Side-Chain Interactions in the C-Peptide Helix: Phe 8 ••• His 12^+

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SYNOPSIS

Previous studies have demonstrated that His 12 plays a major role in the pH-dependent stability of the helix formed by the isolated C-peptide (residues 1–13 of ribonuclease A). Here, amino acid replacement experiments show that His 12^+ stabilizes the C-peptide helix chiefly by interacting with Phe 8. The Phe 8 · · · His 12^+ ring interaction is specific for the protonated form of His 12 (His 12^+) and the interaction is not screened significantly by NaCl, unlike the charged group · · · helix dipole interactions studied earlier in C-peptide. Analogs of C-peptide that are unable to form the Phe 8 · · · His 12^+ interaction show large increases in helix content for Phe → Ala and His → Ala. Therefore, the helical tendencies of the individual residues Phe, His, and Ala are important in determining the result of a replacement experiment. Since the side chains of Phe 8 and His 12 probably interact within the N-terminal helix of ribonuclease A, the existence of the Phe 8 · · · His 12^+ interaction in the isolated C-peptide helix adds to the evidence that the C-peptide helix is an autonomous folding unit.

INTRODUCTION

Recent work indicates that side-chain interactions can make an important contribution to the stability of short α-helices in aqueous solution. There are three lines of evidence. First, the helix formed by the 13-residue C-peptide from RNase A is unexpectedly stable in H_2O[1,2] and two charged side chains at opposite ends of the helix, Glu 2^- and His 12^+, help stabilize the helix.[1-4] Second, the charge on the N-terminal residue of C-peptide affects helix stability by a charged group · · · helix dipole interaction[5] and a similar effect is found when there is a charged group at the C-terminus.[6] Third, Glu^- · · · Lys^+ ion pair interactions help stabilize short alanine-based helices when the Glu^- · · · Lys^+ spacing is $i, i + 4$, as judged by reduced helix stability when the spacing is $i, i + 3$.[7]

The aim of this study is to determine the mechanism by which His 12^+ contributes to the stability of the C-peptide helix. The decrease in helix content for pH titrations from pH 5.3 to pH 9.0 first suggested a charged-group effect involving His 12[1] and chemical synthesis of analogs lacking either a free α-NH_3^+ group or His 12^+ confirmed that this effect is produced by His 12^+.[2] It was surprising to find a charged histidine residue promoting helix formation because His^+ is a considerably stronger helix breaker than His^0 in host–guest studies.[8] Synthesis of an analog lacking Glu 9 showed that a Glu 9^- · · · His 12^+ ion pair interaction could not be responsible[2] and two possible mechanisms were considered next.

Since His 12^+ is close to the negative pole of the helix dipole in the C-peptide helix, His 12^+ might stabilize the helix by interacting with the helix dipole.[2] A fixed charge close to either pole of the dipole strongly affects helix stability as shown either by varying the charge[5,6] or by moving a clus-

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ter of charged residues from one end of the helix to the other.[9] Calculation of the charged group · · · helix dipole interaction[10,11] is more complex than the term implies; it is used here as an abbreviation for the effect.

A second possible explanation for the role of His 12^+ in stabilizing the C-peptide helix is that the imidazolium ring of His 12^+ might interact with the ring of Phe 8, as suggested by the juxtaposition of these rings in the crystal structure of RNase A[12] and the occurrence of other aromatic ring interactions.[13-16] If this explanation is correct, then it adds further evidence that the side-chain structure of the isolated C-peptide helix is closely related to the helix structure in intact RNase A, indicating that the C-peptide helix is an autonomous folding unit.[17] A model peptide study provided nmr evidence for this interaction;[18] it did not show whether or not the Phe 8 · · · His 12 interaction is specific for the protonated form of His 12.

Amino acid replacement experiments (e.g., Phe 8 → Ala) can show whether His 12^+ interacts with Phe 8, with the helix dipole, or with both. Furthermore, if a Phe 8 · · · His 12 interaction is detected, pH titration combined with replacement studies would show if Phe 8 interacts only with the protonated form (His 12^+) or also interacts with His 12^0. To do this it is also necessary to analyze the control replacement Phe 8 → Ala in a peptide with Ala 12 in place of His 12. An independent test for a His 12^+ · · · helix dipole interaction is provided by the NaCl screening test.[5,6] A charged group · · · helix dipole interaction is expected to show substantial screening by 3*M* NaCl; failure to find any screening by 3*M* NaCl would indicate that His 12^+ does not interact strongly with the helix dipole.

MATERIALS AND METHODS

Peptide synthesis and purification procedures have been described previously.[2] The sequences of the peptides studied here are given in Table I. The RN series of peptides were made either on the Stewart Mark V or a Biosearch 9500 automatic synthesizer. Amino acid analysis and fast atom bombardment (FAB) mass spectroscopy were used to determine peptide purity. Peptide DAS2 was synthesized manually and purified as described previously.[7]

Peptide concentration was determined by two methods. The quantitative ninhydrin method[19] was used for all peptides; however, the precision of this method is ±5% and the accuracy is ±10% (at best) in our hands. For peptides with the replacement Phe 8 → Tyr, the concentration was determined spectrophotometrically by the use of tyrosine absorbance. The extinction coefficients for free tyrosine and tyrosine model compounds in water and GuHCl have been determined accurately.[20] The extinction coefficients for acetyl-tyrosyl-ethyl ester were used here, since there appears to be little dependence upon sequence for tyrosine absorbance. A comparison of tyrosine absorbance in water and GuHCl (guanidinium chloride) for tyrosine-containing C-peptide analogues shows no dependence on helix formation and the absorbance spectra appear normal. The accuracy of this method is ±1–2%.

