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Further Studies of the Helix Dipole Model: Effects of a Free α-NH_3^+ or α-COO^- Group on Helix Stability

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ABSTRACT **Interactions between the α-helix peptide dipoles and charged groups close to the ends of the helix were found to be an important determinant of α-helix stability in a previous study.[1] The charge on the N-terminal residue of the C-peptide from ribonuclease A was varied chiefly by changing the α-NH_2 blocking group, and the correlation of helix stability with N-terminal charge was demonstrated. An alternative explanation for some of those results is that the succinyl and acetyl blocking groups stabilize the helix by hydrogen bonding to an unsatisfied main-chain NH group. The helix dipole model is tested here with peptides that contain either a free α-NH_3^+ or α-COO^- group, and no other charged groups that would titrate with similar pK_a's. This model predicts that α-NH_3^+ and α-COO^- groups are helix-destabilizing and that the destabilizing interactions are electrostatic in origin. The hydrogen bonding model predicts that α-NH_3^+ and α-COO^- groups are not themselves helix-destabilizing, but that an acetyl or amide blocking group at the N- or C-terminus, respectively, stabilizes the helix by hydrogen bonding to an unsatisfied main-chain NH or CO group.**

The results are as follows: (1) Removal of the charge from α-NH_3^+ and α-COO^- groups by pH titration stabilizes an α-helix. (2) The increase in helix stability on pH titration of these groups is close to the increase produced by adding an acetyl or amide blocking group. (3) The helix-stabilizing effect of removing the charge from α-NH_3^+ and α-COO^- groups by pH titration is screened by increasing the NaCl concentration, and therefore the effect is electrostatic in origin. (4) Replacing the C-terminal amide blocking group with a methyl-ester blocking group, which cannot donate a hydrogen bond, causes little change in helix stability.

Key words: helix stabilization, helix dipole, charged group, pH titration, electrostatic interaction, hydrogen bonding

INTRODUCTION

The importance of dipole interactions in protein structure and function has been known for some time.[2,3] Helices, in particular, have a large macrodipole because the individual peptide dipoles are aligned almost parallel to the helix axis. Four main-chain NH groups at the N-terminal end of the helix are not hydrogen bonded and carry partial positive charges; likewise, four non-hydrogen-bonded CO groups at the C-terminal end carry partial negative charges. Thus, the α-helix resembles an extended line dipole with a positive pole near the N-terminus and a negative pole near the C-terminus.[3–6] The terms "helix dipole," "positive pole," and "negative pole" are used here as abbreviations. Quantitative calculation of the magnitude of interactions involving a helix dipole requires that the partial charges on individual NH or CO groups be considered.

The resultant helix macrodipole serves a variety of uses in proteins. Binding of negatively charged substrates often occurs near the positive pole of a helix macrodipole.[3,4] The high pK_a of a histidine residue in hemoglobin may result from its position close to the C-terminus of a helix.[7] More recent examples of such sidechain-helix dipole interactions have also been given.[8,9] Dipole–dipole interactions between water molecules and antifreeze peptide helices may act to inhibit the growth of ice nuclei.[10] Calculation of interaction energies for two helix macrodipoles indicates that antiparallel pairing of two helices is favored over parallel pairing, and this could be an important energetic factor in determining the backbone fold of a protein.[6]

Short helices themselves may be stabilized by favorable interactions of the peptide dipoles with appropriately charged residues near either end of the α-helix. This explanation[11,12] may account for the asymmetric distribution of charged residues near the ends of α-helices in proteins.[13–15] It has been suggested[16] that the helix-stabilizing or -destabilizing effects of charged residues near the ends of

