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A Competing Salt-bridge Suppresses Helix Formation by the Isolated C-peptide Carboxylate of Ribonuclease A

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The C-peptide of ribonuclease A (residues 1 to 13) is obtained by cyanogen bromide cleavage at Met13, which converts methionine to a mixture of homoserine lactone (giving C-peptide lactone) and homoserine carboxylate (giving C-peptide carboxylate). The helix-forming properties of C-peptide lactone have been reported. The helix is formed intramolecularly in aqueous solution, is stabilized at low temperatures (0 to 20°C) and also by a pH-dependent interaction between side-chains. The C-peptide lactone helix is about 1000-fold more stable than expected from "host–guest" data for helix formation in synthetic polypeptides.

Here we report the failure of C-peptide carboxylate to form an α-helix in comparable conditions. Formation of a salt-bridge between the α-COO^- group and the imidazolium ring of $His12^+$ appears to be responsible for the suppression of helix formation. The presence of the Hse13-$COO^- \cdots His12^+$ salt-bridge in C-peptide carboxylate is shown by ^1H nuclear magnetic resonance titration of the amide proton resonances of His12 and Hse13, and is expected from model peptide studies. The most probable reason why C-peptide carboxylate does not form an α-helix is that the Hse13-$COO^- \cdots His12^+$ salt-bridge competes successfully with a helix stabilizing salt-bridge ($Glu9^- \cdots His12^+$).

S-peptide (residues 1 to 20 of ribonuclease A) does form an α-helix with properties similar to those of the C-peptide (lactone) helix, which shows that the lactone ring of C-peptide lactone is not needed for helix formation.

These results support the hypothesis that a $Glu9^- \cdots His12^+$ salt-bridge stabilizes the C-peptide (lactone) helix, and they show that specific interactions between side-chains can be important in preventing as well as in promoting α-helix formation.

1. Introduction

Data on the stability of the amide hydrogen bond in water (Klotz & Franzen, 1962; Susi *et al.*, 1964), on α-helix formation in water by synthetic polypeptides (cf. Scheraga, 1978), and on the failure of protein fragments to form stable secondary structure in water (cf. Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969), all

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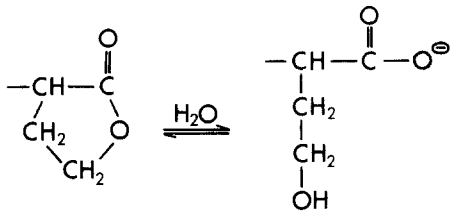

FIG. 1. Hydrolysis of homoserine lactone to homoserine carboxylate. Following cleavage by cyanogen bromide at Met13, a mixture of C-peptide lactone and C-peptide carboxylate is obtained: the 2 species can be separated by ion-exchange chromatography or by HPLC.

support the conclusion that short α-helices are not stable in water at 25°C. Nevertheless, the C-peptide (residues 1 to 13 of RNAase A†) has been found to show partial helix formation in water at low temperatures (0 to 20°C) as judged by circular dichroism (Brown & Klee, 1971). The unexpected stability of the C-peptide helix has been attributed to a specific interaction between side-chains, probably to the salt-bridge $Glu9^- \cdots His12^+$ (Bierzynski *et al.*, 1982). The use of low temperatures to observe the helix may also be important, since α-helix formation by protein fragments may in general be an enthalpy-driven reaction (cf. Bierzynski *et al.*, 1982).

C-peptide exists in two forms, a lactone and an α-carboxylic acid, since the lactone ring of Hse13 is easily cleaved at mild alkaline pH values (Fig. 1). The preparation of C-peptide by cyanogen bromide cleavage at Met13 yields a mixture of the lactone and the carboxylate forms. These have been separated by ion-exchange chromatography on SP-Sephadex C-25 and the purity of each form can be assayed rapidly by high-pressure liquid chromatography (Bierzynski *et al.*, 1982). The lactone form is referred to below as C-peptide and the carboxylate form as C-peptide carboxylate.

We report here on the helix-forming properties of C-peptide carboxylate, because there are major differences between C-peptide and C-peptide carboxylate. For comparison, S-peptide (residues 1 to 20 of RNAase A) has also been studied: it has neither the lactone ring of C-peptide nor the α-COO^- group of C-peptide carboxylate on residue 13.