The pH of the samples was adjusted at room temperature using NaOH or HCl. CD measurements were made on an AVIV 60DS spectropolarimeter using a 1-cm cell. The temperature was controlled using a water bath or a Hewlett-Packard model 89100A temperature controller. Helix content was measured by mean residue ellipticity at 222 nm ($[\theta]_{222}$) and standard conditions were 20 μM peptide, 3°C, 0.1*M* NaCl, and 1 m*M* each of Na borate, Na citrate, and Na phosphate. Best-fit curves for the pH titration data were made by nonlinear least-squares fitting to the Henderson–Hasselbalch equation as described earlier.[6]

Samples for nmr spectroscopy were 0.5–1.0 m*M* peptide, 0.1*M* NaCl in 100% ^2H_2O (Aldrich) with 0.1 m*M* TSP (Na 3-trimethylsilylpropionate-2,2,3,3-^2H; ICN Biochemicals) as an internal chemical shift standard. Nuclear magnetic resonance data were acquired on a General Electric GN-500 spectrometer equipped with a Nicolet 1280 computer. Data were collected as 128–256 summed transients in 8096 time-domain data points. The sweep width was approximately 5000 Hz. Prior to Fourier transformation, data were multiplied by either a decaying exponential function with a line-broadening parameter of 1 Hz or by a double exponential function (a combined Gaussian and decaying exponential) to enhance resolution. Chemical shifts were corrected for the pH dependence of TSP.[21] The pH values in ^2H_2O are reported as pH* without correction for deuterium isotope effects.

Method of Analysis of Replacement Experiments

We have found that three effects must be accounted for in our replacement experiments. These three effects are (1) a Phe 8 · · · His 12^+ side-chain

Table I Peptide Sequences and Helix Contents

	Peptide[a]	Change	$-[\theta]_{222}$ (pH 5.3)	$-[\theta]_{222}$ (pH 9.0)
Phe 8	RN 21	—	13,200	7,600
	RN 29	Ala 12	8,400	8,600
	RN 48	Ala 8	12,900	10,900
	RN 77	Ala 8, Ala 12	3,700	15,800
	RN 24	SucAla 1	15,100	0,400
	DAS 2	SucAla 1, His 11, Ala 12	8,200	10,700
Tyr 8	RN 80	—	12,600	6,400
	RN 103	SucAla 1	16,400	10,400
	RN 104	SucAla 1, His 11, Ala 12	8,000	10,100

[a] The sequence of RN 21 (reference peptide III[5]) is Ac-AETAAAKFLRAHA-NH_2. RN 80 differs from RN 21 by Phe 8 → Tyr.

interaction; (2) a His $12^+ \cdots$ helix dipole interaction; (3) the helical tendency, h_I of the individual residue I in the absence of specific side-chain interactions.

We introduce the empirical term h to describe helical tendency in the C-peptide system. The helical tendency of residue I (h_I), is defined relative to alanine by the relation (valid in the absence of specific side-chain interactions)

$$h_I \equiv K_I/K_A \tag{1}$$

where K_I and K_A are apparent, two-state, equilibrium constants for the coil to helix reaction and the residue of interest (I) and alanine (A; the reference amino acid), are at residue position i. K_I is defined as

$$K_I \equiv f/(1-f) \tag{2}$$

where f is fraction helix as measured by mean residue ellipticity at 222 nm.

For interior residues, the term h is closely related[22] to s_I/s_A, where s is the helix growth parameter of the Zimm–Bragg model. Note that h is a function of residue position whereas s is not, and values of h for end residues[22] are not expected to be close to s_I/s_A. The quantity h_I (or K_I/K_A) is expected to differ somewhat from the ratio (s_I/s_A) for two reasons. The first is that the two-state approximation is a poor approximation to the Zimm–Bragg equation for short α-helices and is especially so for residues close to the ends of α-helices.[23] The second is that helix formation in water by C-peptide and its derivatives is inadequately represented by the Zimm–Bragg model of α-helix formation because position effects as well as side-chain interactions are neglected. A formalism for including some side-chain interactions has been given.[24]

Experimentally, measurement of h_I is straightforward in the case of an uncharged residue such as Phe or His^0, but is more complicated in the case of a charged residue such as His^+ because of the potential for interactions such as a His $12^+ \cdots$ helix dipole interaction. This interaction, as well as the Phe $8 \cdots$ His 12 interaction, can be subtracted from a residue replacement experiment if we assume that these effects are independent of one another. If Δy_{IJ} represents the total change in $[\theta]_{222}$ in a replacement experiment, then the individual contributions to Δy_{IJ} are

$$\Delta y_{IJ} \cong \Delta y_h + \Delta y_{dip} + \Delta y_{int} \tag{3}$$

where Δy_h depends on the different helical tendencies of residues I and J, Δy_{dip} depends on the His $12^+ \cdots$ helix dipole interaction, and Δy_{int} depends on the Phe $8 \cdots$ His 12 interaction. The additive relation of the individual components follows from considering ΔG^0 for this reaction. ΔG^0 for a two-state reaction is approximately linear in f over the range $0.2 \leq f \leq 0.8$, as may be shown by plotting ΔG^0 vs f, using

$$\Delta G^0 = -RT \ln K = -RT \ln[f/(1-f)] \tag{4}$$

By analogy, we suppose that ΔG^0 is also approximately linear in f for helix formation by C-peptide, which is not a two-state reaction. When independent interactions contribute to an overall equilibrium constant, the ΔG^0 values for these interactions are additive, and it follows that the Δy terms in eq. (3) also are approximately additive in the range prescribed ($21{,}600 \geq -[\theta]_{222} \geq 5{,}400$).

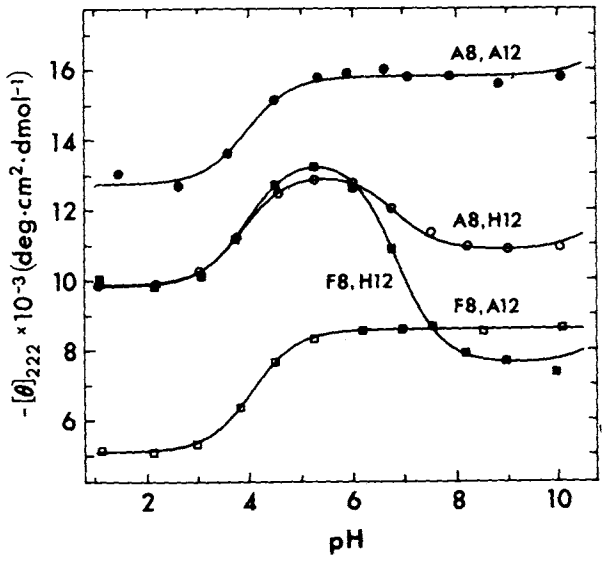

Figure 1. pH dependence of helix content at 3°C, 0.1*M* NaCl, for peptides RN 77 (●), RN 48 (○), RN 29 (□), and RN 21 (■). The sequences of these peptides are given in Table I. $[\theta]_{222}$ is the mean residue ellipticity at 222 nm.

