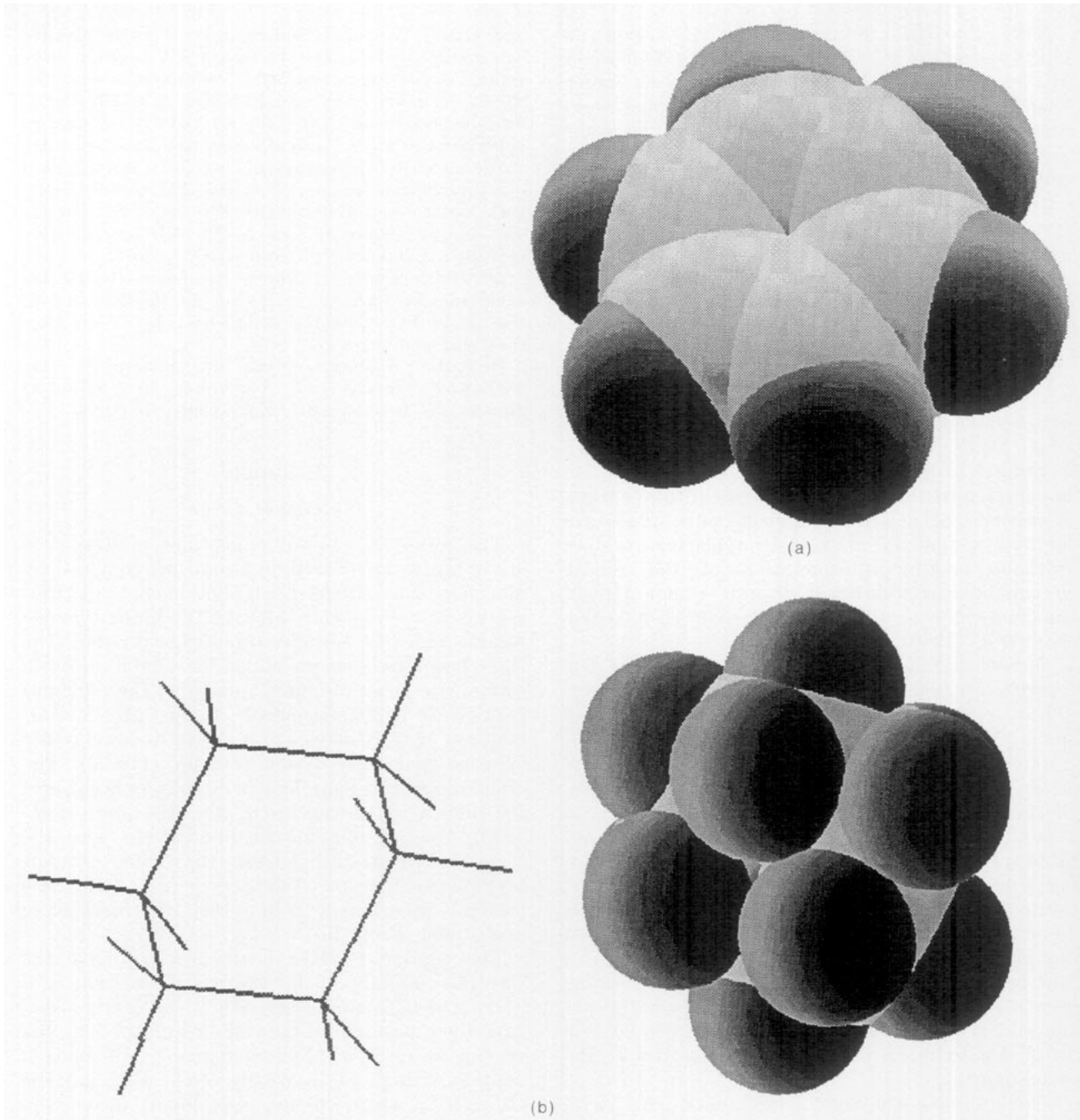

Figure 1. Space filling models of (a) benzene and (b) cyclohexane. The grey area represents carbon atoms, hydrogen atoms are shown in black. The stick figure in (b) gives the orientation of the cyclohexane ring. The chair form is shown; the space filling model of the boat form looks substantially the same as the chair. Models are not to scale.