2. Materials and Methods

Most methods and materials are described either in the preceding paper (Bierzynski & Baldwin, 1982) or by Bierzynski *et al.* (1982). The tripeptide Gly-His-Gly was purchased from Accurate Chemicals and it was acetylated with acetic anhydride by the procedure of Means & Feeney (1971). The His side-chain was deacetylated using conditions described by

† Abbreviations used: RNAase A, bovine pancreatic ribonuclease A; C-peptide, residues 1 to 13 of RNAase A with homoserine lactone at residue 13; C-peptide carboxylate, residues 1 to 13 of RNAase A with homoserine carboxylate at residue 13; S-peptide, residues 1 to 20 of RNAase A; HPLC, high-pressure liquid chromatography; n.m.r., nuclear magnetic resonance; c.d., circular dichroism; $[\theta]$, mean residue ellipticity; p.p.m., parts per million; ImH^+, imidazolium ion; Hse, homoserine.

Jencks & Carriuolo (1959). The product was checked for purity by HPLC and by its proton n.m.r. spectrum. Preparation of C-peptide and C-peptide carboxylate has been described (Bierzynski *et al.*, 1982). S-peptide (Sigma grade XII-PE) was purified on CM Sephadex C-25; the sample was applied in 50 mM-acetate buffer, 0·1 M-NaCl (pH 4·7), and eluted with an NaCl gradient from 0·1 M to 0·25 M. The purity of all peptides was checked by reverse-phase HPLC. Peptide concentrations were determined by the ninhydrin method (Rosen, 1957), using leucine as a standard. Stock solutions of C-peptide carboxylate were kept at pH 10 to prevent conversion to the lactone form. Amide proton spectra were taken in H_2O using the Redfield 21412 pulse sequence technique. Peak assignments are presented elsewhere (Bierzynski & Ribeiro, unpublished data). c.d. spectra were recorded on a Jasco J-500A spectropolarimeter or on a Cary model 60 spectropolarimeter with a model 6001 c.d. accessory in the laboratories of Professor J. T. Yang, University of California Medical School, San Francisco, and Professor I. Tinoco, University of California, Berkeley, respectively. The cell path-length was 1·0 cm. For c.d. studies the peptide concentration was 30 μM and all samples contained 0·1 M-NaCl, 1 mM-Na-citrate, 1 mM-Na-phosphate and 1 mM-Na-borate. The pH was adjusted with HCl or NaOH.

3. Results

(a) *c.d. spectra of C-peptide carboxylate as a function of pH and temperature*

Figure 2(a) shows c.d. spectra (210 nm to 244 nm) of C-peptide carboxylate at 3°C and 42°C (pH 5·01). There are several differences from the spectra shown by C-peptide (Bierzynski *et al.*, 1982). First, the negative ellipticity at 222 nm is small compared to that of C-peptide: $-[\theta]_{222} = 1130$ deg. cm^2 dmol^{-1} at pH 5·01, 3°C, compared to 7000 for C-peptide in similar conditions. Consequently, C-peptide carboxylate shows little if any α-helix in these conditions. C-peptide carboxylate has a smaller value of $-[\theta]_{222}$ at 3°C (1130 deg. cm^2 dmol^{-1}) than C-peptide does at 45°C, where the C-peptide helix is unstable. Second, the minimum in the C-peptide carboxylate spectrum is not at 222 nm, as expected for an α-helix, but is shifted to higher wavelengths (227 to 228 nm). Third, the strong temperature dependence shown by C-peptide is missing. Finally, the strong pH dependence of the c.d. spectrum observed for C-peptide is also missing (Fig. 2(b)).

Curiously, the c.d. spectrum of C-peptide carboxylate decreases in intensity when the pH is raised to 8·23 at 4°C, and this loss in intensity is largely regained when the temperature is raised to 43°C (Fig. 3).

(b) *pH titration of NH resonances of C-peptide carboxylate*

The major reason for the failure of C-peptide carboxylate to form an α-helix appears to be the existence of a salt-bridge between the side-chain of His12$^+$ and the α-COO$^-$ group of the peptide. The evidence for this salt-bridge comes from pH titrations of the ^{1}H n.m.r. amide proton resonances of His12 and Hse13 (Fig. 4(a)), and a comparison of these data with the model peptide studies of Bundi & Wüthrich (1979). The amide protons both of His12 and Hse13 in C-peptide carboxylate show biphasic pH titration curves (Fig. 4(a)), with inflection points close to the pK values of His12 (6·8) and of the α-COO$^-$ group (3·0).

