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Position Effect on Apparent Helical Propensities in the C-peptide Helix

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A search has been made for position effects on apparent helix propensities when another amino acid is substituted for alanine in the C-peptide helix of ribonuclease A. Three internal alanine residues (Ala4, Ala5, Ala6) are used as sites for substitution. Five amino acids, Glu, His, Arg, Lys and Phe, are substituted singly in individual peptides at each of these three positions, and the pH profiles of helix content for the substituted peptides have been determined. The effect of using an acetyl or a succinyl amino-terminal-blocking group has also been determined for each substitution. A strong position effect is found at Ala5: the helix content of the substituted peptide is significantly higher for substitution at position 5 than at positions 4 or 6 in almost all cases. The reason for the position 5 effect is unknown. The results also show that electrostatic interactions often influence substitution experiments, and they provide data on the variability of substitution experiments made with a natural sequence peptide.

Keywords: position 5 effect; helix substitution experiments; C-peptide helix

1. Introduction

The factors that determine the contribution of a particular amino acid to the stability of an α-helix, at a given position in the helix, are not yet well understood. It is clear that there are large differences among the helix propensities of the different amino acids, so that substitution of a single amino acid in a short helix ($\leq$20 residues) is likely to cause a large change in helix content (Padmanabhan *et al.*, 1990; Merutka *et al.*, 1990; Lyu *et al.*, 1990; O'Neil & DeGrado, 1990). It is also clear that specific interactions between pairs of side-chains, such as i, $i+4$ Glu^- . . . Lys^+ ion-pairs (Marqusee & Baldwin, 1987; Lyu *et al.*, 1989), the $Glu2^-$ . . . $Arg10^+$ ion-pair of C-peptide (Fairman *et al.*, 1990) or the Phe8 . . . $His12^+$ side-chain interaction in C-peptide (Shoemaker *et al.*, 1990), can have a strong effect on helix stability, as can the interaction between a charged group and the helix dipole (Shoemaker *et al.*, 1987; Fairman *et al.*, 1989). Other effects remain to be studied. For example, the role of hydrophobic interactions among side-chains in an α-helix has not yet been analyzed experimentally. Another factor might be important: local structural perturbations in the backbone can be induced by particular amino acids and may affect the contribution of a nearby amino acid to helix stability (Barlow & Thornton, 1988).

In order to investigate such position-dependent effects, we undertook the following experiment. The same substitution (Ala → X) was made at each of three positions (Ala4, Ala5, Ala6) in the helix formed by a derivative of the C-peptide (residues 1 to 13) of ribonuclease A. The change in helix

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content upon substitution was measured by circular dichroism (c.d.†), using $-(\theta)_{222}$ as a measure of α-helix content, and the pH dependence of helix content was also measured, to find out how the ionization states of the side-chains affect the apparent helix propensity. In individual peptides, five amino acids (Glu, His, Arg, Lys, Phe) were substituted for Ala at the three positions. Both charged (Glu^-, His^+, Lys^+, Arg^+) and uncharged (Glu^0, His^0, Phe) amino acids have been studied, to determine whether any special effects that emerge are specific for charged amino acids. Both acetyl and succinyl blocking groups were used at the amino terminus to compare the effects of these two blocking groups on the apparent helix propensities.

In quantitative studies of helix propensity made in short peptides, it is important to take into account a general position effect caused by fraying at the ends of an α-helix. This position effect has been studied by Chakrabartty *et al.* (1991) for Ala → Gly substitutions. Helix-fraying causes a substitution to have a smaller effect when the substitution is made near either end than in the center of an α-helix.

The C-peptide derivative used here as a reference peptide has the sequence Ac- (or Suc-) AETAAAKYLRAHA-NH_2. Phe8 in the natural C-peptide helix is replaced by Tyr8 in order to determine peptide concentration accurately by tyrosine absorbance. Tyr8 interacts with $His12^+$ in a similar manner to Phe8 (Shoemaker *et al.*, 1990). Thus, the $Glu2^-$ . . . $Arg10^+$ ion-pair interaction and an interaction similar to the Phe8 . . . $His12^+$ interaction of C-peptide are conserved in this derivative of C-peptide.