His residue, which would have numerous effects on the helix content, including, but not limited to, changes in the strength of the His^+-helix dipole interaction and changes in interaction of His^+ with the Lys+ side-chains.

(b) *Helix formation measured by CD*

CD spectra of AKRef, Cha11H16, F7H11, and F11H16 have the double minima at 208 and 222 nm characteristic of an α-helix. The mean residue ellipticity is independent of peptide concentration (5 to 80 μm) for all peptides checked: F7H11 measured in 10 mM-NaCl (pH 9·05), AKRef in 10 mM-NaCl (pH 3·70) and also in no salt (pH 11·97), and Cha12H16 in 1 M-NaCl (pH 8·20). These results suggest that the helix contents of these peptides are not affected by association in the conditions studied.

(c) *F12H16 behaves similarly to C-peptide*

The pH dependences of the helix contents of peptides F12H16, F11H16 and H16 in 10 mM-NaCl

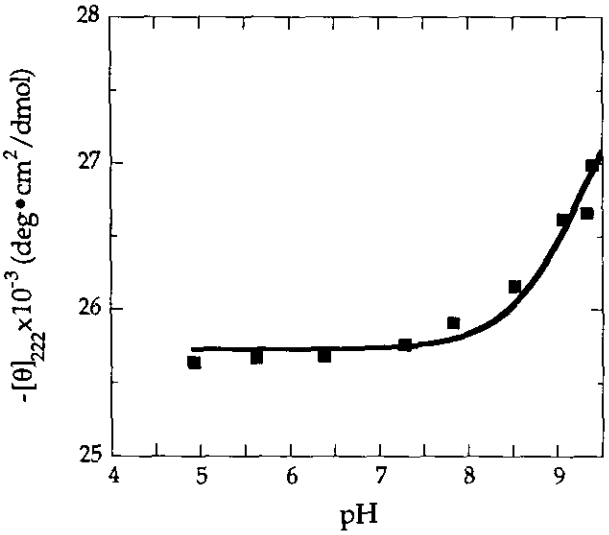

Figure 2. Mean residue ellipticity *versus* pH for the reference peptide in 10 mM-NaCl at 0°C. Continuous line represents the data fitted to the Henderson–Hasselbalch equation using 1 pK_a value for the titration of the 3 lysine and one tyrosine residues.

and in 1 M-NaCl are shown in Figure 3. F12H16 is much more helical than F11H16, especially at low pH; Phe and His spaced (i, $i+4$) stabilize the helix. For F12H16, as the His titrates from pH 5 to pH 8, the helix content dramatically decreases, reminiscent of C-peptide (Bierzynski *et al.*, 1982). Above pH 8·5 where histidine is completely uncharged, there is still some stabilization of the helix when Phe and His are spaced (i, $i+4$), but it is substantially weaker than at pH 5. Subtraction of the curve for F11H16 from F12H16 (see Fig. 5(a)) shows the

Table 2

Relative helix forming tendencies of Ala, Cha and Phe (measured in 10 mM-NaCl at 0°C (pH 7))

Peptide	$-[\theta]_{222}$ (deg·cm^2/dmol) × 10^{-3} ± S.D.
AKRef	25·7 ± 0·4
Cha12	25·6 ± 0·6
Phe12	18·2 ± 0·7

pH dependence of the strength of the interaction of (i, $i+4$) Phe–His relative to the (i, $i+5$) spacing (see Discussion, below).

The pH curve for H16 shows the effect on helix content of titrating His16. When His16 is deprotonated, the stabilizing interaction between His^+16 and the helix dipole is lost. In addition, the helix propensity of His^+ may be different from the helix propensity of uncharged His^0. These two effects are present in all peptides containing His16.