The expected titration shifts for these amide protons in the *absence* of a salt-bridge, based on model peptide studies (Bundi & Wüthrich, 1979) are as follows. (1) The amide proton of Hse13 would be expected to show a large, inductive upfield

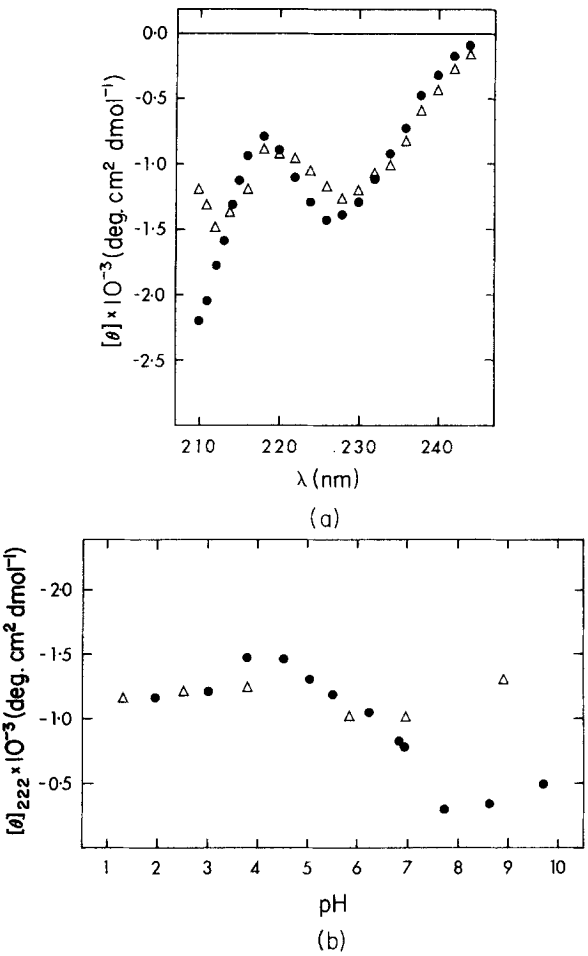

FIG. 2. (a) c.d. spectra of C-peptide carboxylate at pH 5·01 (0·1 M-NaCl): (●) 3·2°C; (△) 42·2°C. (b) pH dependence of $[\theta]_{222}$ for C-peptide carboxylate (0·1 M-NaCl) at 6·7°C ± 0·2 deg.C (●) and 45·0°C ± 0·1 deg.C (△).

shift (−0·4 to −0·6 p.p.m.) as the α-COOH group is deprotonated ($pK = 3·0$). (2) The amide proton of His12 would *not* be expected to titrate with the α-COOH group. This is clear from the data on model peptides (Bundi & Wüthrich, 1979), which show that the penultimate amide is too far (6 bonds) to experience through-bond effects. Additionally, no through-space interaction between the α-COO^- group and the penultimate amide was observed by Bundi & Wüthrich (1979), who did not, however, study peptides containing histidine. (3) The amide protons of both His12 and Hse13 might be expected to titrate with the imidazole side-chain unless the ring is too far from the amide protons of His12 and Hse13 (4 bonds and 5 bonds, respectively) to transmit an inductive effect. This has not yet been studied.

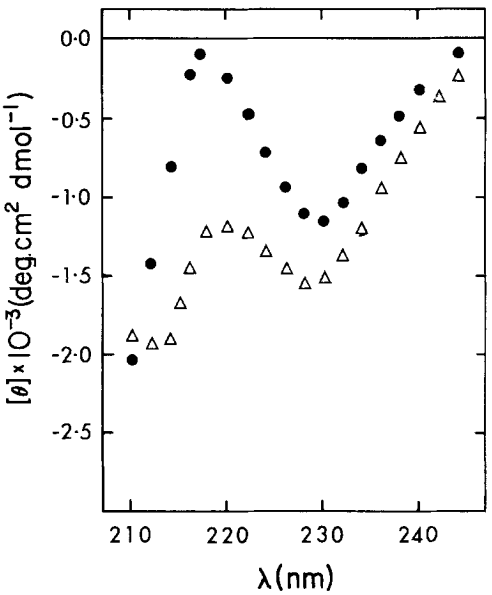

FIG. 3. c.d. spectra of C-peptide carboxylate at pH 8·23 (0·1 M-NaCl): (●) 3·6°C; (△) 43·5°C.