RESULTS

Helical Tendencies of Individual Residues

Table I lists the peptides studied here and their helical contents at pH 5.3 and pH 9.0. Figure 1 shows the pH profile of helix content for four peptides: the reference sequence (RN 21, with Phe 8, His 12) and peptides with either or both of the replacements Phe 8 → Ala, His 12 → Ala. For peptides containing Glu 2, Phe 8, Arg 10, and His 12, the pH profile of helix content is governed by formation of a Glu 2^- ··· Arg 10^+ ion pair interaction in going from pH 2 to pH 5.3 (Shoemaker et al., manuscript in preparation) and by breaking a Phe 8 ··· His 12^+ interaction in going from pH 5.3 to pH 9.0, as we show here.

After completion of some of the work described here, we found that the accuracy and reproducibility of the peptide concentrations, and hence the values of $-[\theta]_{222}$, could be improved from ±10% (ninhydrin) to ±1–2% by replacing Phe 8 with tyrosine and then measuring peptide concentration by tyrosine absorbance. This change in procedure markedly improves the accuracy of a replacement experiment, which involves two concentration measurements, one for each peptide. Peptides with Tyr 8 are also listed in Table I. The helix-forming properties of RN 80 (with Tyr 8) and RN 21 (with Phe 8) are similar up to pH 9.0, where the ionization of tyrosine begins to affect the results. The helical tendency of Phe 8 is evaluated by making the replacement Phe 8 → Ala in peptides with Ala 12. The result (Table II) is that Phe 8 has a surprisingly low helical tendency relative to Ala: $h = 0.49$ whereas $s_F/s_A = 0.98$. To find the helical tendency of His 12^0, a similar analysis is used. The replacement experiment His 12 → Ala is made in peptides with Ala 8, and h is measured at pH 9.0, where His 12 is uncharged. Then, to find the helical tendency of His 12^+, it is necessary to measure the difference between His^+ and His^0 in conditions where His^+ does not interact significantly with the helix dipole. In Table II, the replacement $His^+ \rightarrow His^0$ has been made by pH titration for a peptide with His 11 (peptide DAS 2), in which the interaction of His 11^+ either with Phe 8 or with the helix dipole is believed to be small (see Discussion).

Residue Interactions

The Phe 8 ··· His 12 interaction has been studied by an interchange experiment: Ala 11 and His 12 have been interchanged both in DAS 2 and in RN 104. This interchange experiment directly measures the minimum strength of the Phe 8 ··· His 12 (or Tyr 8 ··· His 12) interaction provided (1) the helical tendency of His 11 is the same as that of His 12 and (2) the interaction of His 11^+ in DAS 2, either with Phe 8 or with the helix dipole, is small enough to be neglected. In assigning the result of the interchange experiment to the Phe 8 ··· His 12

Table II Helical Tendencies of Individual Residues

Residue	h	s/s_A[a]	Replacement	pH	Peptides	$-\Delta[\theta]_{222}$
Phe 8	0.49	0.98	Phe 8 → Ala	5.3, 9.0	RN 29 → 77	7,300
His 12^0	0.48	0.91	His 12^0 → Ala	9.0	RN 48 → 77	4,900
His 12^+	0.32	0.64	His 12^+ → Ala[b]	5.3	[b]	7,400

[a] Ratio of the host–guest value of s^8 for this residue to s for alanine.
[b] Computed for His 12^+ by combining the result of pH titration of His $11^+ \rightarrow$ His^0 in DAS 2 ($-\Delta[\theta]_{222} = 2{,}500$) with the data for His 12^0 → Ala above.

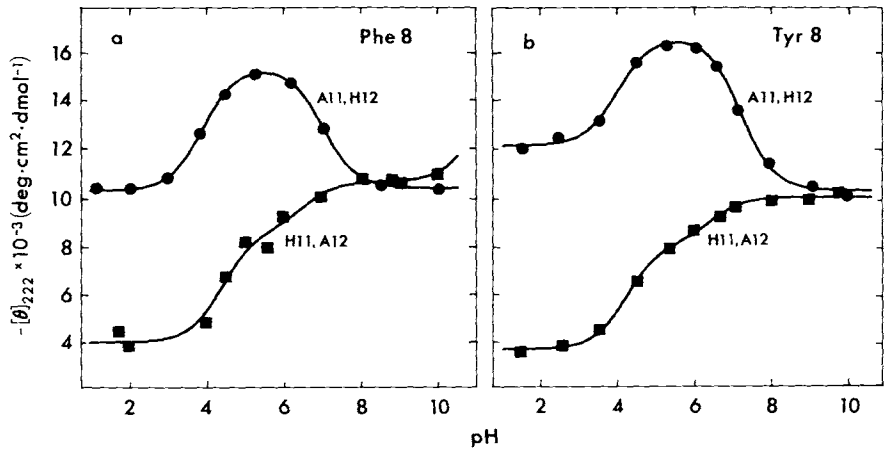

Figure 2. The pH dependence of helix content at 3°C, 0.1*M* NaCl (a) for peptides with Phe 8—RN 24 (●) and DAS 2 (■)—and (b) for peptides with Tyr 8—RN 103 (●) and RN 104 (■). Their sequences are given in Table I.

interaction, we rely on experiments (see below) showing that the Phe 8 · · · His 12 interaction is dominant and the His 12^+ · · · helix dipole interaction is minor in peptides with Phe 8 and His 12. Because of its importance, the interchange experiment has been repeated using peptides with Tyr 8, for which the peptide concentrations are known accurately, and both sets of results are given in Figure 2 and in Table III.

These results indicate that there is a strong interaction between Phe 8 and His 12^+ ($-\Delta[\theta]_{222}$ = 6,900), but no significant interaction between Phe 8 and His 12^0 (Figure 2, Table III). Thus, the Phe 8 · · · His 12 interaction is specific for the protonated form of His 12.

In peptide RN 48 with Ala 8, His 12^+ interacts strongly with the helix dipole. This can be seen from the pH profile of helix content for RN 48 (Figure 1), in which the helix content drops as His $12^+ \rightarrow$ His^0. To quantitate the interaction of His 12^+ with the helix dipole in RN 48, we subtract the effect of His $11^+ \rightarrow$ His^0 in DAS 2 from that of His $12^+ \rightarrow$ His^0 in RN 48. The result shows that forming the His 12^+ · · · helix dipole interaction in RN 48 corresponds to $-\Delta[\theta]_{222}$ = 4,500 (Table III).

