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^{1}H NMR and CD evidence of the folding of the isolated ribonuclease 50–61 fragment

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In our search for potential folding intermediates we have prepared and characterized the fragment of RNase A corresponding to residues 50–61. Proton chemical shift variations with temperature, addition of stabilizing (TFE) or denaturing agents (urea) provide a strong experimental basis for concluding that in aqueous solution this RNase fragment forms an α-helix structure similar to that in the intact RNase A crystal. This conclusion lends strong support to the idea that elements of secondary structure (mainly α-helices) can be formed in the absence of tertiary interactions and act as nucleation centers in the protein folding process.

Peptide folding; ^{1}H-NMR; Protein fragment; Helical structure; RNase A

1. INTRODUCTION

Short linear peptides (<30 residues) in general do not show marked conformational preferences in aqueous solution, and thus a single favored conformation has been detected and well characterized for only a few special peptides. However, in relation to the protein folding problem [1], it is very important to establish whether, as a general rule, short protein fragments with a defined structure in the protein are able to adopt the same native structure when isolated. This will only be possible if such potential kinetic 'folding intermediates' are experimentally detected and unambiguously characterized, which is not easy in view of their inherent instability [2].

As far as we know, the N-terminal peptides from RNase are the only known examples of short protein fragments, the structure of which has been well characterized and found to be very similar to the native one [3,4]. As will be shown in this paper, another fragment of the same protein, that corresponding to residues 50–61, forms an α-helix structure (the population of which can be increased by TFE addition) in aqueous solution that involves approximately the same residues which are helical in the RNase A crystal. CD spectra and ^{1}H NMR chemical shift variations with temperature and addition of denaturing (6 M urea) and stabilizing (TFE) agents lend strong support to the latter conclusion. These results show that the S-peptide folding is not a singular case and that probably other short linear protein fragments adopt as a rule native-like secondary structures in aqueous solution which have not previously been detected due to their low populations.

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Abbreviations: CM-RNase, *S*-carboxymethylated bovine pancreatic ribonuclease A (EC 3.1.27.5); SAP, *Staphylococcus aureus* protease (EC 3.4.21.19); TFE, trifluoroethanol; σ, standard deviation; COSY, two-dimensional homonuclear correlated spectroscopy; CD, circular dichroism; HPLC, high-performance liquid chromatography

2. EXPERIMENTAL

S-carboxymethylation and SAP digestion (cleavage after E residues) of RNase A (Sigma, type XII-A) were performed as in [5]. The CM-RNase 50–86 fragment was purified by reverse-phase HPLC, scaled up to a preparative range (Polygosil C-18, 1.6 × 25 cm column). The 50–61 peptide was obtained by subsequent digestion of

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the 50–86 peptide with trypsin at 37°C in NH_4HCO_3 buffer, and was purified in the same way. All the reactions were controlled by analytical HPLC to detect the point of maximum yield and to avoid unwanted secondary reactions. Amino acid analysis gave the expected composition.

CD spectra were recorded on a Rousel-Jouan CD185 dichrograph equipped with a thermostatted cell holder. A path length of 1 cm, peptide concentrations (measured by UV absorbance) of about 0.05 mg/ml and 2 mM NaH_2PO_4 buffer were used. A mean residue weight of 110 was employed in the computation of $[\theta]$.

Details on the NMR spectra recording conditions (360 MHz) and sample manipulation can be found in [6]. The concentration of NMR samples was maintained as low as possible (1–1.5 mM) to minimize eventual aggregation effects. Double resonance difference spectroscopy was used to establish the intra-residue assignment. Phase-sensitive COSY spectra were obtained using the time-proportional phase-incrementation method [7], and presaturation of the HDO solvent line.

3. RESULTS

CD spectra of CM-RNase 50–61, obtained under different experimental conditions, are shown in fig.1, where the relative effects of the addition of urea, TFE and temperature decrease can be seen.

Recent NMR sequential assignment techniques [7] are not generally applicable to short linear peptides unless a unique or largely preferential structure is present in solution. Therefore, the conventional manner of assignment based on characteristic δ values of reference peptides [8], COSY connectivity patterns, double resonance and pH titration behavior has been followed to assign the CM-RNase 50–61 ^1H NMR spectrum. Amide signals from D53 and terminal K61 residues were easily identified on the basis of their large titration shifts. Repeated residues were assigned as follows: the upfield (0.25 ppm) H_α S signal was assigned to S50 on the basis of the well-known behavior of N-terminal H_α signals; amide signals from A52 and V54 were distinguished from A56 and V57 because of titration of the former two with a pK_a value of 3.8, corresponding to their adjacent residue D53, a typical behavior of flexible peptides [9,10]; only