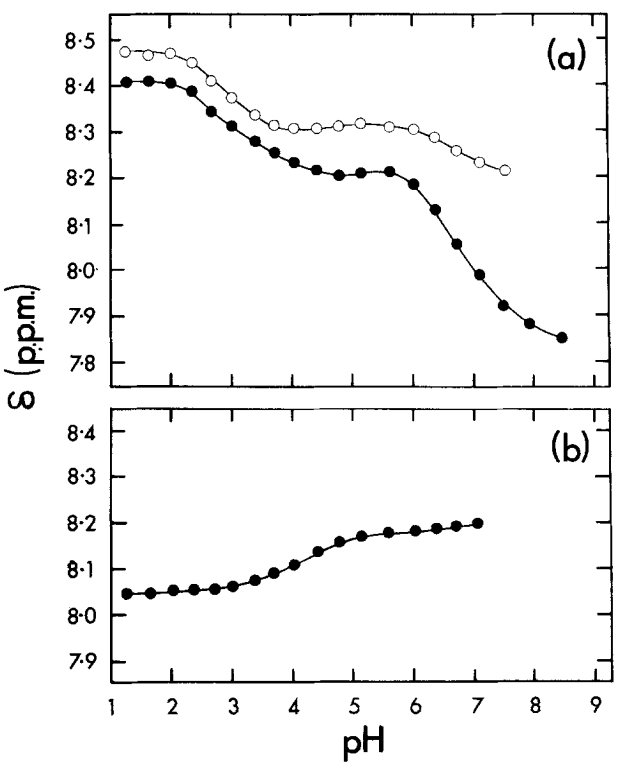

FIG. 4. pH dependence of chemical shifts of C-peptide carboxylate at 1°C in H_2O (no added salt). (a) Amide proton of His12 (○) and amide proton of Hse13 (●). (b) Amide proton of Glu9 (●).

Figure 4(a) suggests that intrinsic shifts may be present†. In any case, the major titration shift of the Hse13 amide resonance should occur as the α-COOH group is deprotonated, and not when $His12^+$ is deprotonated.

The results shown in Figure 4(a) are clearly different from those expected in the absence of specific interactions. The amide proton of Hse13 shows a lesser (−0·18 p.p.m.) upfield shift with deprotonation of the α-COOH group and a greater shift (−0·38 p.p.m.) with deprotonation of His12. The amide proton of His12 titrates with its imidazole side-chain group, but also with the α-COOH group; this is in marked contrast to the model peptide data of Bundi & Wüthrich (1979). We deduce that the biphasic titration curves of the His12 and Hse13 amide resonances (Fig. 4(a)) are caused by an interaction between the two residues, most likely involving a salt-bridge between ImH^+ and the α-COO^- group.

If this salt-bridge explanation is correct then, in conditions where the C-peptide helix cannot be formed, the amide resonance of His12 in C-peptide should not shift near pH 3, since the lactone form of the peptide does not have an α-COOH group. Figure 5 shows that the amide resonance of His12 in C-peptide is not affected by pH values near pH 3, as expected. The measurements were made at high temperatures (57°C) where the C-peptide helix is unstable, to avoid chemical shifts due to the pH dependence of helix content (Bierzynski *et al.*, 1982). The His12 amide resonance of C-peptide carboxylate still titrates around pH 3 at 57°C (Fig. 5), although the change is smaller than at 1°C. The amide titration curves

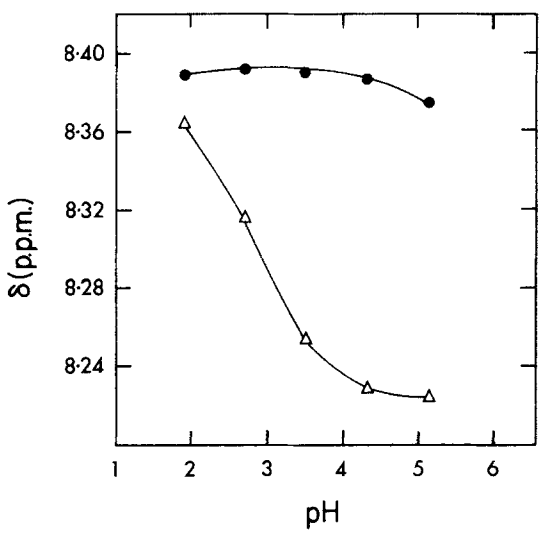

FIG. 5. pH dependence of chemical shifts for the amide proton of His12 at 57°C in H_2O (0·1 M-NaCl, 0·02 M-Na acetate buffer) in C-peptide (lactone) (●) and C-peptide carboxylate (△).

† Previous attempts to study the effects of the His ring titration on amide proton resonances (cf. Bundi & Wüthrich, 1979) have been hampered by the fast intrinsic exchange rates of amides adjacent to the His side-chain (Molday *et al.*, 1972). The low temperature (1°C) used here and the presence of the α-COO^- group (cf. Molday *et al.*, 1972) both contribute to slowing down the exchange rate of the Hse13 amide proton, so that the titration curves shown in Fig. 4(a) could be measured.

could not be measured in the pH range where His12 titrates at 57°C, because the exchange rates are too fast.

The titration curve of the Glu9 amide resonance of C-peptide carboxylate at 1°C is shown in Figure 4(b). There is a downfield shift (0·13 p.p.m.) at the pK of Glu9. This results from a charge-stabilized hydrogen bond made between the ionized side-chain of Glu9 and the Glu9 amide proton; this bond is broken when the Glu9 side-chain is protonated (Bundi & Wüthrich, 1979).