NaCl Screening Test for a His 12^+ ··· Helix Dipole Interaction

Earlier experiments with C-peptide analogues[5,6] show that the interaction between the helix dipole and a fixed charge close to one end of the helix can be screened by NaCl. Screening is substantial, but not complete, by 3*M* NaCl. This result agrees with careful screening experiments done with a different peptide helix system,[9] when those experiments are interpreted as measuring a helix dipole interaction. Application of this test is shown in Figure 3 by comparing the pH profiles of helix content in 0.1*M* vs 3.0*M* NaCl.

The individual features of these experiments are as follows: (1) There is a His 12^+ · · · helix dipole interaction in peptide RN 48, with Ala 8, as shown by substantial screening in 3*M* NaCl (Figure 3b). (2) The Phe 8 · · · His 12^+ interaction in RN 21

Table III Residue Interactions

Interaction	pH	Peptides	$-\Delta[\theta]_{222}$
Phe 8 · · · His 12^+	5.3	DAS 2 → RN 24	6,900
Phe 8 · · · His 12^0	9.0	DAS 2 → RN 24	−300
His 12^+ · · · dipole[a]	5.3 → 9.0	RN 48, DAS 2	4,500
Tyr 8 · · · His 12^+	5.3	RN 104 → RN 103	8,200
Tyr 8 · · · His 12^0	9.0	RN 104 → RN 103	300

[a] The His 12^+ · · · helix dipole interaction is measured in peptides in which the Phe 8 · · · His 12 interaction is absent.

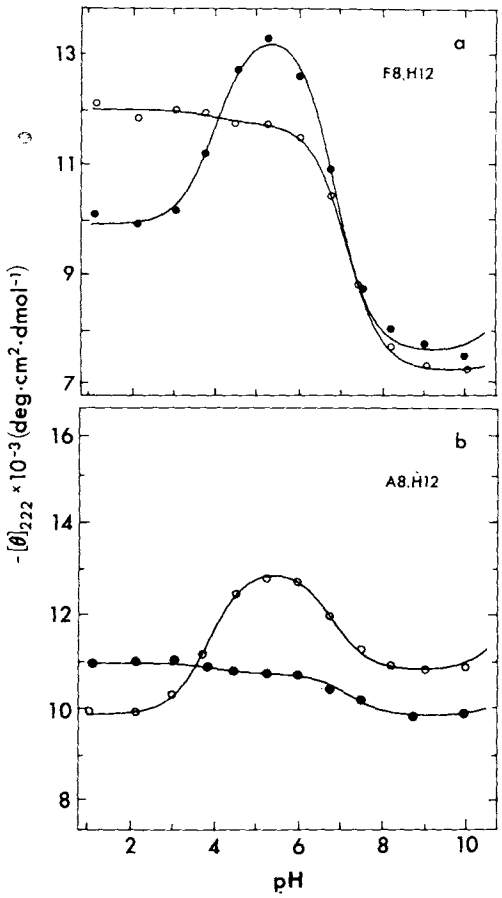

Figure 3. The pH dependence of helix content at 3°C, 0.1*M* NaCl (a) for peptides RN 21 in 0.1*M* (●) and 3.0*M* (○) NaCl, and (b) RN 48 in 0.1*M* (○) and 3.0*M* (●) NaCl.

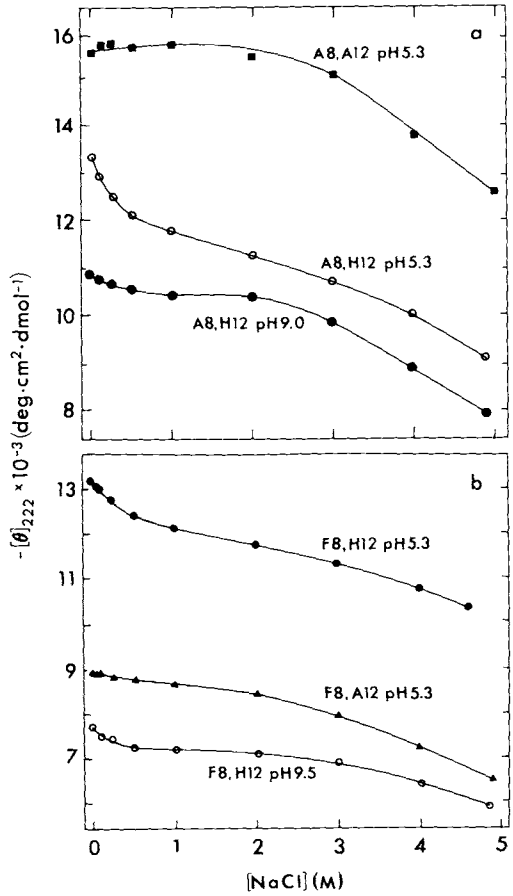

Figure 4. NaCl dependence of helix content at 3°C for (a) peptides RN 48 [pH 5.3 (○) and pH 9.0 (●)] and RN 77 [pH 5.3 (■)] and (b) peptides RN 21 [pH 5.3 (●) and pH 9.5 (○)] and RN 29 [pH 5.3 (▲)].

shows little screening in 3*M* NaCl (Figure 3a), and there is, at best, a minor interaction of His 12^+ with the helix dipole when Phe 8 is present, as judged by NaCl screening. (3) The Glu $2^- \cdots$ Arg 10^+ ion pair interaction is screened by 3*M* NaCl (see peptide RN 21, Figure 3a, for which this interaction can be studied in isolation). Studies of this interaction will be described in more detail (Shoemaker et al., in preparation).