2. Materials and Methods

Peptide synthesis and purification techniques have been described (Shoemaker *et al.*, 1985, 1987). Peptide purity was determined by amino acid analysis and FAB mass spectrometry. Peptide concentration was determined by tyrosine absorbance (Shoemaker *et al.*, 1990). c.d. measurements were made on an AVIV 60DS spectropolarimeter and have been described (Shoemaker *et al.*, 1985). Non-linear least-squares analysis was used to fit the pH titrations of $[\theta]_{222}$ to the Henderson–Hasselbalch equation, as described by Fairman *et al.* (1989). The buffer used for c.d. measurements is 0·1 M-NaCl with 1 mM each of Na borate, Na citrate and Na phosphate. The peptide concentration is about 20 μM.

3. Results

(a) *Method of comparing substitution results*

The effect of a substitution is reported here simply as the change in helix content as measured by the difference between $[\theta]_{222}$ and $[\theta]_{400}$. It would be preferable to report the change in Gibbs free energy, but this is not yet possible: the two-state equation is a poor approximation to the helix-coil transition even for short α-helices (see Chakrabartty *et al.*, 1991), and the Zimm–Bragg (or Lifson–Roig) parameters are not yet known accurately. For reasons discussed earlier, it is desirable that the initial and final values of $-[\theta]_{222}$ be in the middle part of the unfolding transition curve so that $\Delta[\theta]_{222}$ is approximately proportional to the change in ΔG° (Shoemaker *et al.*, 1990). The comparison of different substitutions will be distorted if this is not true. Figure 1 shows thermal unfolding curves of the acetyl and succinyl reference peptides at pH 5·3. Most of the substitutions studied here are helix-destabilizing. Since the succinyl reference peptide has a higher helix content than the acetyl reference peptide, the succinyl peptide might be more useful

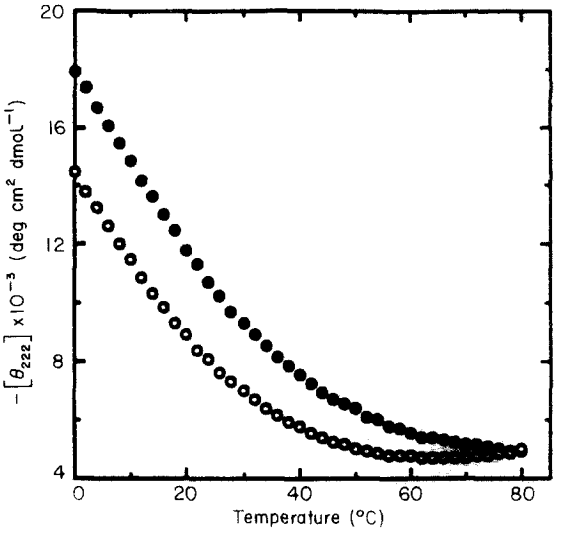

Figure 1. Thermal unfolding curves, measured by c.d. of the (○) acetyl and (●) succinyl reference peptides, at pH 5·3. See Materials and Methods for buffer. $[\theta]_{222}$ is the mean residue ellipticity at 222 nm.

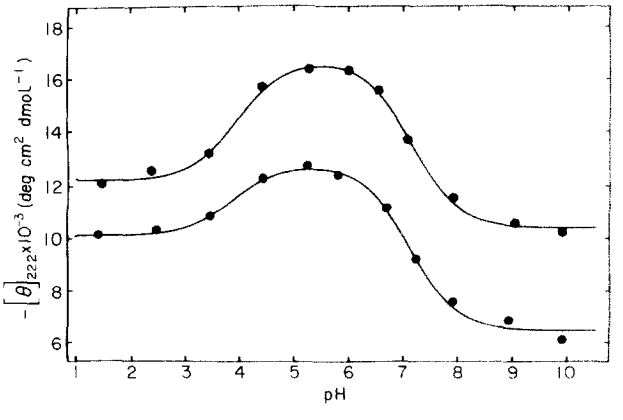

Figure 2. pH profiles of helix content, measured by $-[\theta]_{222}$, for the (●) acetyl and (●) succinyl reference peptides at 3°C. The titration curves are a non-linear least-squares best fit to the data points using the Henderson–Hasselbalch equation (Fairman *et al.*, 1989).

