Low-temperature ^{1}H-NMR evidence of the folding of isolated ribonuclease S-peptide

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The temperature (−7°C to 45°C, pH 5.4) and pH (0°C) dependence of ^{1}H chemical shifts of ribonuclease S-peptide (5 mM, 1 M NaCl) has been measured at 360 MHz. The observed variations evidence the formation of a partial helical structure, involving the fragment Thr-3–Met-13. Two salt-bridges stabilize the helix: those formed by Glu-9$^-$...His-12$^+$ and Glu-2$^-$...Arg-10$^+$. The structural features deduced from the ^{1}H-NMR at low temperature for the isolated S-peptide are compatible with the structure shown by the same molecule in the ribonuclease S crystal.

^{1}H-NMR *Ribonuclease S-peptide* *Peptide folding* *Helical structure* *Sidechain interaction*

1. INTRODUCTION

In [1,2], we reported the ^{1}H-NMR parameters of ribonuclease S-peptide (N-terminal 19 fragment) and C-peptide (13 fragment) at room temperature and pH 1.5–11.5. Under the conditions studied, no evidence was found for the existence of any preferred structure. We therein announced further studies in more favourable conditions for helix formation; i.e., low temperature and high saline concentration. We here report the results obtained in such a study, which clearly substantiate the fact that the S-peptide forms a partial helical structure (Thr-3–Met-13) at <20°C.

Authors in [3–6] examined the helix formation properties of C-peptide lactone (13-terminal homoserine lactone), C-peptide carboxylate (13-terminal homoserine) and S-peptide, by CD and ^{1}H-NMR. They confirmed the observations in [7] according to which the C-peptide forms intramolecularly a partial helical structure in water at 1°C in 0.1 M NaCl. From the C-peptide CD spectra dependence on pH they suggested that the salt bridge Glu-9$^-$...His-12$^+$ stabilizes the helix and that the lactone form is essential, since otherwise the terminal carboxylate competes with the one from Glu-9$^-$ and suppresses helix formation.

Our work, which involves a thorough analysis of the S-peptide ^{1}H-NMR spectra, provides a means of simultaneously monitoring all regions of the peptide in the folding process. The analysis of the pH and temperature dependence of chemical shifts provides information about the main features of the structure formed at low temperature. They are compatible with the structure shown by the S-peptide in native ribonuclease S [8].

We provide additional evidence about the important fact that a small peptide forms a folded

Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TSP, sodium 2,2,3,3-tetradeutero-3-trimethylsilylpropionate; abbreviations for the amino acids are those recommended by the IUPAC–IUB commission on biochemical nomenclature; Im, imidazole; Gnd, guanidino

Definitions: Ribonuclease S, a derivative of ribonuclease A (EC 3.1.27.5) obtained by cleavage with subtilisin (EC 3.4.21.14); S-peptide, the ribonuclease S fragment containing the N-terminal amino acids of ribonuclease A; C-peptide, the N-terminal peptide of ribonuclease A obtained by cyanogen bromide cleavage

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structure at low temperature. Assuming that the behaviour of the S-peptide is not singular, our work shows that low temperature high field ^{1}H-NMR can be of great value on investigating the structure of functional peptides in the absence of their receptors, or in measuring the helix formation propensities of some given sequences of amino acid residues in the search for a code of helix formation in proteins.

2. MATERIALS AND METHODS

The S-peptide was purchased from Sigma (lot no.72F-8000) and used without further purification. As described in [1], the S-peptide was identified as 70% S-peptide 1–19 and 30% S-peptide 1–20. For the NMR measurements, a 5-mM solution of the S-peptide in D_2O, 1 M NaCl was used. Shifts are given with reference to internal TSP. The pH-values given are uncorrected pH-meter readings. Spectra were taken in the FT mode on a Bruker WM-360 spectrometer. Probe temperatures were calibrated with a methanol thermometer.

3. RESULTS

The variation with temperature of the chemical shifts of H_α protons of S-peptide, 5 mM, 1 M NaCl (pH 5.4) is given in fig.1. The spectral assignments have been reported in [1]. Resonances of Ala-4, Ala-5 and Ala-6 have been unequivocally assigned by preliminary titration experiments of the peptide 3–19. Fig.1 shows the different behaviour of H_α proton resonances with temperature, depending on their position in the S-peptide sequence. Those belonging to the fragment Lys-7–His-12 show a steep descent with temperature (below 15°C), whereas those in the terminal residues show, with the exception of Ala-4, no, or very modest slopes. The H_α signals shift upfield, in the same way that they do in homopolymers undergoing the random coil to helix transition [9,10].