Complete NaCl screening curves show these effects in more detail. The principle is that two peptides that differ in only one residue will show parallel NaCl screening curves if they have the same screenable interactions, but if the replacement gives one peptide an additional screenable interaction, that peptide will show additional NaCl screening, especially between 0 and 1*M* NaCl. Thus, in Figure 4a the NaCl screening curve for RN 48 (with Ala 8, His 12) shows a sharp change in $-[\theta]_{222}$ between 0 and 1*M* NaCl at pH 5.3 (His 12^+) as compared either to pH 9.0 (His^0) or to peptide RN 77 (Ala 12). On the other hand, in Figure 4b the screening curve for peptide RN 21 (Phe 8, His 12) shows little change in shape between 0 and 1*M* NaCl in going from pH 5.3 (His$^+$) to pH 9.5 (His^0), and is also similar to the curve for peptide RN 29 (Ala 12). Thus, little NaCl screening of the Phe 8 $\cdots$ His 12^+ interaction is observed

Proton NMR Studies

Most of the well-resolved resonances in the ^{1}H-nmr spectra of RN 21, RN 29, and RN 48 were assigned by comparison with the spectrum of RN 24.[25] RN 24 differs from RN 21 in the presence of a succinyl group instead of an acetyl group at the N-terminus. Phe 8 ring proton resonances for RN 21 and RN 29 were assigned through analysis of their spin-coupling multiplicities and relative intensities; a triplet with single proton intensity was assigned to ζ, a

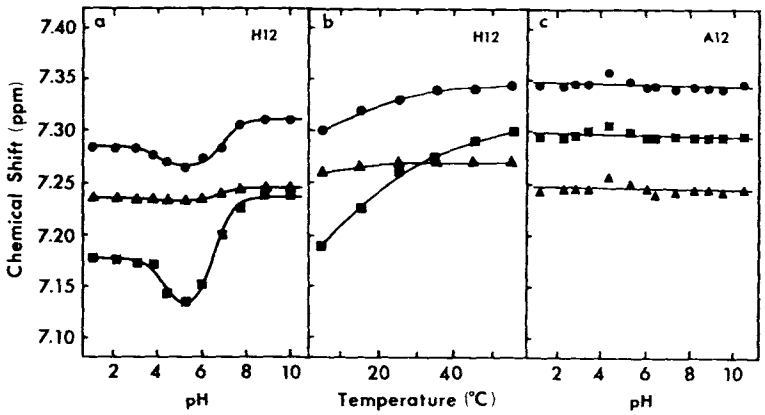

Figure 5. Chemical shifts of three sets of Phe 8 ring proton resonance lines as a function of pH and temperature: (a) and (b) peptide RN 21 (F8, H12); (c) peptide RN 29 (F8, A12) for Phe 8 δ (▲), ϵ (●), and ζ (■). Standard conditions are (see Methods) 3°C (a, c) and pH 5.3 (6).

triplet with two-proton intensity to ϵ, and a doublet with two-proton intensity to δ.

In C-peptide, the chemical shifts of side-chain resonances in Phe 8 and His 12 reflect the pH dependence of helix content,[1] indicating that these side-chain conformations are different in the helix and in the random coil. The His 12 C2H and C4H resonances of RN 21, at pH* 5.3, are approximately 0.2 ppm upfield of their positions in the spectrum of RN 48, a peptide in which Phe 8 → Ala, showing that the Phe 8 and His 12 rings are near each other. This result was seen earlier in a study of a similar C-peptide analogue.[18] The chemical shift difference is observed at low pH as well. Above pH* 5.3, the upfield shifts of the C4H and C2H resonances in RN 21 are smaller, about 0.15 and 0.1 ppm, respectively.

Comparison of the Phe 8 ring proton resonances in RN 29, in which His 12 → Ala, with those in RN 21 provides further evidence for a pH-dependent interaction between Phe 8 and His 12 (Figure 5). The ζ resonance of RN 21 is shifted about 0.2 ppm upfield at pH* 5.3 relative to its position in RN 29, the ϵ resonance is approximately 0.08 ppm upfield, and the δ resonance is hardly shifted. The ζ and ϵ chemical shifts in RN 21 show marked pH dependencies, and their upfield shifts relative to RN 29 show minima at pH* 5.3. The δ chemical shift is nearly independent of pH. In RN 29 the Phe 8 ring proton chemical shifts show no pH dependencies (Figure 5c). It is noteworthy that the chemical shift of the ζ proton provides a sensitive probe of the Phe 8 $\cdots$ His 12^+ interaction. In peptide RN 29, where this interaction is absent, the chemical shift of the ζ proton falls midway between those of the δ and ϵ protons. In peptide RN 21, where this interaction is present, the chemical shift of the ζ proton falls below that of δ at pH 5.3 but approaches the noninteracting position seen in RN 29, midway between the δ and ϵ protons, either as the temperature increases (Figure 5b) or as His $12^+ \rightarrow$ His^0 (Figure 5b).

The chemical shift inequivalence between the two His 12 Hβ resonances is large (Figure 6), indicating restricted motion of the His 12 side chain, and there is also a substantial change caused by the replacement Phe 8 → Ala, in agreement with an earlier study.[18] At pH* 5.3, one of the Hβ resonances of RN 21 is approximately 0.3 ppm upfield of the highest field Hβ resonance of RN 48. This high-field resonance moves downfield below pH* 5.3 and has no observable pH dependence above this pH. The high pH behavior is probably a consequence of compensatory changes in chemical shift due to deprotonation of the imidazolium ring, which is expected to result in an upfield shift and loss of helix content, which probably brings the chemical shift back downfield toward the RN 48 chemical shifts.

The magnitude of a three-bond coupling constant (^3J) is dependent on the torsion angle between the coupled protons.[26] In peptides and proteins, coupling between Hα and Hβ ($^3J_{\alpha\beta}$) can provide a measure of χ_1, the torsion angle between C_α and C_β, provided stereospecific assignments of the two Hβ resonances can be made. In the absence of such assignments, the coupling constants still provide information on whether or not χ_1 has a unique value. The His 12 Hα resonance in RN 21 is a doublet of doublets ($^3J_{\alpha\beta}$ = 5.0 and 9.5 Hz) at

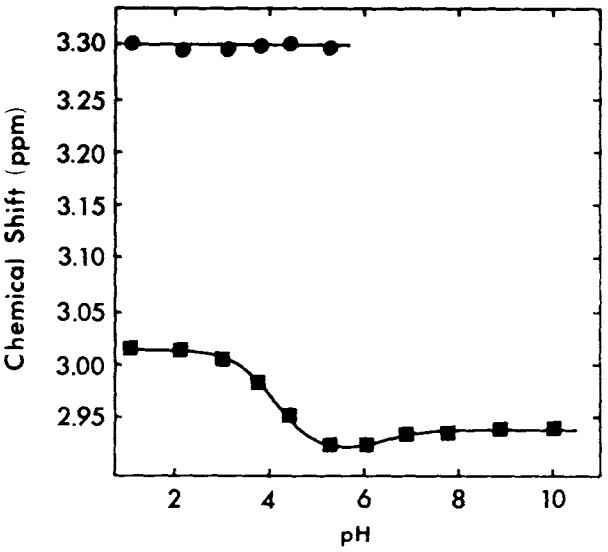

Figure 6. Chemical shift of His 12 Hβ resonances as a function of pH for peptide RN 21 (F8, H12).

and below pH* 5.3. Such a coupling pattern suggests that one χ_1 is favored when His 12 is protonated. The coupling pattern collapses to a triplet at alkaline pH. In RN 48, the His 12 Hα is a triplet over the whole pH range studied. The presence of Phe 8 thus appears to restrict the motion of the protonated His 12 side chain.