† Abbreviations used: c.d., circular dichroism; $[\theta]$, mean residue ellipticity; Ac, acetyl; Suc, succinyl; FAB, fast atom bombardment; n.m.r., nuclear magnetic resonance.

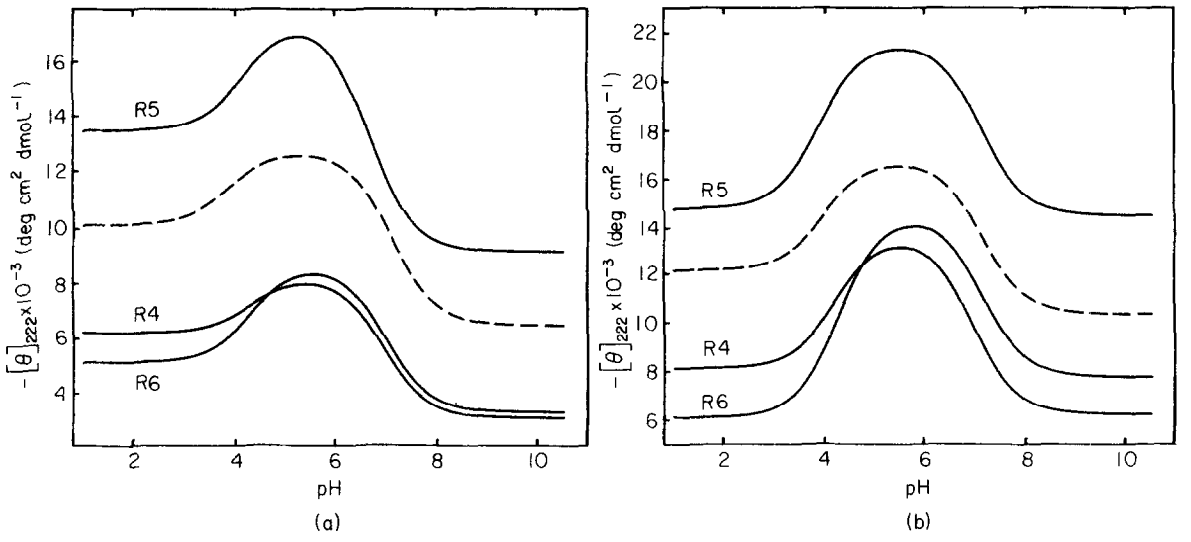

Figure 3. pH profiles of helix content at 3 °C for the Arg peptides with either an (a) acetyl or a (b) succinyl blocking group. R4, R5 and R6 are the peptides with arginine at positions 4, 5 and 6, respectively. The broken line curves are the pH profiles for the reference peptides. The data points have been omitted for clarity. (See the legend to Fig. 2.)

for these substitution experiments, since the final value of $-[\theta]_{222}$ for the substituted peptide is more likely to be in the linear range.

To compare the same substitution (A → X) at three different positions, pH profiles of the three peptides (with amino acid X at position 4, 5 or 6) are given on the same graph, together with the pH profile of the reference peptide. This allows the three substituted peptides to be compared over a wide pH range, and it can be seen whether the differences are uniform or restricted to a particular pH range. The data points have been omitted from these figures for clarity. The data were fitted to the Henderson–Hasselbalch equation by a non-linear least-squares method, as described earlier (Fairman *et al.*, 1989). For all substituted peptides except the His substitutions, only two pK_a values were used: one for His12 and one for all types of carboxyl groups present (either on Glu2, on the succinyl blocking group, or on any substituted glutamate residue). For some of the His-substituted peptides, a third pK_a value was used for the substituted histidine residue.