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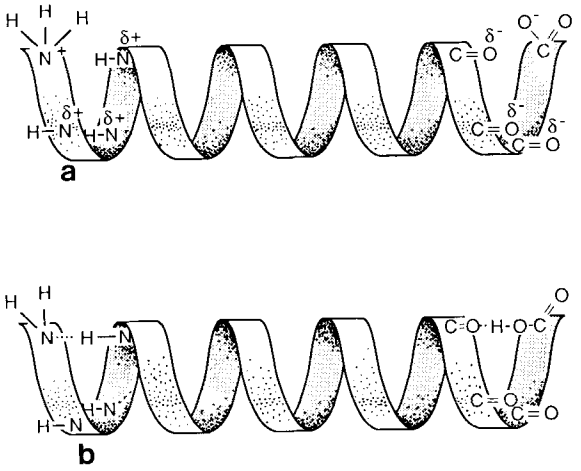

Fig. 1. Helix ribbon diagrams illustrating **a:** the charged group interaction with the peptide dipoles and **b:** the hydrogen bonding interaction between end groups and main-chain peptide groups at the ends of helices. The helix dipole model is represented at neutral pH, illustrating helix-destabilizing interactions, whereas the hydrogen bonding model is represented at an acid pH for the C-terminus and an alkaline pH for the N-terminus illustrating helix-stabilizing interactions.

helices can be taken into account by modifying the values of s (the helix growth parameter of the Zimm–Bragg model) in a position-dependent manner. A formalism for extending the Zimm–Bragg theory to include charged group–helix dipole interactions, and also other specific sidechain interactions, has been given.[17]

Charged residues near the ends affect the stability of an isolated α-helix in aqueous solution as predicted for their interaction with the peptide dipoles, according to earlier reports from our laboratory.[1,18–20] There is, however, a possible alternative explanation for some of those results, which has been brought into focus by a recent report that negatively charged ligands bind to the N-terminus of an α-helix via hydrogen bonding[21] and by statistical evidence that side chains close to the ends of helices in proteins often hydrogen bond to unsatisfied main-chain NH or CO groups.[22] Hydrogen bonding at helix ends may occur during folding even if the hydrogen bonds in question are not found in the final folded protein.[23] Thus, in the experiments of Shoemaker et al.,[1] the increase in helix content upon blocking the α-NH_3^+ group of Ala-1 may result from hydrogen bonding of the acetyl and succinyl blocking groups to an unsatisfied main-chain NH group.

There are several basic distinctions between the helix dipole and hydrogen bonding explanations. In the helix dipole model, the charge on the α-NH_3^+ group destabilizes the helix through a repulsive electrostatic interaction with the peptide dipoles (Fig. 1a). The α-NH_2 and acetyl groups, lacking a charge, should have no effect on helix stability. In the hydrogen bonding model, the α-NH_3^+ group is not itself helix-destabilizing, but it cannot form a helix-stabilizing hydrogen bond to a main-chain NH group since it is not a hydrogen bond acceptor. The unprotonated α-NH_2 group, however, may now stabilize the helix by hydrogen bonding to a main-chain NH group (-N · · · HN-) (Fig. 1b). Likewise, in this model, the acetyl blocking group makes a similar helix-stabilizing hydrogen bond between two nonionizing groups (-CO · · · HN-).

Our present experiments are designed to determine whether α-NH_3^+ and α-COO^- groups are helix-destabilizing and, if so, whether the responsible interaction is electrostatic in origin. Peptides were synthesized for which any change in helix content upon ionization of α-NH_2 and α-COOH groups could easily be followed. These peptides are analogs of the C-peptide from ribonuclease A so that comparison can be made with earlier work; their amino acid sequences are given in the legends to Figures 2, 4, and 6. The test for whether a given interaction that affects helix content is electrostatic is to determine if the interaction can be screened by mobile salt counterions. The data presented here should also be useful in testing algorithms for calculating the magnitude of electrostatic interactions in proteins or in isolated helices.