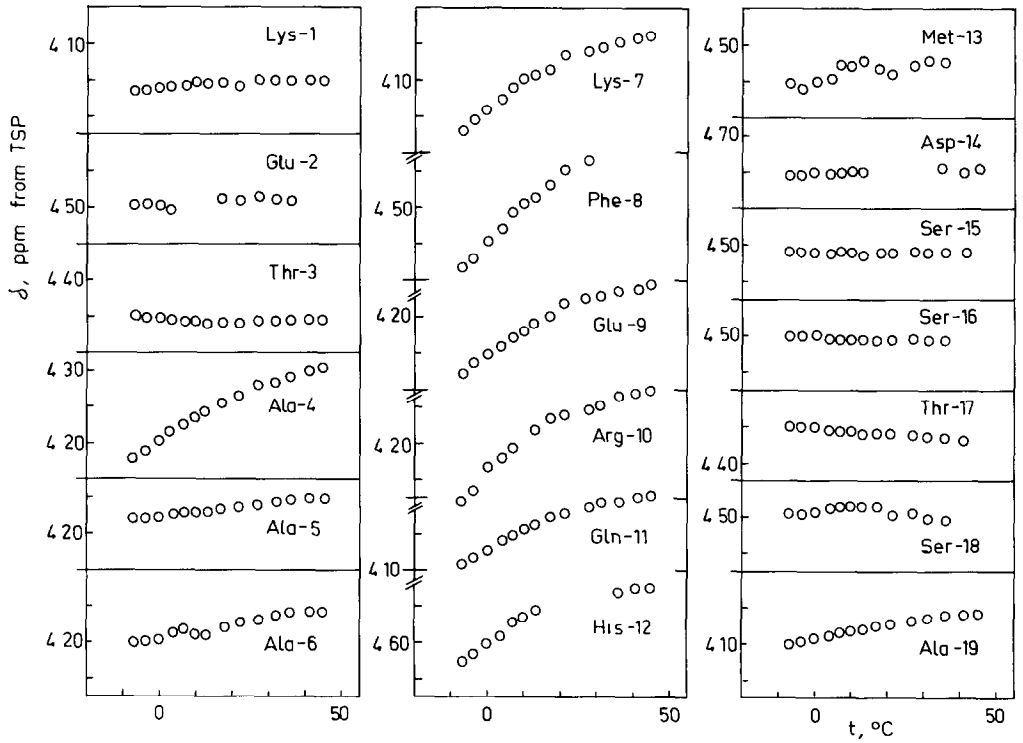

Fig.1. Temperature dependence of H_α proton chemical shifts of ribonuclease S-peptide (N-terminal 19 fragment), 5 mM solution in D_2O, 1 M NaCl, pH 5.4, 360 MHz. The spectral assignment follows that given in [1].

In table 1 we report the shift temperature coefficients between −7°C and +7°C for all protons whose resonances could be sorted out from the surrounding overlapping signals. After a proper scaling in magnitude and sign, all shift-temperature curves nearly coincide, clearly pointing towards a two-state concerted transition. Contrary to the H_α protons, most H_β and H_γ signals shift downfield with decreasing temperature. Maximal slopes were for Thr-3–H_β and His-12–$H_{\beta'}$ (upfield signal). It is to be noted that the equivalence of Arg-10–H_δ–$H_{\delta'}$ signals is broken below 20°C and individual signals emerge with a continued splitting

Table 1

Shift temperature coefficients ($\alpha . 10^4$, ppm/°C) between −7 and 7°C, of proton resonances of S-peptide (pH 5.4), 1 M NaCl