(b) *pH profiles of helix content*

Figure 2 shows the fit of the computer-drawn curves to the data points for the acetyl and succinyl reference peptides. This comparison between data points and the fitted curve provides a basis for estimating the accuracy of the pH profiles in the following figures, which are given as computer-drawn curves without data points. For the acetyl reference peptide, the increase in helix content from pH 2 to pH 5·3 is caused by the ionization of Glu2 and formation of the helix-stabilizing

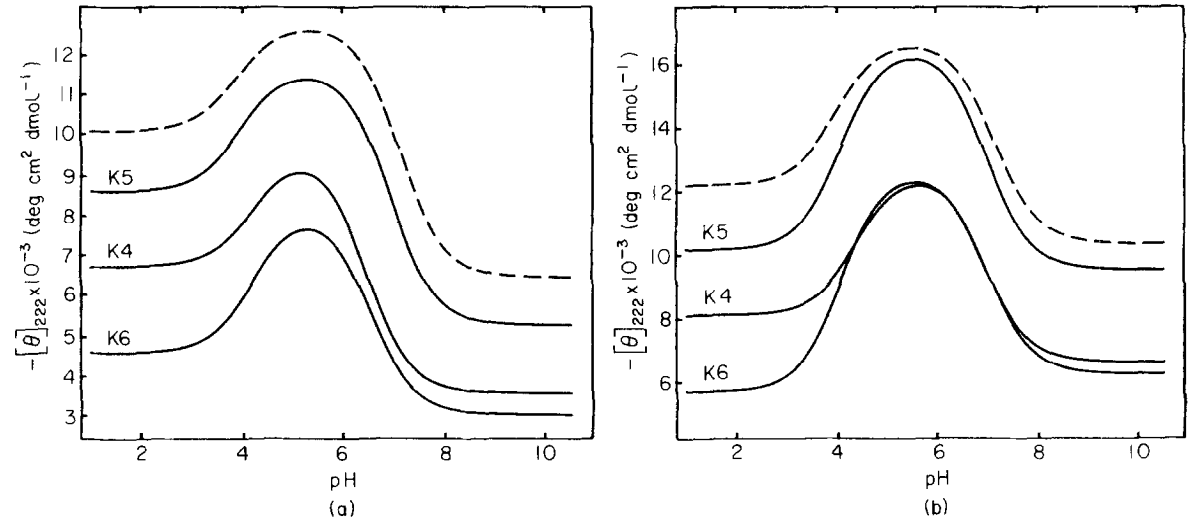

Figure 4. pH profiles of helix content at 3 °C for the Lys peptides. (See the legend to Fig. 3.).

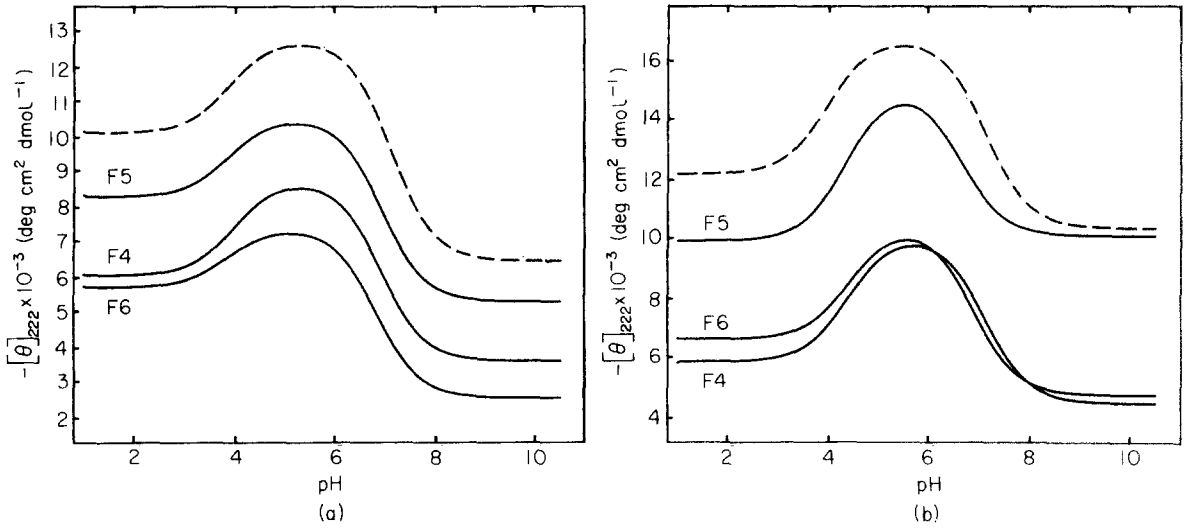

Figure 5. pH profiles of helix content at 3°C for the Phe peptides. (See the legend to Fig. 3.).