(c) *Amide proton titrations and existence of a salt-bridge in N-acetyl-Gly-His-Gly*

Tran *et al.* (1977) have demonstrated a salt-bridge (charge-pair stabilized H-bond) between the α-COO^- group and the ImH^+ side-chain in peptides of the type X-His-Gly. They used proton n.m.r. to measure vicinal coupling constants and non-equivalence of chemical shifts of side-chain protons (His2 $\beta\beta'CH_2$ and Gly3 $\alpha\alpha'CH_2$). They also determined the c.d. spectra of the peptides as a function of pH. They found that a particular rotamer (III, *gauche–gauche*) of the His side-chain is stabilized when both the ImH^+ and the α-COO^- groups are ionized, and by model building they showed that rotamer III can be stabilized by a hydrogen bond between the ImH^+ and α-COO^- groups.

We measured the amide proton titration curves for *N*-acetyl-Gly-His-Gly to check whether the His2 amide resonance titrates at the α-COO^- pK, as expected from data on C-peptide carboxylate (Fig. 4(a)). By studying the *N*-acetyl peptide, the amide titration curve of Gly1 can be measured as well as those of His2 and Gly3. The results are shown in Figure 6. The amide resonances of His2 and Gly3 both show upfield shifts at the α-COO^- pK ($-0{\cdot}13$ and $-0{\cdot}12$ p.p.m. with apparent pK values of 3·1 and 3·2, respectively), whereas the amide resonance of Gly1 is independent of pH in this range. Exchange was too rapid to permit the amide titration curves to be measured at the His2 pK (7·1). We conclude that a charge-pair stabilized hydrogen bond, formed by the penultimate ImH^+ side-chain and the α-COO^- group, causes an upfield titration shift of the His amide resonance at the α-COO^- pK, and that the titration shift can be used to detect this particular salt-bridge in longer peptides, such as C-peptide carboxylate.

Since the titration shift of the His amide resonance is upfield, one may ask whether this is a through-bonds or through-space effect of titrating the salt-bridge. Bundi & Wüthrich (1979) found that all intrinsic (i.e. through-bond) titration shifts are upfield, although not all upfield shifts are intrinsic. If the titration shift is through-bonds, then an upfield inductive shift must be observed in the titration curves of all proton resonances at bonds connecting the His2 amide proton and the salt-bridge. Figure 7 shows that this is not the case for the His2 $\beta\beta'CH_2$ resonances at the α-COO^- pK. A substantial upfield shift is observed for each resonance at the His2 pK, but at the α-COO^- pK, one of the His2 βCH_2 resonances shows a *downfield* shift, and the other βCH_2 resonance shows no significant change (Fig. 7). We conclude that the shifts in the His2 amide resonance at the pK of the α-COO^- group arise predominantly from through-space effects. This agrees with the analysis of Tran *et al.* (1977).

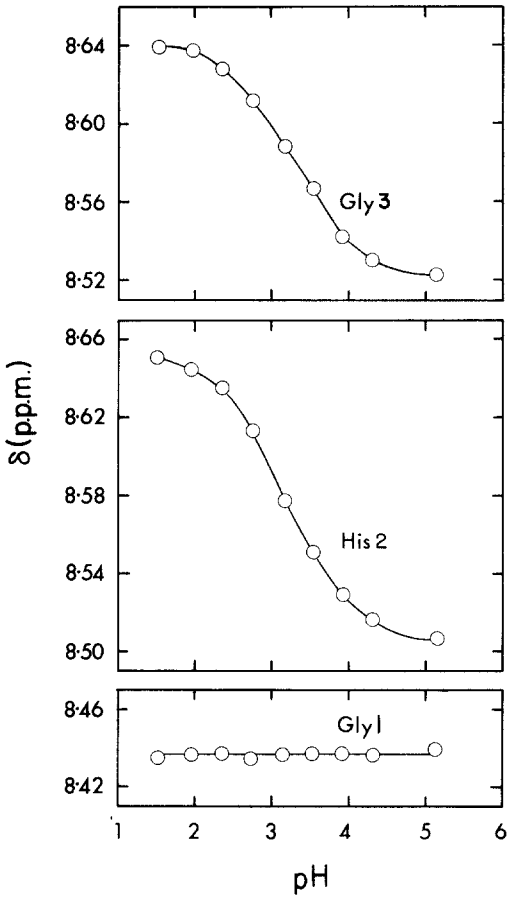

FIG. 6. pH dependence of amide proton chemical shifts in *N*-acetyl-Gly-His-Gly at 4°C in H_2O (0·1 M-NaCl, 20 mM-Na acetate buffer), Gly3 (top), His2 (middle) and Gly1 (bottom). The average chemical shifts for the glycine amide triplets and the histidine amide doublet are plotted.