Amino acid	Position					
	α	β	γ	δ	ϵ	ζ
Lys-1	7	6	2	2	0	
Glu-2	−9		−56[a]			
			14			
Thr-3	−6	−118	−43			
Ala-4	33	−18				
Ala-5	4	−6				
Ala-6	10	−41				
Lys-7	42	−53		−4	4	
		−17		−4		
Phe-8	50	−18		*ortho*	*meta*	*para*
		−26		12	41	66
Glu-9	33		−37			
			−24			
Arg-10	51			−30		
				7		
Gln-11	33		−56			
			11			
His-12	37	1		H2	H5	
		106		65	49	
Met-13	8		−15		0	
			−29			
Asp-14	2	−3				
		1				
Ser-15	1	−9				
		4				
Ser-16	−2	1				
		1				
Thr-17	−4	0	0			
Ser-18	6	−9				
		4				
Ala-19	11	−1				

[a] Downfield signal, up; upfield signal, down

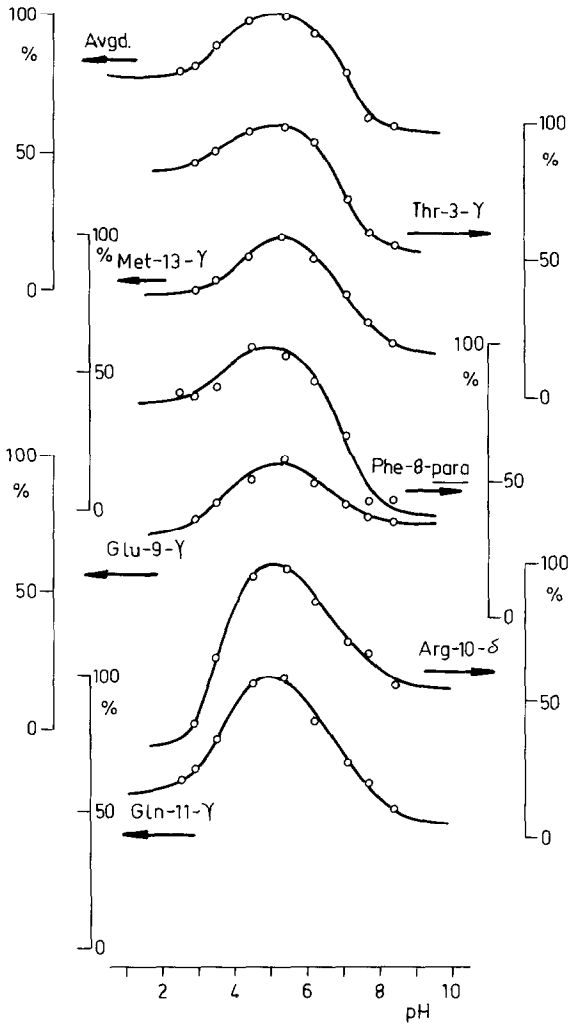

Fig.2. pH Dependence of relative chemical shifts of some proton resonances of ribonuclease S-peptide, 5 mM solution in D_2O, 1 M NaCl, at 0°C. Shift differences are relative to the ones measured at 45°C (pure coil data, zero in the percent scale). Curves were scaled to give an arbitrary 100% for the maximum shift differences. Top, weighted averaged curve for the 32 resonances measured. Below, curves for Thr-3-γ, Met-13-γ, Phe-8-*para*, Glu-9-γ, Arg-10-δ and Gln-11-γ. Maximum shift differences for these protons were, respectively, 0.098, 0.037, −0.141, 0.068, 0.067 and 0.106 ppm.

as temperature decreases. A similar situation is found for Glu-2–H_γ–$H_{\gamma'}$ signals.

Fig.2 illustrates the pH dependence of chemical shifts at 0°C. Chemical shifts are relative to the ones at 45°C (considered in principle as pure coil data) so as to eliminate changes with pH not caused by the conformational change. Therefore, assuming a rapidly interconverting two-state equilibrium:

$$H \rightleftharpoons C$$

where H and C refer, respectively, to the folded (H) and coil (C) species, these curves show the pH dependence of the relative populations of the helical state. At the top of fig.2, the weighted average curve for 32 proton resonances is shown. Curves were scaled to give an arbitrary 100% for the maximum shift difference value. Weights were given according to the reliability of the measurements. The average curve is an asymmetrical bell-shaped one with apparent pK_a of 3.6 and 7.0. Helix formation is maximal at pH ~5 and helix population reduces to 77% and 57% of its maximal value at pH < 3 and pH > 8, respectively. Although the general shape of our pH dependence curve resembles the one reported in [4] obtained by CD spectra, some differences are noteworthy. We have not found any evidence for the inflexions at pH ~3.3 and 4.5 nor for the maximum at pH 3.7. Also, our curves show for pH < 3 and pH > 8 substantially larger helix populations.