The interaction of His^+16 with the helix dipole is screened slightly by 1 M-NaCl, as demonstrated by the relative sizes of the change in helix content on titrating His^+ to His^0 for peptide H16 in 10 mM and 1 M-NaCl. The salt screening experiment shows that the Phe–His interaction is not significantly screened by NaCl, the same result as in C-peptide (Shoemaker *et al.*, 1990). The difference curves for F12H16–F11H16 in 10 mM and 1 M-NaCl are identical (data not shown).

(d) *Interaction does not depend on His being close to the C terminus*

To determine whether the Phe–His interaction requires proximity of His to the C terminus of the

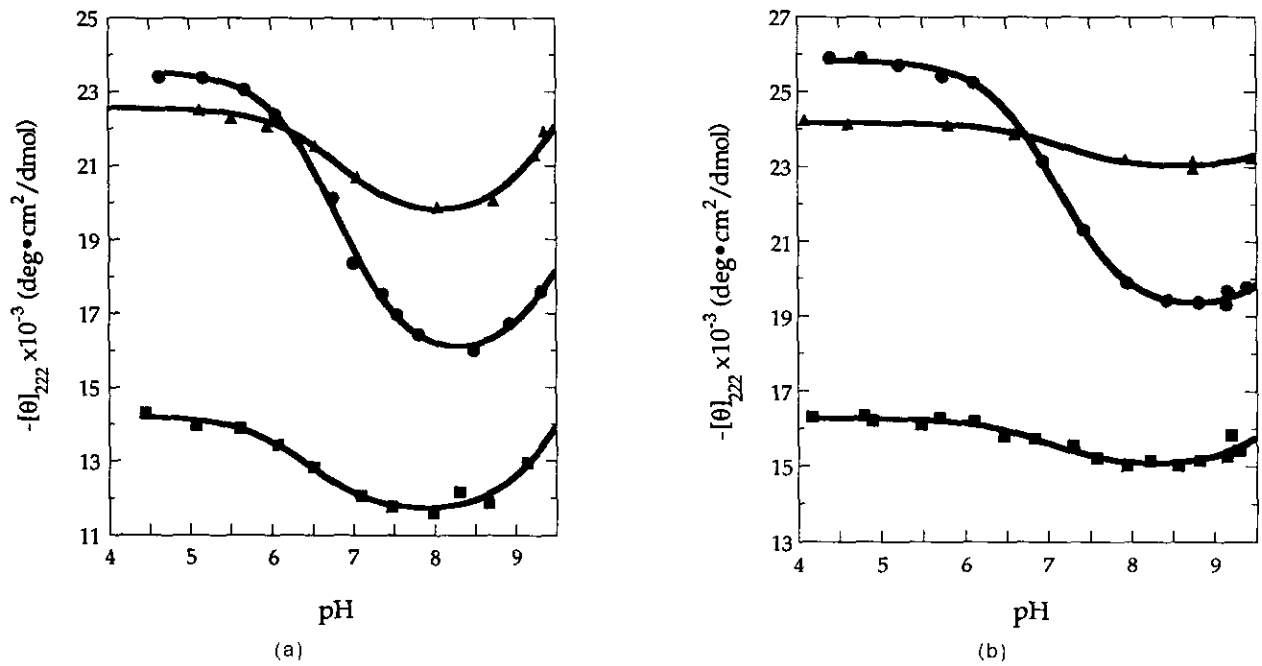

Figure 3. pH dependence of the mean residue ellipticity of peptides F12H16 (●), F11H16 (■) and H16 (▲) (a) in 10 mM-NaCl, (b) in 1 M-NaCl at 0°C.