MATERIALS AND METHODS

Peptide synthesis and purification procedures are described in Shoemaker et al.,[1,18] All peptides were synthesized on a Biosearch 9500 automatic synthesizer. Peptides with C-terminal amides were synthesized on *p*-methylbenzhydrylamine resin. Peptides RN59 and RN90 were synthesized using hydroxymethyl poly(styrene/1% divinyl benzene) resin. To form the methylester blocked peptide, RN90 was cleaved from the resin by transesterification in methanol–dimethylformamide–triethylamine (25:25:12) at 40–45°C for 5 days giving an insoluble mixture of protected peptide–ester and peptide–resin. After HF cleavage and countercurrent distribution, the ester was further purified and separated from the free carboxylic acid by HPLC on a YMC-PACK D-ODS-5 C_{18} reverse-phase column. Peptide purity was confirmed by HPLC, TLC, and high-voltage paper electrophoresis. Amino acid analysis gave the correct composition and the molecular weight was determined by FAB mass spectroscopy. Peptide–resins were acetylated or succinylated at the N-terminus with acetic anhydride or succinic anhydride, respectively, in dimethyl formamide containing an equivalent of triethylamine. Peptide concentration was determined by the quantitative ninhydrin method.[24] CD methods are described in Shoemaker et al.;[1,18] measurements were made on an AVIV 60DS spectropolarimeter. Helix content was measured by mean residue ellipticity at 222 nm, $[\theta]_{222}$. Apparent pK_a's were determined by fitting the pH titrations of helix content to the Henderson–

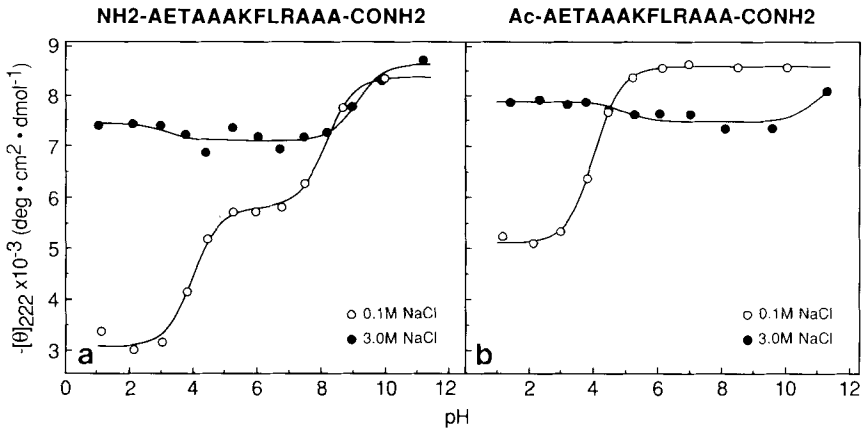

Fig. 2. pH dependence of helix content at 3°C for peptides **a:** RN33 and **b:** RN29 in 0.1 M (○) and 3.0 M (●) NaCl. The sequence of RN33 is AETAAAKFLRAAA-CONH$_2$. RN29 is RN33(Ala 1 → acetyl-Ala). Standard conditions are 20 μM peptide and 1 mM each of Na borate, Na phosphate, and Na citrate. The change in $[\theta]_{222}$ for complete helix formation at 3°C is near 30,000 deg cm^2 dmol^{-1}, when the baseline value for 0% helix is estimated as +3,000 deg cm^2 dmol^{-1}.[32,33] The curves were generated by nonlinear least-squares fit to the Henderson–Hasselbalch equation.

Hasselbalch equation using a program for performing nonlinear least-squares analysis written by David Whitman and modified by us.