4. DISCUSSION

The sharp changes in chemical shift with decreasing temperature undergone for some particular protons in the molecule of S-peptide, as opposed to the gradual and small variation above 20°C, demonstrate that the molecule is undergoing a major change in its conformation. This is confirmed by the fact that the variation of the chemical shifts of the various protons in the molecule, are very different in magnitude and sign, reflecting specific changes in local environments.

The upfield chemical shift changes of H_α signals between residues 7–12 with decreasing temperature are compatible with helix formation. The helix probably extends further towards the N-terminal end, as can be inferred from preliminary ^{13}C-NMR studies, where the carbonyl signals of Ala-4, Ala-5 and Ala-6 show significant downfield shifts with decreasing temperature [11]. This would mean that the hydrogen bond of the neighbour NH significantly contributes to the upfield shift of H_α in the helix. The very large upfield shift of $H_{\beta'}$ (high field signal) of His-12 can be rationalized as arising from the Phe-8 phenyl ring current effect, since it lies close to the face of the ring (see fig.3). Conversely, the ring current effect of the histidine ring on the aromatic phenyl protons would shift to high field the resonances of the *ortho-*, *meta-* and *para-*protons in increasing order, which is actually observed. From these features, and taking into account the fact that the shift dependence on temperature in residues 14–19 is very small, we may conclude that the structure whose population is growing with decreasing temperature is not far from the one observed in the crystal ribonuclease S-system, where the S-peptide forms a quite regular right-handed helix involving residues 3–13.

As put forward in [3] for the closely related C-peptide molecule, helix formation is an enthalpy-driven process in which a salt bridge Glu-9^-...His-12^+ stabilizes the helix formed by residues 3–13, which involves 7 intramolecular hydrogen bonds. The main evidence for the Glu-9^-...His-12^+ stabilizing factor comes from the pH dependence of helix content as followed by CD spectra. Our results lend further support to this interpretation, since for the S-peptide the minimum helix content is obtained for pH > 8,

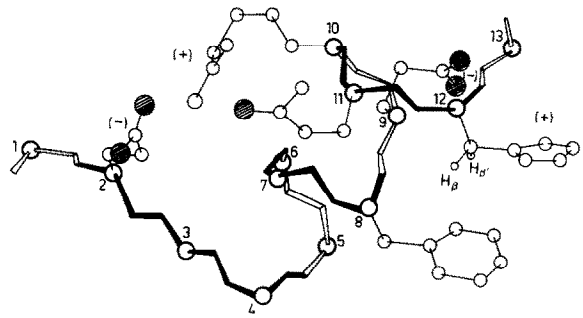

Fig.3. Schematic drawing of ribonuclease S-peptide based on the crystal structure of the peptide in ribonuclease S (α-carbons are shown as circles). Side chain torsion angles are as follows: Phe-8, χ_1, 180°, χ_2, 90°; Glu-9, χ_1, 180°, χ_2, 60°, χ_3, 90°; Arg-10, χ_1, 180°, χ_2, 180°, χ_3, 60°, χ_4, 60°; Gln-11, χ_1, 300°, χ_2, 0°; His-12, χ_1, 180°, χ_2, 90°.

when the imidazolium group is deprotonated. If the sidechain conformation of His-12 in fig.3 is accepted, the only reasonable partner for the formation of a salt bridge stabilizing a helix turn is obviously the Glu-9 carboxylate.

The search for actual sidechain-specific interactions contributing to helix stability would be greatly helped by studying the pH dependence of chemical shifts in the pure helix. Unfortunately, the temperature for 100% conversion to the helix form is too low to be experimentally accessible. We can, however, approach that situation by analyzing the differences between a normalized curve of helix content dependence on pH and the ones corresponding to particular protons for which a long-range interaction with a titrating group is suspected. As stated above, a normalized curve (0°C, fig.2, top) has been built by averaging all proton shift data, the majority of which can be thought to be unaffected by the titration of a given group. Large deviations from this curve for a particular proton must be interpreted as differences in the titration properties of this proton in the helix *vs* the coil. For protons which do not titrate in the coil, those deviations evidence the proximity to a titrating group originated by helix formation.