In order to assess the contribution of helix content to the anomalous Phe 8 and His 12^+ chemical shifts in RN 21, chemical shifts were monitored as a function of temperature. As the temperature is increased, the Phe 8 and His 12^+ chemical shifts move towards the values observed in RN 29 and RN 48, respectively (Figure 5b; other data not shown). Therefore, the chemical shift differences observed at pH* 5.3, 3.0°C, are dependent on helix content and not just on the absence of Phe 8 or His 12.

DISCUSSION

Effect on Helix Stability of the Helical Tendencies of Individual Residues (Phe, His, Ala)

A surprising result of these replacement experiments is the large effect of Phe 8 → Ala in peptides with Ala 12 (Table II). The effect of His 12 → Ala is also large in peptides with Ala 8 (Table II). The large effect of His → Ala is not surprising, because His^+ has a low host–guest value of s [0.69 at 0°C, relative to 1.08 for Ala[8]]. In the case of Phe, whose host–guest value of s is 1.06 at 0°C, the effect is surprising. The helical tendency of a residue relative to Ala is expected to be closely related to its s value divided by that of Ala [see the discussion of eq. (1)]. Our result for Phe → Ala does not agree with the host–guest values of s for these residues. A study of Ala → Gly replacements at positions 4, 5, and 6 in RN 21 showed[22] that the helical tendency of Gly is predicted correctly, to a first approximation, from the ratio of the s values of Gly and Ala found by the host–guest method. On the other hand, a study of alanine-based peptides of de novo design, solubilized by insertion of three or more Lys^+ residues, found that the helical stability of these peptides is much higher than predicted by their host–guest values of s.[27]

Mechanism by Which His 12^+ Stabilizes the C-Peptide Helix

A ring interaction between Phe 8 and His 12^+ is the primary mechanism by which His 12^+ stabilizes the C-peptide helix. This conclusion is based on two experiments. First, studies of the replacement Phe 8 → Ala show that Phe 8 contributes to helix stability. Although $-\Delta[\theta]_{222}$ is small for Phe 8 → Ala when His 12^+ is present (RN 21 → RN 48, Table I), the effect of breaking the Phe 8 · · · His 12^+ interaction is masked by two compensating effects. First, Phe 8 has a much lower helical tendency than Ala 8 in peptides with Ala 12 ($-\Delta[\theta]_{222}$ = 7,300 for Phe 8 → Ala, Table II). Second, in a peptide with Ala 8, His 12^+ makes a new interaction with the helix dipole ($-\Delta[\theta]_{222}$ = 4,500, Table III). As explained in the section on analysis of replacement experiments, these values, which are formed by subtracting the result of one replacement experiment from another, are not very accurate because of the uncertainty in peptide concentration. Consequently, the strength of the Phe 8 · · · His 12^+ interaction is estimated by a different route, using an interchange experiment, and the interchange experiment has been repeated with peptides containing Tyr 8, so that the peptide concentrations are known accurately from tyrosine absorbance (Table III). Tyr 8 interacts with His 12^+ somewhat differently than Phe 8 interacts with His 12^+, as judged by the pK_a of His 12 in RN 80 and in RN 103 (7.1) as compared to the pK_a in RN 21 and RN 24 (6.3).

The interchange experiment by itself can be interpreted either in terms of a Phe 8 · · · His 12^+ or of a His 12^+ · · · helix dipole interaction, since either interaction could be broken by the interchange of His 12 with Ala 11. The replacement Phe

8 → Ala shows that the Phe 8 · · · His 12^+ interaction is the major effect, and this conclusion is confirmed by the failure to find a NaCl-screenable His 12^+ · · · helix dipole interaction in RN 21. Other experiments with C-peptide analogues show that charged group · · · helix dipole interactions are screened substantially with NaCl[5,6] and the lack of screening here argues against a His 12^+ · · · helix dipole interaction. Unfortunately, the Phe 8 → Ala replacement experiment is complex: in addition to breaking the Phe 8 · · · His 12^+ interaction (RN 21 → RN 48), it is necessary to take account of the large difference in helical tendency between Phe 8 and Ala 8 (RN 29 → RN 77) and of the helix-stabilizing His 12^+ · · · helix dipole interaction in RN 48.

The interchange experiment measures the minimum strength of the Phe 8 · · · His 12^+ interaction because it neglects any helix-stabilizing interaction of His 11 either with Phe 8 or with the helix dipole. Since His^+ is more strongly helix breaking than His^0 in host–guest studies, an increase in helix content is expected for $His^+ \rightarrow His^0$ if the His residue interacts neither with the helix dipole nor with Phe 8. This increase in helix content is observed when His $11^+ \rightarrow His^0$ both in peptides DAS 2 and RN 104, (Figure 2), which suggests that any interaction of His 11^+ with either the helix dipole or Phe 8 is small.

The interchange experiment shows that the Phe 8 · · · His 12^+ interaction is entirely dependent on His 12 being protonated (Table III). This fact suggests that the interaction is electrostatic in nature even though it is not screened significantly by NaCl. An electrostatic model for predicting the relative strengths of interaction between H-bond donors and aromatic rings[28] would, if applied here, predict that the Phe 8 · · · His 12^+ interaction is strongly dependent on protonation of His 12. Quantum mechanical calculations[29] for an $(i, i + 4)$ Phe · · · His interaction predict that the interaction should be stronger when His is protonated.