RESULTS

Effect of an α-NH_3^+ Group

Shoemaker et al.[1] showed that a free α-NH_3^+ group in a C-peptide analogue is helix-destabilizing relative to an α-amino group blocked by acetylation: this result was interpreted in terms of an unfavorable electrostatic interaction of the positive charge with the positive pole of the helix dipole. Figure 2 shows data that further test the helix dipole interpretation of the destabilizing effect of a free α-NH_3^+ group, by comparing the pH dependence of helix content for peptides with either a free or blocked amino terminus in both 0.1 and 3.0 M NaCl. In 0.1 M NaCl, the pH titration curve for the peptide with a free α-NH_2 terminus (RN33, Fig. 2a) shows that deprotonation of this group (pK_a 8.2) causes a substantial increase in helix content. At pH 10, the helix content of RN33 is nearly equal to that of the peptide with an acetyl blocking group (RN29 in 0.1 M NaCl, Fig. 2b). Thus, pH titration and acetylation both remove the charge on the α-NH_3^+ group and both increase helix stability to a similar extent.

Comparison of the pH titration curves for RN33 in 0.1 and 3.0 M NaCl (Fig. 2a) shows that the helix-destabilizing effect of protonating the α-NH_2 group is substantially reduced in 3.0 M NaCl. RN29 shows no change in helix content between pH 6 and pH 10 in either 0.1 or 3.0 M NaCl (Fig. 2b).

For both peptides, Glu-2 gives rise to the acid limb of the pH titration curve, as reported earlier.[18] Protonation of Glu-2 (pK_a 4.0) lowers the helix content, primarily by weakening a Glu -2^- ··· Arg -10^+ ion pair.[19,25,26] Apparent loss of the acid limb in 3.0 M NaCl indicates significant screening of the Glu-2^- ··· Arg-10^+ interaction (Shoemaker et al., in preparation).

The electrostatic nature of the helix-destabilizing effect of the α-NH_3^+ group is also illustrated by comparing the helix contents of RN33 and RN29 as a function of NaCl concentration (Fig. 3). Comparison of either the pH 5.3 or pH 9.5 results for RN29 in Figure 3b to the pH 9.5 results for RN33 (free α-NH_2 group) in Figure 3a shows that they are all similar. In the range from 0 to 3 M NaCl, there is a slight decrease in helix content. In contrast, when RN33 has a charged amino terminus (pH 5.3, Fig. 3a), the peptide shows a dramatic increase in helix content with increasing NaCl concentration. Helix-destabilizing effects above 3.0 M NaCl are qualitatively similar for all peptides which we have studied (Fairman et al., unpublished results) and are presumably the result of Hofmeister effects.

Effect of an α-COO^- Group

The negative charge on an α-COO^- group in C-peptide is also known to be helix-destabilizing,[27,28] and this effect was recently interpreted in terms of an unfavorable interaction with the helix dipole.[19] We tested this interpretation using peptides that contain either a free C-terminus or an amide or methylester blocking group at the C-terminus. The experiment is identical in design to that in Figures 2 and 3 and the results are shown in Figures 4 and 5.

Figure 4 compares the pH dependence of helix content in 0.1 and 3.0 M NaCl for three peptides: one with a free C-terminus (RN59, Fig. 4a), another with an amide blocking group (RN54, Fig. 4b), and the last with a methylester blocking group (RN90, Fig. 4c). In the 0.1 M NaCl pH profile, RN59 shows an acid limb (pK_a 3.2) caused by titration of the free

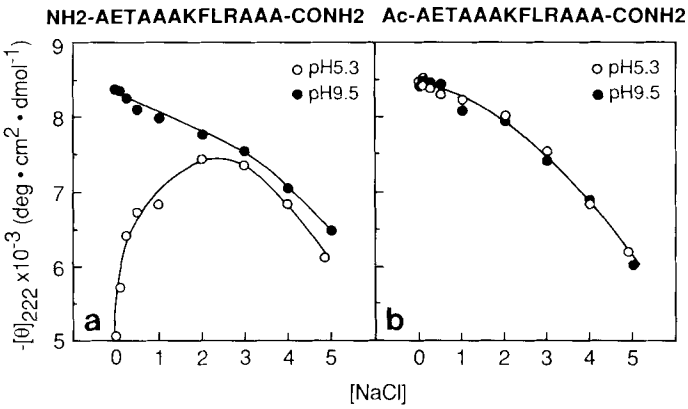

Fig. 3. NaCl dependence of helix content at 3°C for peptides **a:** RN33 and **b:** RN29 at pH 5.3 (○) and pH 9.5 (●).The lines through the data points for all NaCl dependence figures were drawn by inspection.