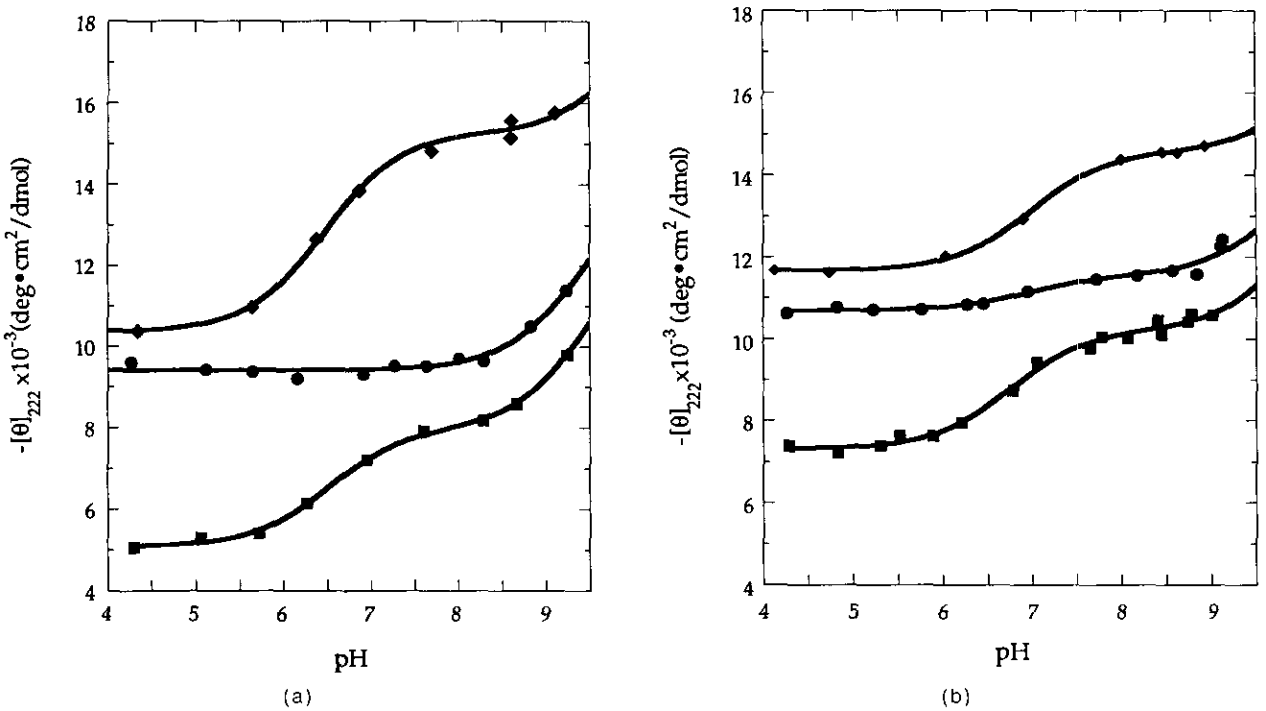

Figure 4. F7H11 (●) F6H11 (■) and H11 (◆) (a) in 10 mM-NaCl, (b) in 1 M-NaCl at 0°C.

helix, helicity was measured as a function of pH for peptides F6H11, F7H11 and H11 (Fig. 4). These peptides were designed using the same principles as for the F11H16–F12H16 pair. The existence of the interaction does not depend on its location in the helix: F7H11 is more helical than F6H11 at every pH. Subtraction of the curve for F6H11 from F7H11 yields a curve that is qualitatively similar to the difference curve for the interaction at the C terminus in that the interaction is more stabilizing at low pH than at high pH (Fig. 5(a)). The difference curves for F7H11–F6H11 in 10 mM and