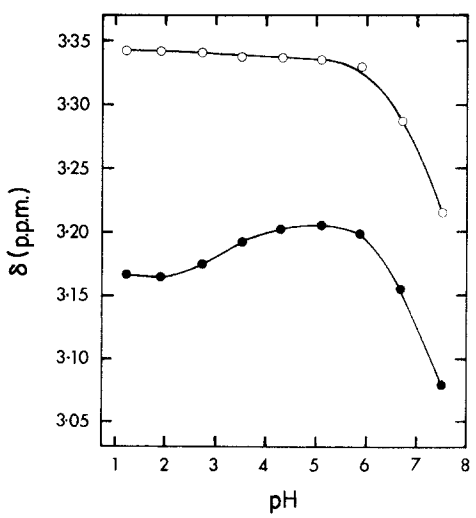

FIG. 7. pH dependence of chemical shifts for the His2 $\beta\beta'CH_2$ resonances in *N*-acetyl-Gly-His-Gly at 4°C in ^2H_2O. The average chemical shift for each β proton is plotted.

(d) *Helix formation by S-peptide*

The c.d. spectra of S-peptide resemble those of C-peptide, and not those of C-peptide carboxylate, in being strongly dependent upon pH and temperature. The S-peptide results are more complex, and the c.d. evidence of α-helix formation is less clearcut, than in the case of C-peptide. Figure 8 shows the c.d. spectrum of S-peptide at pH 4 and 1°C; these conditions are nearly optimal for helix formation. There is a broad minimum in the spectrum between 220 and 225 nm and the value of $-[\theta]_{222}$ is only 4200 deg. cm^2 dmol^{-1} as compared to 7100 for C-peptide at pH 5 and 3°C. The curve of $[\theta]_{222}$ *versus* pH at 3·5°C shows at least three components (Fig. 9), with a maximum near pH 3·7, a shoulder around pH 5·8, and a lesser shoulder around pH 2. Unlike C-peptide, S-peptide does not show measurable helix formation at pH 7·5 (3·5°C). We use the high-temperature c.d. spectrum at 40°C as a baseline for helix formation, as in the case of C-peptide (Bierzynski *et al.*, 1982), because $-[\theta]_{222}$ shows the same type of strong dependence upon temperature in both cases (Fig. 10).

4. Discussion

(a) *Hydrogen bonds stabilized by salt-bridges*

When hydrogen-bond stability in water is discussed, it is important to distinguish between uncharged H-bonds (such as the amide H-bond N—H $\cdots$O=C), singly charged stabilized H-bonds (such as the H-bond made by the

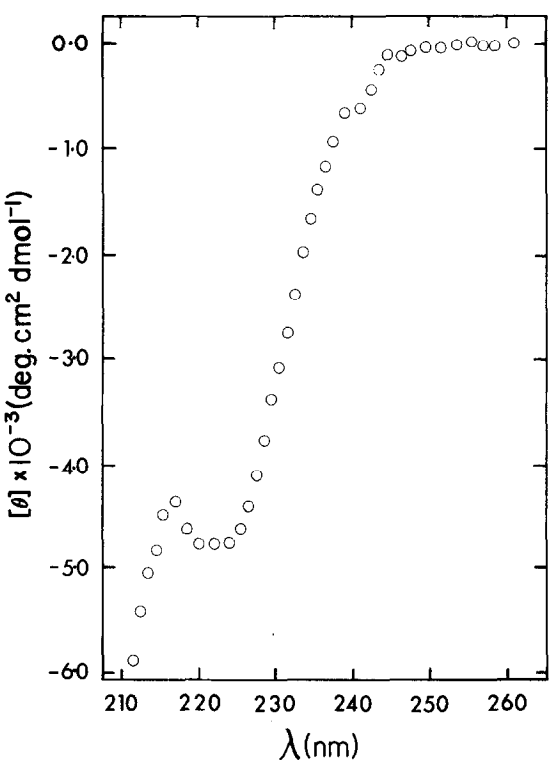

FIG. 8. c.d. spectrum of S-peptide at pH 4·03 and 1·0°C (0·1 M-NaCl).