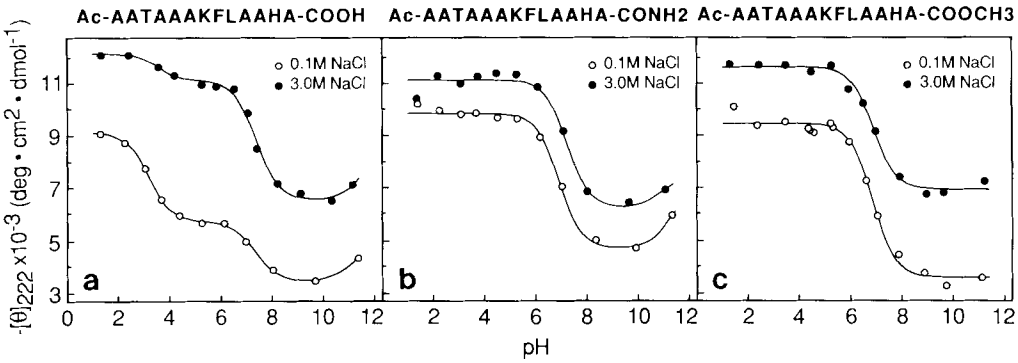

Fig. 4. pH dependence of helix content at 3°C for peptides **a:** RN59, **b:** RN54, and **c:** RN90 in 0.1 M (○) and 3.0 M (●) NaCl. The sequence of RN59 is acetyl-AATAAAKFLAAHA. RN54 is RN59(α-COOH → α-$CONH_2$). RN90 is RN59(α-COOH → α-$COOCH_3$).

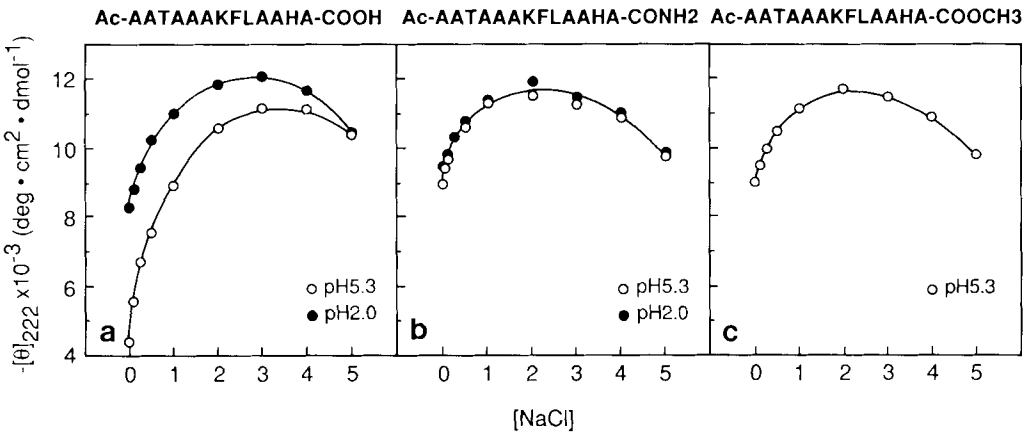

Fig. 5. NaCl dependence of helix content at 3°C for peptides **a:** RN59, **b:** RN54, and **c:** RN90 at pH 5.3 (○) and pH 2.0 (●).