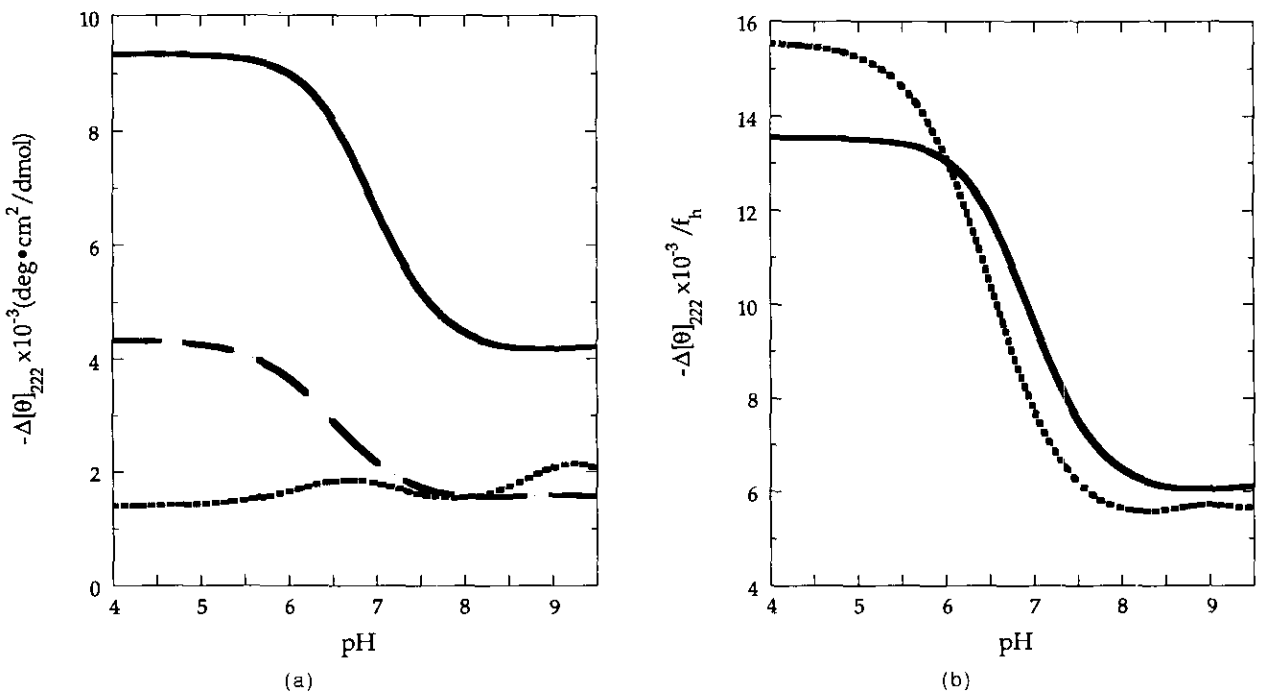

Figure 5. (a) Differences in helix content *versus* pH for peptides spaced (i, $i+4$) and (i, $i+5$) in 10 mM-NaCl. F12H16−F11H16 (———), F7H11−F6H11 (— — —), Cha12h16−Cha11H16 (- - - -). The curves were generated by subtracting the curvefit lines for the (i, $i+5$) peptides from the lines for the (i, $i+4$) peptides. (b) Difference curves normalized for the helix content of the (i, $i+4$) peptide at low pH show that the strength of the Phe–His interaction is comparable at the C-terminus and in the middle of the helix. Dotted line is (F7H11–F6H11)/f_h, solid line is (F12H16-F11H16)/f_h. f_h is the fraction helix measured by CD.

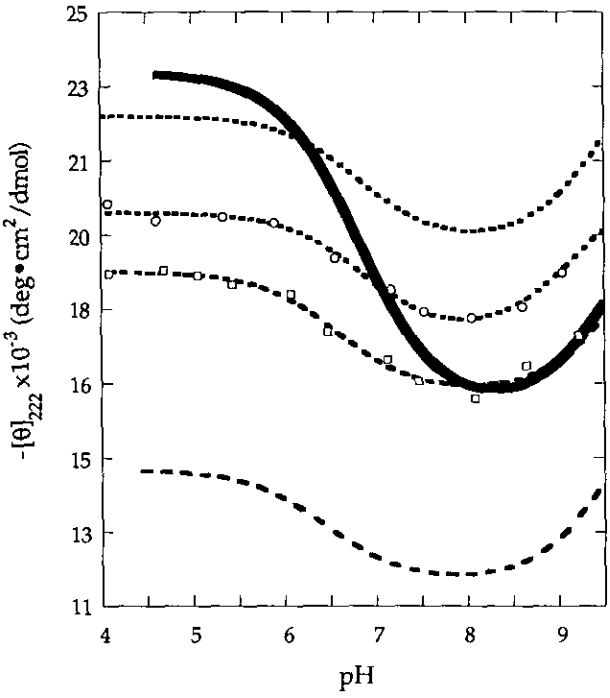

Figure 6. Dependence of helix content on pH for peptides Cha12H16 (– –○– –) and Cha11H16 (– –□– –) in 10 mM-NaCl. Curve-fitted lines for H16 (- - - -) F11H16 (– – –) and F12H16 are reproduced for comparison.