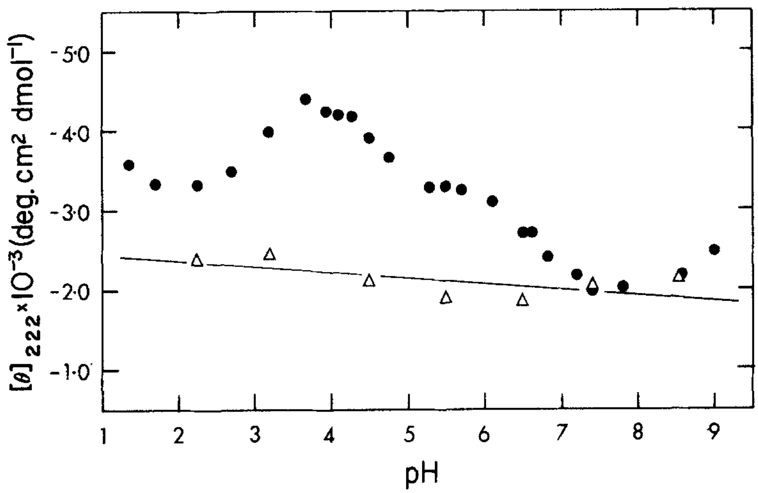

FIG. 9. pH dependence of $[\theta]_{222}$ for S-peptide (0·1 M-NaCl) at 3·5°C ± 0·2 deg.C (●) and at 40·0°C ± 0·1 deg.C (△).

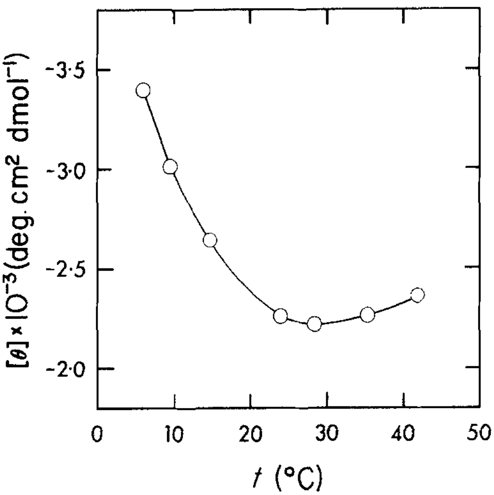

FIG. 10. Temperature dependence of $[\theta]_{222}$ for S-peptide (0·1 M-NaCl) at pH 5·03.

γ-COO^- group of a Glu residue to its own NH proton; Bundi & Wüthrich, 1979; cf. Fig. 4(b)), and charge-pair stabilized H-bonds such as the $ImH^+ \cdots {}^-OOC$ H-bond discussed here. Bundi & Wüthrich (1979) observed that the single charge stabilized H-bond $COO^- \cdots H{-}N$ is stronger than the uncharged bond

$$\overset{\diagup OH}{C{=}O} \cdots H{-}N$$

in the case of a glutamate side-chain bending back to H-bond to its own NH. The charge stabilized H-bond is easily measurable in water, but the H-bond is broken by protonating the COO^- group. Likewise, we note here that the $ImH^+ \cdots {}^-OOC$ H-bond, which is stabilized by a charge pair, is stronger than the singly charged H-bond, $ImH^+ \cdots O{=}\overset{HO \diagdown}{C}$. When the αCOO^- group of

N-acetyl-Gly-His-Gly is protonated, the chemical shifts of the Gly3 $\alpha\alpha'CH_2$ resonances become equivalent (data not shown), indicating that protonation breaks the charge-pair stabilized H-bond between His2 and the α-COO^- group (cf. Tran *et al.*, 1977).

(b) *A charge-pair stabilized hydrogen bond between the ImH^+ and the α-COO^- group prevents helix formation in C-peptide carboxylate*

The evidence that C-peptide carboxylate fails to form an α-helix because it contains the charge-pair stabilized H-bond $ImH^+\cdots\alpha$-COO^- is as follows. First, the c.d. spectra of C-peptide carboxylate show clearly the absence of the pH and temperature-dependent helix formation shown by C-peptide (lactone). Second, the amide proton titration curves of His12 and Hse13, taken together with the work of Tran *et al.* (1977) and the data presented here for *N*-acetyl-Gly-His-Gly, show the existence of this H-bond in C-peptide carboxylate. Third, S-peptide does form an α-helix resembling that of C-peptide in its strong dependence on pH and temperature, and S-peptide has neither the lactone ring of C-peptide nor the α-COO^- group of C-peptide carboxylate on residue 13. We can therefore rule out the possibility that the lactone ring is essential for helix formation in these peptides. Finally, a logical explanation for these observations is provided by the study of C-peptide by Bierzynski *et al.* (1982), who conclude that the C-peptide helix is stabilized by a pH-dependent interaction between side-chains, probably by the salt-bridge $Glu9^-\cdots His12^+$. Consequently, a competing charge-pair stabilized H-bond can destabilize the helix.

The $His12^+\cdots\alpha$-COO^- interaction is likely to be stronger than that of $Glu9^-\cdots His12^+$, because it closes a smaller ring (an 11-membered ring, compared to a 19-membered ring for $Glu9^-\cdots His12^+$), and the entropy loss on ring closure decreases with the size of the ring (cf. Flory & Semlyen, 1966).