The Phe 8 · · · His 12^+ interaction in the isolated peptide helix was first proposed on the basis of ^{1}H-nmr studies of both model peptides[30] and S-peptide.[4] It was concluded from nmr data[18] that Phe 8 interacts with His 12^0 as well as with His 12^+. The latter conclusion was based on an analysis of chemical shifts of ring protons in both residues. Our data are consistent with this deduction, but the interchange experiment shows clearly that the Phe 8 · · · His 12^0 interaction contributes much less to helix stability than the Phe 8 · · · His 12^+ interaction.

The C-Peptide Helix as an Autonomous Folding Unit: Role of the Phe 8 ··· His 12^+ Interaction

In the crystal structure of RNase A,[12] the side chains of Phe 8 and His 12 are positioned so as to form an aromatic interaction of the type described by several workers.[13–16] The two aromatic rings adopt an approximately edge (His 12)-to-face (Phe 8) alignment characteristic of these interactions, with a closest approach of 2.5 Å between the HD1 atom of Phe and the HD2 atom of His 12. A Phe 8 · · · His 12^+ interaction probably underlies the use of the His 12 C2H resonance as a probe in detecting partial formation of the N-terminal helix in otherwise unfolded RNase A.[31–33] In the folded protein, the imidazole ring of His 12 may be primarily fixed by a hydrogen bond between His 12 HD1 and Thr 45 main-chain CO.[34,35] Although this interaction is obviously missing in the C-peptide helix, the results here indicate that it is not required for the Phe 8 · · · His 12^+ interaction.

An autonomous folding unit has sufficient information in its sequence to direct its own folding to a structure resembling that found in the intact protein.[17] By these criteria, the C-peptide helix appears to be an autonomous folding unit. As we show here, the Phe 8 · · · His 12^+ interaction contributes significantly to the stability of the isolated helix. The coupling constant of the His 12 peptide NH is substantially larger than others in the C-peptide helix,[25] suggesting that the helix stops at His 12. The detailed geometry of the Phe 8 · · · His 12^+ ring interaction in the isolated C-peptide helix and its role in helix termination need to be investigated.

APPENDIX

By studying tyrosine-containing peptides whose concentrations are known accurately, it is possible to determine better values of $-[\theta]_{222}$ for 0 and 100% helix content than were available previously. To obtain the value for 100% helix, we take a strong helix former, RN 103, and use the TFE (trifluoroethanol) titration method.[27,36,37] For reasons that are not well understood, TFE promotes cooperative helix formation only until about 12 mole % TFE is added. A break in the curve of $-[\theta]_{222}$ vs mole % TFE occurs near 12% TFE whether or not complete helix formation is obtained.[36,37] Consequently, to be confident that a plateau level of $-[\theta]_{222}$ reflects complete helix formation,[27] the curve must break below 12 mole %. The results for RN 103 (Figure 7) break just below 10 mole %, and the value obtained by extrap-

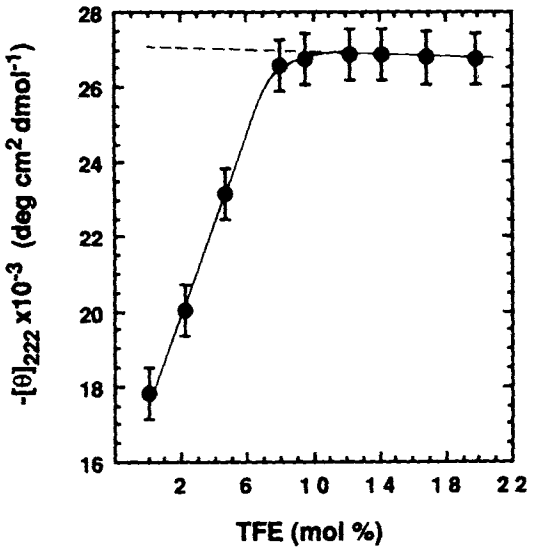

Figure 7. TFE titration of helix content for peptide RN 103 at 3°C, 0.1*M* NaCl. See appendix.

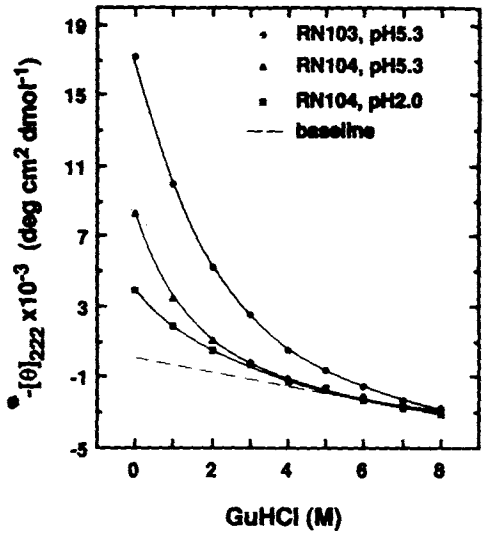

Figure 8. Determination of the baseline curve (for 0% helix content) of $-[\theta]_{222}$ vs *M* GuHCl. GuHCl-induced unfolding curves are shown at 3°C, 0.1*M* NaCl, for three cases: strong helix formation (● RN 103, pH 5.3), moderate (▲ RN 104, pH 5.3), and weak (■ RN 104, pH 2.0) helix formation.

olating back to 0% TFE (27,000 ± 700 deg cm^2 dmol^{-1}) represents a reasonable value for 100% helix. In any case, this should be a reliable minimum value of $-[\theta]_{222}$ for 100% helix. It agrees satisfactorily with an early rough estimate[1] of 26,500 ± 1,500 and with a study[38] made by adding derivatives of S-peptide (1–15) to S-protein: the increase in $-[\theta]_{222}$ was attributed solely to a change in helix content of S-peptide (1–15). A value of 29,000 ± 1000 deg cm^2 dmol^{-1} was obtained for $-\Delta[\theta]_{222}$, the change in $-[\theta]_{222}$ between 0 and 100% helix content; the value of $-[\theta]_{222} = -2{,}500$ deg cm^2 dmol^{-1} for 0% helix content was taken from another study,[39] and the value of $-[\theta]_{222}$ for 100% helix is 26,500 deg cm^2 dmol^{-1}. They made measurements on analogues of S-peptide (1–15), which have 15 residues in contrast to the 13-residue peptides studied here.