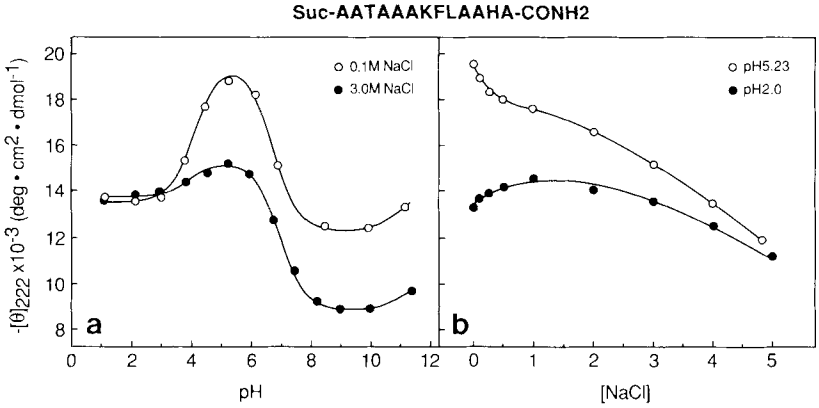

Fig. 6 **a:** pH dependence of helix content at 3°C for peptide RN44 in 0.1 M (○) and 3.0 M (●) NaCl. The sequence of RN44 is succinyl-AATAAAKFLAAHA-$CONH_2$. **b:** NaCl dependence of helix content at 3°C for peptide RN44 at pH 5.3 (○) and pH 2.0 (●).

α-COOH group. The helix content decreases with increasing pH as the α-COOH group ionizes. When this group is blocked with an amide group (RN54, Fig. 4b), the acid limb disappears. At pH 1.5, the helix contents for both RN59 and RN54 are about the same.

In addition, Figure 4a shows the pH titration curve for RN59 in 3.0 M NaCl. The helix-destabilizing effect of deprotonating the α-COOH group is strongly decreased at the higher NaCl concentration. The helix content of RN54 is unaffected between pH 2 and pH 5, both in 0.1 and 3.0 M NaCl (Fig. 4b).

The alkaline limb (pK_a 6.9) in the pH titration curves for both RN59 and RN54 is assigned to His-12.[18] According to substitution analysis,[19] $His\text{-}12^+$ stabilizes the helix by an interaction with Phe-8, as suggested earlier,[29,30] as well as by an interaction with the α-helix peptide dipoles. A further study of the Phe-8 · · · $His\text{-}12^+$ interaction will be described in a separate manuscript (Shoemaker et al., in preparation).

The effect of NaCl is also demonstrated in Figure 5 where the helix contents for RN59 and RN54 are measured as a function of NaCl concentration. When the α-COOH group is blocked (RN54 at pH 2.0 and pH 5.3, Fig. 5b) or uncharged (RN59, pH 2.0, Fig. 5a), the salt dependences are similar. There is a moderate increase in helix content from 0 to 3 M NaCl. In contrast, when the α-COOH group is charged (RN59, pH 5.3, Fig. 5a), there is a substantially larger increase between 0 and 3 M NaCl, again indicating that the helix-destabilizing effect of the $\alpha\text{-}COO^-$ group is electrostatic in origin.

Effect of a Non-Hydrogen-Bonding Blocking Group

The results presented above show the importance of electrostatic interactions for helix stability and thereby support the helix dipole model, but these experiments do not show directly whether or not hydrogen bonds between the blocking groups and main-chain NH or CO groups affect helix stability. This problem is addressed in the comparison of peptides RN54 and RN90 in Figures 4 and 5. Whereas the amide blocking group in RN54 has the potential to form a helix-stabilizing hydrogen bond with a free carbonyl group near the C-terminus (-CO · · · HN-), RN90 has a methylester blocking group which cannot hydrogen bond to a free carbonyl group. The helix contents for RN54 and RN90 are similar in the range from pH 1 to pH 5 in both 0.1 and 3.0 M NaCl (Fig. 4b and c). Moreover, the dependence of helicity on NaCl concentration is quite similar for RN54 and RN90 (Fig. 5c). This result shows that the difference between the NaCl curves for the peptides with a free and an amidated $\alpha\text{-}COO^-$ group (Fig. 5a and b) cannot be ascribed to hydrogen bonding between the NH_2 blocking group and main-chain CO groups, since the same difference is observed for the peptide with a methylester blocking group. The variability in the magnitude of the alkaline limb of the titration curve, which shows the effect of titrating His 12, is unexpected but does not affect the comparison of helix content at pH 2.