At pH 2 and at pH 8, both the charge pairs $Glu9^-\cdots His12^+$ and $His12^+\cdots\alpha$-COO^- are discharged. Nevertheless, C-peptide still shows measurable helix formation at pH 2 and at pH 8, but C-peptide carboxylate does not. At these pH values, a charge-stabilized H-bond may still form in place of the salt-bridge, since only one partner of the interaction is uncharged. Thus, the $Glu9\cdots His12$ interaction may still stabilize the C-peptide helix at pH 2 or pH 8. Above pH 7 and at low temperatures, the C-peptide carboxylate adopts a conformation with a lower (absolute) value of $[\theta]_{222}$ (Fig. 3). This structure, which is clearly not α-helical, and melts out with increased temperature (Fig. 3), has not yet been studied in detail. A possible explanation for the failure of C-peptide carboxylate to show partial helix formation at pH 2 is that the ImH^+ of His12 can form a charge-stabilized H-bond with the oxygen (γ-OH) of the Hse13 side-chain in C-peptide carboxylate. Model building shows that this is a linear (180°) H-bond that would, of course, compete with the Glu9 COOH group for the His12 ImH^+ group. The analogous H-bond in C-peptide (lactone) between the ImH^+ and the oxygen of the lactone ring, is not sterically feasible. Further work is needed to test for the existence of this H-bond in C-peptide carboxylate.

(c) *Helix formation by S-peptide*

It is clear from Figures 8 to 10 that S-peptide does show partial α-helix formation in water near 1°C, and that the strong dependence upon pH and temperature of helix stability resembles that of C-peptide. This is in contrast to earlier studies on the S-peptide, which reported that the conformation of the S-peptide (as judged by c.d.) was independent of both temperature and pH (Klee, 1968). However, in these earlier studies (Klee, 1968), the pH dependence was checked at 26°C, where the helix is unstable (Fig. 10), and the temperature dependence was investigated at high pH (11·5), where the helix is also unstable (Fig. 9).

Helix formation in S-peptide is more complex and less well-documented than for C-peptide. The average extent of helix formation is smaller for S-peptide (as estimated from mean residue ellipticities) and the c.d. spectra do not show as clearly the presence of an α-helical component. The minimum near 222 nm, which should be sharply defined for an α-helix, is broad (Fig. 8). Also, the pH dependence of helix formation is complex (Fig. 9). We will not analyze here the interactions affecting the stability of the S-peptide helix, except to point out that the β-COO^- group of Asp14 can make a charge-pair stabilized H-bond to $His12^+$ when residue 12 is in an α-helix and residue 14 is not. (Residues 3 to 12 form an α-helix in RNAase S and RNAase A.) Model building indicates that this interaction would not itself affect the helix stability. The $His12^+ \cdots Asp14^-$ interaction would, however, compete with the $Glu9^- \cdots His12^+$ interaction, and thereby destabilize the helix.

Without knowing the number of residues that participate in forming the helix, it is difficult to use c.d. data to estimate the fraction of S-peptide molecules in a helical form, since the complete helix–random coil transition cannot be measured. If the helix is limited to residues 3 to 12 as in RNAase S (i.e. ten helical peptide groups), then the mean residue ellipticity should be increased by a factor of 20/10. When this is done, and the same assumptions made previously (Bierzynski *et al.*, 1982) are made here†, we calculate a helix content of 15% at 3·5°C and pH 3·7 (the pH optimum for S-peptide, Fig. 9).

(d) *Stability of isolated α-helices and the mechanism of protein folding*

This work, together with studies on the C-peptide (Bierzynski *et al.*, 1982), demonstrates that side-chain interactions within an isolated α-helix are crucial in determining helix stability in water for short helices. The C-peptide helix is at least 1000-fold more stable than expected from the host–guest parameters for helix formation. C-peptide carboxylate shows only trace amounts of helix in conditions where the C-peptide helix is easily measurable. It is apparent that side-chain interactions within an isolated α-helix may also play a critical role in stabilizing initial folding intermediates. There is now substantial evidence that hydrogen-bonded intermediates, probably containing α-helices and β-sheets, are present at early stages in protein folding (for a review, see Kim & Baldwin, 1982) but the

† In addition to assuming that the helix is limited to residues 3 to 12, we assume that the helix is completely melted out at high temperatures, and use $[\theta]_{222}$ at 40°C as a baseline for 0% helix. A value of $-26{,}500$ deg. cm^2 dmol^{-1} is used for complete helix formation. These assumptions have been discussed previously (Bierzynski *et al.*, 1982).

structures of these intermediates have not yet been characterized. Much more work is needed to determine whether isolated α-helices and β-sheets play a dominant role in the initial stages of folding, stabilized by intra-helix or intra-sheet side-chain interactions, or whether tertiary interactions play a critical role in selecting the sequences that are stabilized in α-helical or β-sheet form (cf. Lim, 1974).

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