$Glu2^-$ . . . $Arg10^+$ ion-pair interaction (Fairman *et al.*, 1990). The decrease in helix content from pH 5·3 to pH 9 is caused by the titration of His^+ to His^0 and consequent loss of the Tyr8 . . . $His12^+$ side-chain interaction (Shoemaker *et al.*, 1990). For the succinyl reference peptide there is, in addition, an increase in helix content between pH 2 and pH 5·3 caused by the ionization of the succinyl group resulting in formation of a $succinyl^-$ . . . helix dipole interaction (Shoemaker *et al.*, 1987; Fairman *et al.*, 1989). This causes a larger change in $[\theta]_{222}$ between pH 2 and pH 5·3 than is observed for the acetyl peptide. The ionization of the succinyl group has an apparent pK_a value which is practically equivalent to that for the ionization of Glu2 (Fairman *et al.*, 1989), so that the computer-drawn curve was calculated with only a single pK_a value in this range.

The pH profiles for $Arg4^+$ and $Arg6^+$ in the acetyl peptides are quite similar to each other, both in shape and in value (Fig. 3): there is, however, a significant difference in the magnitudes of the acid limbs in the succinyl peptides. Although the actual values of $-[\theta]_{222}$ of Arg5 at different pH values are strikingly different from those of the $Arg4^+$ and $Arg6^+$ peptides, all three peptides have pH profiles that are similar in shape to each other and to the reference peptide. Thus, the surprising difference in results found for the substitution Ala → Arg^+ at position 5 *versus* positions 4 and 6 is shown over the entire pH range studied, and cannot be explained by a helix-stabilizing ion-pair interaction between $Arg5^+$ and either $Glu2^-$ or the succinyl group, since both interactions would be broken by titration to pH 2.

The same comments apply, but less forcefully, to the Lys^+ peptides (Fig. 4). The acetyl and succinyl $Lys5^+$ peptides, although showing substantially higher helix contents than $Lys4^+$ and $Lys6^+$, are slightly below the curves shown by the reference peptides, in marked contrast to $Arg5^+$. Again, as seen in the pH profiles for the succinyl peptides with $Arg4^+$ and $Arg6^+$, the magnitudes of the acid limbs

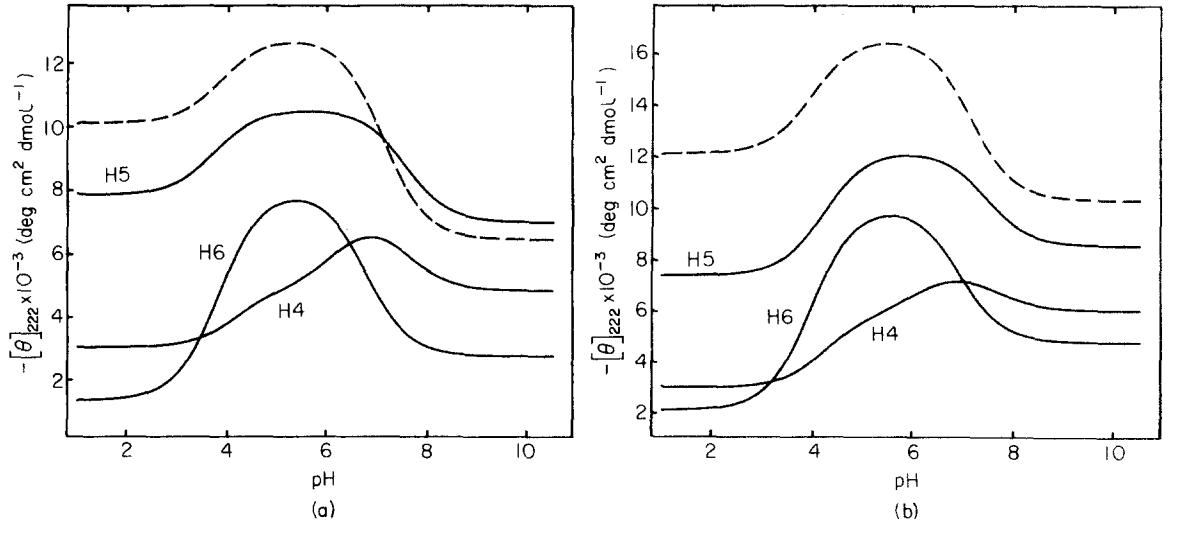

Figure 6. pH profiles of helix content at 3°C for the His peptides. (See the legend to Fig. 3.).