1 M-NaCl are the same within experimental error (data not shown), further evidence that the Phe–His interaction is not screened by salt.

(e) *Studies with cyclohexylalanine*

To determine whether or not the Phe–His interaction depends on the aromaticity of the phenylalanine side-chain, we replaced it with the non-aromatic analog cyclohexylalanine (Cha: side-chain $CH_2(C_6H_{11})$. The pH curves for Cha11H16 and Cha12H16 and the curve-fitted lines for H16, F12H16 and F11H16 are shown in Figure 6. Curves for H16, F11H16, Cha11H16 and Cha12H16 are practically parallel, but the curve for F12H16 is strikingly different. These results indicate that His16 interacts in a pH-dependent manner with Phe12, but not with Phe11, Cha12 or Cha11. Subtraction of the curve for Cha11H16 from Cha12H16 yields a nearly horizontal line over the pH region in which histidine titrates (Fig. 5(a)). The similarity between the pH dependences of the helix contents for Cha11H16 and Cha12H16 shows that Cha is not able to promote helix stability in the same manner as Phe.

In order to interpret more clearly the results of Phe to Cha substitutions, it is necessary to know the relative helix propensities of Phe and Cha. The mean residue ellipticities of peptides Phe12 and Cha12 and the reference peptide are given in Table 2. Phe is quite helix-destabilizing but Cha is about as helix-stabilizing as Ala.

4. Discussion

(a) *Properties of the Phe–His interaction*

The Phe–His interaction, defined by enhanced helix stability in the presence of Phe and His residues spaced (i, $i+4$), is present in an alanine-based peptide and depends only on the correct spacing of Phe and His. The presence of the interaction is independent of its position in the helix. The Phe and His side-chains interact whether His is charged or not, although the interaction is about two times stronger with His^+ than with His^0. Helix stabilization by the Phe–His^0 interaction indicates that the interaction is an aromatic interaction. Further evidence comes from peptides that have Phe replaced with Cha: Cha12H16 does not have enhanced helix stability compared to Cha11H16. The small difference between the helix contents of Cha12H16 and Cha11H16 is a position effect caused by fraying of the ends of the helix (Chakrabartty *et al.*, 1991).

(b) *Factors affecting the change in helix content with pH*

Three factors determine the change in helix content with pH for all of the peptides in this study: the interaction of His^+ with the helix dipole, the difference in helix propensity between His^+ and His^0, and the Phe–His interaction determine the shapes of the pH curves. All the pH curves for peptides containing His16 have the same basic shape (Fig. 6), which indicates that the helix is more stable when His16 is charged. All the pH curves for peptides containing His11 (Fig. 4) also have the same basic shape (different from the shape for the His16 peptides) but they show an increase in helix content as $His^+ \rightarrow His^0$, which indicates that His^+11 is helix-destabilizing relative to $His11^0$. This is a consequence of a less favorable His^+-helix dipole interaction in His^+11 than in His^+16.

The overall helix content is affected by the helix propensities of the substituted residues and their positions (Chakrabartty *et al.*, 1991). The peptides containing His11 are much less stable than the peptides with His16 both for the reason discussed above and because His is a helix-destabilizing residue and position 11 is closer to the center of the helix than position 16. The Lifson–Roig theory for the helix-coil transition (Lifson & Roig, 1961) predicts that helices are frayed at the ends (see Rohl *et al.*, 1992) and, as a result, helix-destabilizing residues have a lesser destabilizing effect near the ends of the helix than in the middle (Chakrabartty *et al.*, 1991). Within the set of peptides containing His16, the overall helix content is determined by whether Phe, Ala or Cha is located at position 11 or 12 (Fig. 6, Table 2). The different helix propensities of these residues cause a pH-independent change in the mean residue ellipticity. In Figure 6, the shapes of the curves for H16, F11H16, Cha12H16 and Cha11H16 are all the same. H16 is the most helical