Effect of a $Succinyl^-$ Group

In addition to showing that an $\alpha\text{-}NH_3^+$ group is helix-destabilizing relative to an acetyl amino group, Shoemaker et al.,[1] showed that the negative charge on a succinyl blocking group acts to stabilize a helix. Figure 6a shows the pH dependence of helix content for peptide RN44 in 0.1 and 3.0 M NaCl. As the succinyl group ionizes (pK_a 4.2), the helix content increases. This pH dependent helix-stabilizing effect is greatly reduced in 3.0 M NaCl. The effect of NaCl confirms that the helix-stabilizing interaction is chiefly electrostatic and, in this case, NaCl acts to screen a favorable interaction. The NaCl dependence of helix stability for RN44 (Fig. 6b) shows that there is a drop in helix content from 0 to 3 M NaCl

when the succinyl group is charged, although there is an increase in helix content when the blocking group is neutral (cf. the results for RN54 in Figs. 4b and 5b). As in RN54 and RN59, the alkaline limb of the pH titration curves for RN44 is caused by His-12.

DISCUSSION

Interpretation of these results by the helix dipole model is straightforward. Both the α-NH_3^+ and α-COO^- groups are found to be helix-destabilizing, as predicted by the model. Essentially the same increase in stability is found by pH titration as by use of a chemical blocking group. The helix-destabilizing effect of an α-NH_3^+ or α-COO^- group is predominantly electrostatic in origin, as shown by screening the interaction with NaCl. Also as predicted by this model, an N-terminal, negatively charged, succinyl group is helix-stabilizing and exerts its effect by an electrostatic interaction, as shown by screening with NaCl.

The helix-stabilizing effect of blocking an α-COO^- group with an amide group cannot be explained by hydrogen bonding between the amide and a nearby main-chain CO group, because the same increase in helix stability is found with a methylester blocking group, which cannot serve as a hydrogen bond donor. In addition, the electrostatic screening tests (pH titration in 3.0 M NaCl and NaCl dependence) show little difference between these peptides. These results indicate either that the hydrogen bond is not present in the peptide with an amide group or that this hydrogen bond is not sensitive to NaCl concentration. It should be possible to design experiments with model compounds that show whether or not hydrogen bond strength in aqueous solution is strongly dependent on ionic strength.

Both NMR evidence[26] and a study of Ala $\rightarrow$ Gly substitutions[31] in C-peptide indicate that the C-peptide helix terminates at Thr-3 and possibly at His-12, causing residues 1 and 13 to be outside the helix and thus causing the C-terminal α-$CONH_2$ group to be in a conformation unsuitable for hydrogen bonding to a main-chain CO group. Both for this reason and to test the generality of our results, it would be desirable to test, in other helix-forming peptides, whether groups that block an α-NH_3^+ or α-COO^- group can stabilize the helix by hydrogen bonding to main-chain NH or CO groups.

Our data for the decreased helix content of peptides with a free, as compared to a blocked, α-NH_3^+ or α-COO^- group strongly support the helix dipole model. Moreover, the increase in helix stability caused by blocking an α-COO^- group with a methylester group cannot be explained by the hydrogen bonding model. Recent evidence from Brian Matthews and co-workers supports these results.[9] They have shown that an aspartate sidechain can affect the thermal stability of T4 lysozyme by interacting with a nearby helix dipole and without the formation of a hydrogen bond. Although our data strongly support the helix dipole model, both in general and in the C-peptide system in particular, they do not rule out the possibility that polar side chains at the ends of other helices may increase helix stability by hydrogen bonding to unsatisfied main-chain NH or CO groups.[22,23]

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