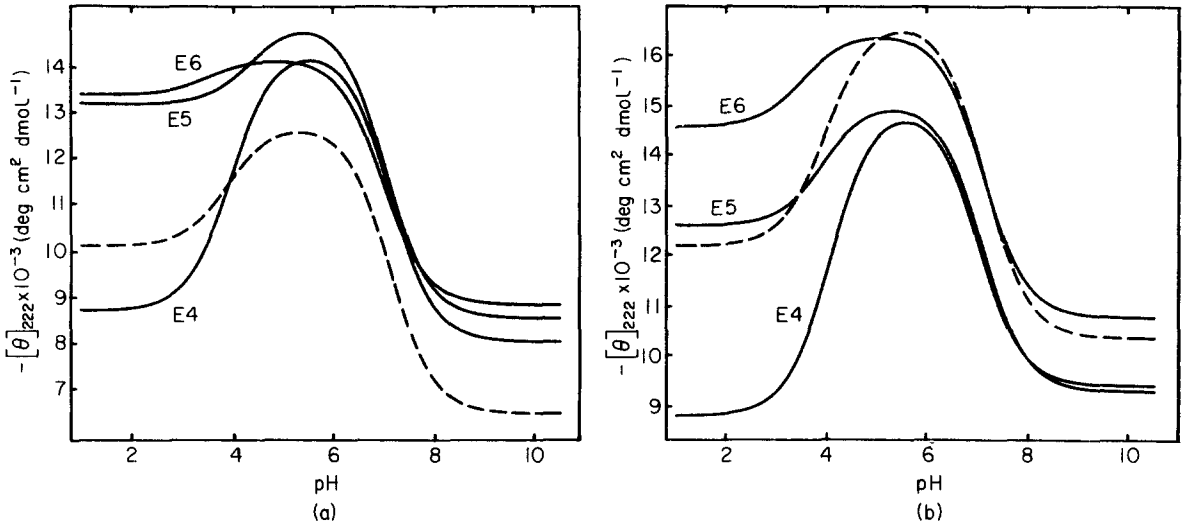

Figure 7. pH profiles of helix content at 3 °C for the Glu peptides. (See the legend to Fig. 3.).

are significantly different in the succinyl peptides with $Lys4^+$ and $Lys6^+$ but a curious feature of these peptides is that the differences between $Lys4^+$ and $Lys6^+$ are largest at pH 2, where differences might be least expected. The $Lys5^+$ curve is quite similar in shape to the reference peptide, both for the acetyl and succinyl peptides.

The Phe peptides show similar behavior (Fig. 5) to the Lys^+ and Arg^+ peptides. The increase in helix content of Phe5 over Phe4 and Phe6 is more striking for the succinyl than for the acetyl peptides, and the curves for Phe4 and Phe6 are closely similar in the case of the succinyl peptides.

Since the His peptides each have two histidine residues, which are likely to have different pK_a values, and since titration of a His^+ residue to His^0 usually changes the helix content whether or not the His^+ residue participates in a specific helix-stabilizing interaction (Shoemaker *et al.*, 1990; Armstrong *et al.*, unpublished results), the pH profiles of the His peptides (Fig. 6) are more complex than those of the Arg^+, Lys^+, or Phe peptides. The pH zone in which histidine titration affects the helix content is usually broader than in the reference peptide, because there are two His residues with differing pK_a values. An increase in helix content accompanying titration of His^+ to His^0 can be seen for His4 between pH 6 and pH 7, in contrast to the uniform decrease between pH 5·3 and pH 9 seen for His6 and for the reference peptide. Both the acetyl and succinyl His5 peptides have significantly higher helix contents than His4 or His6. The curve for His5 is not displaced uniformly downwards from the reference peptide but rises above pH 7. This is the expected behavior if the substituted His5 residue is non-interacting, and if it is more helix-destabilizing in the His^+ than in the His^0 form (see Discussion). The curves for His6 and His4 show substantial differences both in the acetyl and succinyl peptides. A good part of the divergence evidently arises from the difference between the pK_a values of the His6 and His4 residues, caused probably by electrostatic interaction with one or more other charged residues. This interpretation for the His6 peptide is likely to hold for the effects seen for the succinyl peptides with $Arg6^+$ and $Lys6^+$ substitutions.