of these peptides because it contains Ala at positions 11 and 12. Cha12H16 and Cha11H16 are next because cyclohexylalanine appears to be mildly helix-destabilizing relative to Ala, and F11H16 is the least stable because Phe is highly helix-destabilizing relative to Ala. The radically different shape of F12H16 compared to F11H16, H16, Cha12H16 and Cha11H16 indicates that Phe12 interacts with His16 whereas Cha12, Cha11, Phe11, Ala11 and Ala12 do not. This difference in behavior demonstrates a pH-dependent interaction between Phe and His when they are spaced $(i, i+4)$.

The Phe–His^+ interaction causes a change in $[\theta]_{222}$ that is twice as large when the Phe–His pair is at the C terminus as when it is in the middle of the helix. One reason is that in F7H11, the helix content is much lower than in F12H16 because the two helix-destabilizing residues are near the center of the helix. This causes the change in helix content resulting from protonation of His11 to be smaller. Figure 5(b) shows what the change in helix content of F12H16 and F7H11 would be if normalized for the lower helix content. This is done only for illustration: there is no simple way of comparing accurately the changes seen in F7H11 and F12H16.

(c) *Control peptides*

In the design of the control peptides, F11H16 and F6H11, His was not moved to avoid changing the strength of the His^+-dipole interaction. Subtracting the curve for F11H16 from the curve for F12H16 should effectively cancel both the helix dipole effect and the effect of different s-values for charged and uncharged His, leaving only the contribution from the Phe–His interaction. Although this analysis is not exact because of the different helix contents of the $(i, i+4)$ peptides and the $(i, i+5)$ peptides, it gives a qualitative measure of the pH dependence of the strength of the Phe–His interaction.

The lower helix content of F6H11 relative to F7H11 must result from moving Phe from 7 to 6 (the only difference between these 2 peptides). Moving Phe (a helix-destabilizing residue) farther from the center of the peptide will stabilize the helix in a pH-independent manner. This cannot explain the difference between the curves for F6H11 and F7H11 for two reasons: the helix fraying effect should be quite small at these positions, and F7H11 is more helical than F6H11, opposite to the result expected for the helix fraying effect alone. Therefore, the lower helix content of F6H11 relative to F7H11 must be caused by loss of the helix-stabilizing Phe–His interaction.

(d) *Mechanism of the Phe–His interaction*

All our results are consistent with the H-bond model of Burley & Petsko (1986) and of Levitt & Perutz (1988), and these same results make the helix dipole model unlikely. (1) There is a significant interaction between uncharged His and Phe; this interaction is consistent with the H-bond model but is not predicted by the helix dipole model. (2) The nature of the Phe–His^+ interaction is the same in the middle of the helix as it is close to the C terminus. The helix dipole model predicts a very weak interaction near the middle of the helix, but by normalizing for the helix content, we show that the strength of the Phe–His interaction is comparable in the middle of the helix and at the C terminus. (3) Replacing Phe by the bulky Cha group does not yield a helix-stabilizing His–Cha interaction. If His^+ is constrained to make a stronger interaction with the helix dipole solely by a steric constraint from Phe, Cha might be expected also to have a strong effect on the interaction of His^+ with the helix dipole.

An interaction similar to the Phe–His interaction was discovered recently in barnase (Loewenthal *et al.*, 1992) where His18 stabilizes the protein *via* a tertiary interaction with Trp94. The His–Trp interaction is similar to the Phe–His interaction described here in that it stabilizes the protein more at low pH than at high pH and is not screened by salt. Loewenthal *et al.* (1992) find that when Trp94 is replaced by Phe or Tyr, the Tyr–His interaction is more stabilizing than the Phe–His interaction, in agreement with the findings in C-peptide (Shoemaker *et al.*, 1990).

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