To obtain a value for 0% helix, we use tyrosine-containing peptides to repeat an earlier experiment.[39] The GuHCl unfolding curves are measured for three peptides: strong, moderate, and weak helix formers, respectively. Their unfolding curves coincide at GuHCl concentrations where complete unfolding is obtained, and the baseline for the weak helix former is extrapolated back to 0*M* GuHCl to give $-[\theta]_{222} = 200 \pm 500$ deg cm^2 dmol^{-1} at 0% helix content (Figure 8). Our data agree reasonably with the earlier results[39] for these three peptides whose concentrations were not known accurately. Consequently, it was not possible to determine where their GuHCl unfolding curves coincided. Instead, it was observed that the curve of $-[\theta]_{222}$ vs *M* GuHCl is independent of *M* GuHCl for P-peptide, an 8-residue fragment of C-peptide, and the horizontal P-peptide curve yielded a value of $-[\theta]_{222} = -2{,}500 \pm 500$ deg cm^2 dmol^{-1}. The P-peptide value agrees satisfactorily in 8*M* GuHCl with the values shown in Figure 8. When GuHCl-induced unfolding is measured at high temperatures (45–80°C), where the helix content is strongly reduced, the results show clearly that $-[\theta]_{222}$ decreases with increasing *M* GuHCl (data not shown). Consequently, we now regard the P-peptide data[39] as anomalous and adopt 200 ± 500 deg cm^2 dmol^{-1} for the value of $-[\theta]_{222}$ at 0% helix, 3°C.

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REFERENCES

1. Bierzyński, A., Kim, P. S. & Baldwin, R. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2470–2474.
2. Shoemaker, K. R., Kim, P. S., Brems, D. N., Marqusee, S., York, E. J., Chaiken, I. M., Stewart, J. M. & Baldwin, R. L. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2349–2353.
3. Rico, M., Gallego, E., Santoro, J., Bermejo, F. J. Nieto, J. L. & Herranz, J. (1984) *Biochem. Biophys. Res. Comm.* **123**, 757–763.
4. Rico, M., Santoro, J., Bermejo, F. J., Herranz, J., Nieto, J. L., Gallego, E. & Jiménez, M. A. (1986) *Biopolymers* **25**, 1031–1053.
5. Shoemaker, K. R., Kim, P. S., York, E. J., Stewart, J. M. & Baldwin, R. L. (1987) *Nature* **326**, 563–567.
6. Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M. & Baldwin, R. L. (1989) *Proteins* **5**, 1–7.
7. Marqusee, S. & Baldwin, R. L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8898–8902.
8. Sueki, M., Lee, S., Powers, S. P., Denton, J. B., Konishi, Y. & Scheraga, H. A. (1984) *Macromolecules* **17**, 148–155.
9. Ihara, S., Ooi, T. & Takahashi, S. (1982) *Biopolymers* **21**, 131–145.
10. Hol, W. G. J. (1985) *Prog. Biophys. Mol. Biol.* **45**, 149–195.
11. Godzik, A. & Wesolowski, T. (1988) *Biophys. Chem.* **31**, 29–34.
12. Wlodawer, A., Svensson, L. A., Sjölin, L. & Gilliland, G. L. (1988) *Biochemistry* **27**, 2705–2717.
13. Burley, S. K. & Petsko, G. A. (1985) *Science* **229**, 23–28.
14. Singh, J. & Thornton, J. M. (1985) *FEBS Lett.* **191**, 1–6.
15. Blundell, T., Singh. J., Thornton, J., Burley, S. K. & Petsko, G. A. (1986) *Science* **234**, 1005.
16. Burley, S. K. & Petsko, G. A. (1986) *FEBS Lett.* **203**, 139–143.
17. Shoemaker, K. R., Fairman, R., Kim, P. S., York, E. J., Stewart, J. M. & Baldwin, R. L. (1987) *Cold Spring Harbor Symp. Quant. Biol.* **LII**, 391–398.
18. Dadlez, M., Bierzyński, A., Godzik, A., Sobocinska, M. & Kupryszewski, G. (1988) *Biophys. Chem.* **31**, 175–181.
19. Rosen, H. (1957) *Arch. Biochem. Biophys.* **67**, 10–15.
20. Brandts, J. F. & Kaplan, L. J. (1973) *Biochemistry* **12**, 2011–2024.
21. DeMarco, A. (1977) *J. Magn. Reson.* **26**, 527–528.
22. Strehlow, K. G. & Baldwin, R. L. (1989) *Biochemistry* **28**, 2130–2133.
23. Schellman, J. A. (1958) *J. Phys. Chem.* **62**, 1485–1494.
24. Vásquez, M. & Scheraga, H. A. (1988) *Biopolymers* **27**, 41–58.
25. Osterhout, J. J., Baldwin, R. L., York, E. J., Stewart, J. M., Dyson, H. J. & Wright, P. E. (1989) *Biochemistry* **28**, 7059–7064.
26. Karplus, M. (1959) *J. Phys. Chem.* **30**, 11–15.
27. Marqusee, S., Robbins, V. M. & Baldwin, R. L. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5286–5290.
28. Levitt, M. & Perutz, M. F. (1988) *J. Mol. Biol.* **201**, 751–754.
29. Bermejo, F. J., Rico, M., Santoro, J., Herranz, J., Gallego, E. & Nieto, J. L. (1986) *J. Mol. Struct.* **142**, 339–342.
30. Bierzyński, A., Dadlez, M., Sobocinska, M. & Kupryszewski, G. (1986) *Biophys. Chem.* **25**, 127–134.
31. Blum, A. D., Smallcombe, S. H. & Baldwin, R. L. (1978) *J. Mol. Biol.* **118**, 305–316.
32. Bierzyński, A. & Baldwin, R. L. (1982) *J. Mol. Biol.* **162**, 173–186.
33. Swadesh, J. K., Montelione, G. T., Thannhauser, T. W. & Scheraga, H. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 4606–4610.
34. Wlodawer, A. & Sjölin, L. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2853–2855.
35. Wlodawer, A. & Sjölin, L. (1983) *Biochemistry* **22**, 2720–2728.
36. Nelson, J. W. & Kallenbach, N. R. (1986) *Proteins* **1**, 211–217.
37. Merutka, G. & Stellwagen, E. (1989) *Biochemistry* **28**, 352–357.
38. Mitchinson, C. & Baldwin, R. L. (1986) *Proteins* **1**, 23–33.
39. Shoemaker, K. R., Fairman, R., York, E. J., Stewart, J. M. & Baldwin, R. L. (1988) in *Proceedings of the American Peptide Symposium, 10th*, pp. 15–20.

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