The pH profiles of the Glu peptides (Fig. 7) do not fit in with the behavior described above for the other residues. Curiously, the differences from each other and from the reference peptide are more striking at pH 2, where the Glu peptides are in the Glu^0 form. Probably the Glu peptides, unlike the Arg^+ and Lys^+ peptides, show pH profiles that are not uniformly like that of the reference peptide in shape because one or more of the substituted Glu residues participates in a specific interaction, but further research is needed to resolve the question.

4. Discussion

(a) *Position effect at residue 5*

The results show clearly that when Arg^+, Lys^+, Phe, His^+ or His^0 is substituted for Ala5, the helix content of the substituted peptide is significantly higher than when the substitution is made at Ala4 or Ala6. The same effect is observed either with an acetyl or succinyl blocking group. Thus, there appears to be a position effect which is specific for position 5 and is general for several amino acids. Such a position effect is unexpected and is not easy to explain.

Model building indicates that the side-chain of a substituted amino acid might interfere sterically with the $Glu2^- \ldots Arg10^+$ salt bridge seen in the X-ray structure of RNase A when the substitution is made at position 6, but not at position 4 or 5 (Strehlow & Baldwin, 1989). Since it is position 5, not position 6, that shows the unusual effect of substituting another amino acid for alanine, this possible explanation is not of much help. Moreover,

the same position effect is found at pH 2, where Glu2 is protonated and the salt bridge interaction should be broken, as at pH 6, where the salt bridge is intact.

A ^{1}H-n.m.r. study indicates that both an extended helix and a kinked helix form of C-peptide can be observed in solution, in addition to the random coil or extended form (Osterhout *et al.*, 1989). All three conformations should be considered in analyzing the results of substitution experiments. The kinked helix results from the presence of the $Glu2^- \ldots Arg10^+$ salt bridge. Thus, the position 5 effect might result from a shift in the proportions of extended helix *versus* kinked helix when a substitution is made either at position 4, 5 or 6. This explanation suffers, however, from the difficulty mentioned above: the position 5 effect is found both at pH 2, where Glu2 is protonated and the kinked helix should disappear, and at pH 6. An H-bond between the γOH group of Thr3 and the peptide CO group of Ala6 is also involved in the kink (Baker & Hubbard, 1984) and this H-bond might remain at pH 2, so it is possible that the kinked helix persists at pH 2.

There are few other clues as to the origin of the position 5 effect. It is not shown by glycine for alanine substitutions (Strehlow & Baldwin, 1989). Curiously, Ala5 is strongly conserved in evolution whereas Ala4 and Ala6 are not (Blackburn & Moore, 1982), although substitution of Ala5 by another amino acid is helix-stabilizing compared with the same substitution made at Ala4 or Ala6.

(b) *Non-uniformity of substitution effects in C-peptide*

Some general conclusions can be drawn. First, it is evident that substitution of a charged residue is likely to have a position-specific effect, either through general electrostatic interactions or through formation of specific ion-pairs. This can be seen clearly in the pH profiles of the His peptides, which show different pK_a values for His4, His5 and His6 and likewise different helix contents. An electrostatic effect is also evident when Lys^+, Arg^+ and His^+ are present at position 6 in the succinyl peptides. Curiously, however, the differences in helix content are at least as large for His^0 as for His^+, and this is true also of Glu^0 *versus* Glu^-. Thus, the second general conclusion is quite surprising: non-uniform effects at different positions are as frequent and as large for uncharged as for charged residues. The third conclusion is equally surprising. Non-uniformity in substitution effects appears to be largest at pH 2, where the $Glu2^- \ldots Arg10^+$ ion-pair interaction is broken. Why this should be so is a complete mystery, and deserves to be studied.

The results indicate that the C-peptide helix of ribonuclease A is not a good system in which to measure relative helix propensities. A similar conclusion may apply to other natural sequence peptides. The position-dependent effects observed here provide a cautionary note concerning the study of helix propensities.

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