In fig.2, the curves for Thr-3-β and Met-13-γ protons are given as a sample of resonances which follow in a satisfactory way the shape of the averaged curve. Below, we show 4 curves belonging to some particular sidechain end protons, which deviates from the averaged curve. In table 2 we give the tentative titration shifts in the helix deduced from the comparison of these 4 single proton curves with the averaged one.

Table 2

Titration shifts in the helix (0°C) for selected sidechain end protons as deduced from the comparison of their titration curves with the weighted averaged one (see text)

	$pK_a \sim 4$	$pK_a \sim 7$
Phe-8-*para*		+
Glu-9-γ		+
Arg-10-δ	+	
Gln-11-γ	+	

A (+) sign means a downfield shift at a pH higher than the pK_a

The fact that the Phe-8-*para* as well as the Glu-9$^-$ resonances feel the pK_a of dissociation of the imidazolium group of His-12, agrees with the local structure shown in fig.3 and with the suggestion of the salt bridge stabilizing interaction His-12$^+$...Glu-9$^-$.

The titration shift undergone by the Arg-10-δ proton (table 2) with a $pK_a \sim 4$ in the helix provides evidence for a Glu-2$^-$...Arg-10$^+$ salt bridge. The two sidechain end groups are in close proximity in the ribonuclease S-crystal, and an actual interaction has been postulated in that system [12] and supported experimentally [13]. A preliminary observation of the NH(ϵ)–Arg-10 signal in H_2O at 5°C confirms that this resonance titrates with a $pK_a \sim 3.5$. Sidechains of Glu-2$^-$, Arg-10$^+$ and Gln-11 can be accommodated as depicted in fig.3, thus providing an explanation for the titration shift detected for Gln-11 in the helix (table 2).

Then, in addition to the 7 intramolecular hydrogen bonds, the helix content in each pH region will be determined by the balance of the following interactions:

pH < 3.6 Im^+(12)...COOH(9), Gnd^+(10)...COOH(2)

3.6 < pH < 7.0 Im^+(12)...COO^- (9), Gnd^+(10)...COO^- (2)

pH > 7.0 Im (12)...COO^- (9), Gnd^+(10)...COO^- (2)

The fraction of helix content under the studied conditions remains to be quantified. Authors in [3] have evaluated the helix percentage for C-peptide lactone, at pH 5, 1°C, from mean residue ellipticity (222 nm) measurements, as 29 ± 2%. By comparing the ^1H chemical shifts reported by these authors for Thr-3-γ and Phe-8-*meta* of C-peptide with those obtained by us for the S-peptide, a rough value of 30% is obtained under the conditions pH 5.4, 0°C, 1 M NaCl. We may use this value for calculating apparent equilibrium constants as a function of temperature for the two-state equilibrium H $\rightleftharpoons$ C and thus to obtain the values for $\Delta G°$, $\Delta H°$ and $\Delta S°$. Using the curves for protons Thr-3-γ, Phe-8-*meta*, His-12-β' and Lys-7-α, the following averaged values were obtained: $\Delta G° = -1.31 \pm 0.04$ kcal/mol, $\Delta H° = 8.6 \pm 0.4$ kcal/mol and $\Delta S° = 33 \pm 2$ cal/degree.mol, for 25°C, pH 5.4, 1 M NaCl. Actual uncertainties in these estimates are larger than the reported ones,

especially those arising from the estimation of the helix content which can be particularly severe. However, it is worth pointing out that our value for ΔH° is about 50% of that found in [3] for the C-peptide lactone from CD measurements. Assuming that all interactions (7 H-bonds and 2 salt bridges) contribute evenly to the enthalpy of helix formation a very reasonable value of -1 kcal/mol/interaction is found.

Finally, a further contribution of this work is that of providing secondary structure shift values, which can be of use in determining the effect of peptide groups on the chemical shifts of neighbour protons. In this respect, the secondary shifts of Thr-3-β and Ala-4-α are striking. We have not yet found a rationale for them. Further work on this system, particularly a low temperature ^{1}H-NMR study of NH resonances in H_2O as well as a low temperature ^{13}C-NMR study are in progress.

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