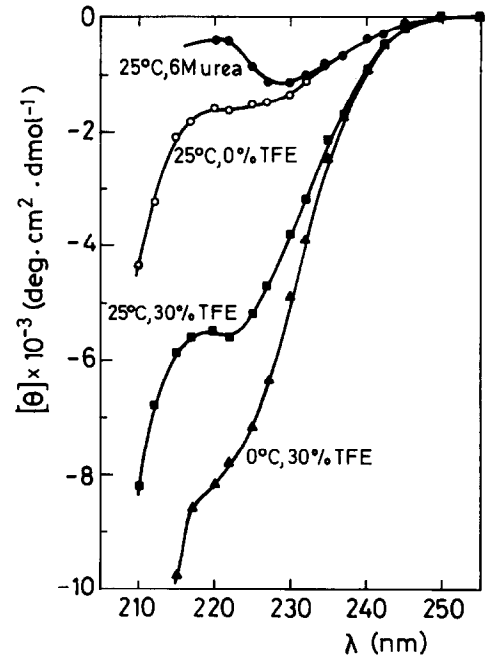

Fig.1. CD spectra of CM-RNase 50–61 at pH 5.4, under different experimental conditions.

one of the two Q signals titrated with a pK_a value corresponding to D53 (3.8), so it was assigned with the same proximity criterium to Q55.

Table 1 summarizes the δ values of CM-RNase 50–61 resonances at 22°C and pH 5.4. Amide $^3J_{HNCH}$ coupling constants, measured under the

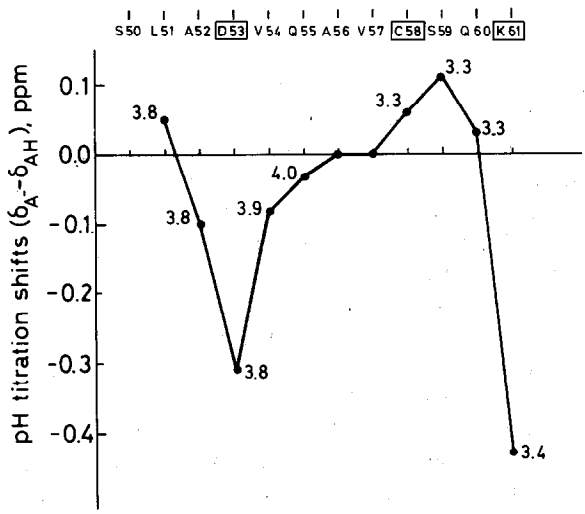

Fig.2. pH titration shifts of amide signals of CM-RNase 50–61, plotted vs residue number. The corresponding pK_a values are also shown.

Table 1

Chemical shifts (δ, ppm from TSP) and NH shift temperature coefficients (α, ppb/K) of the CM-RNase 50–61 fragment (22°C, pH 5.4, D_2O or H_2O/D_2O, 90:10)

Residue	NH	H_α	H_β	H_γ	Others	α	$\alpha^{a}_{urea} - \alpha$
S50	–	4.21	4.04	–	–	–	–
L51	8.74	4.37	1.66	1.68	$\delta\delta'$ 0.96 $\delta\delta'$ 0.92	−5.9	−0.1
A52	8.30	4.29	1.38	–	–	−6.5	0.7
D53	8.08	4.61	2.77 2.69	–	–	−5.0	1.6
V54	8.02	4.11	2.13	0.97 0.96	–	−8.5	−0.1
Q55	8.31	4.33	2.01	2.40	δNH_2 7.64[c] δNH_2 6.86	−8.6	0.5
A56	8.43	4.35	1.41	–	–	−8.1	0.0
V57	8.18	4.13	2.10	0.97 0.96	–	−9.2	−0.1
C58[b]	8.62	4.60	3.01	–	$\delta\delta'$ 3.30	−7.9	0.2
S59	8.59	4.47	3.90	–	–	−8.8	0.4
Q60	8.38	4.37	2.17 2.01	2.40	δNH_2 7.60[c] δNH_2 6.86	−6.6	0.5
K61	8.05	4.20	1.86 1.73	1.43	$\delta\delta'$ 1.68 $\epsilon\epsilon'$ 3.01	−9.2	0.2

[a] α values measured in 6 M urea
[b] Carboxymethylcysteine
[c] The reverse assignment is also possible

same conditions, were within the range 5.7–8.0 Hz. Amide shift temperature coefficients measured in the absence and presence of denaturing agents are also included in table 1, since they are useful data for detecting the eventual formation of peptide secondary structure in solution. Titration shifts of amide signals, together with the corresponding pK_a values, are shown as a function of the sequence residue number in fig.2.

Changes in δ values of peptide resonances originated from TFE addition are of the utmost significance in detecting and characterizing increasing populations of folded structures in solution. To evaluate the magnitude of TFE δ shifts not related to conformational changes, the $\Delta\delta$ values arising from addition of 30% TFE were measured in random tetrapeptides [11] at 22°C and pH 5.4. The mean and σ values obtained for TFE shifts on H_α and H_β resonances, excluding those arising from protons adjacent to carboxylates which are ascribed to the known TFE effect on the pK_a values of these groups, are shown in fig.3. Analogous data were measured for the CM-RNase 50–61 fragment with the help of COSY spectra taken at three different TFE/D_2O proportions (0, 15, 30%, v/v) which allowed us to follow unambiguously the course of all H_α and H_β resonances. The differences in δ values of such resonances between 30 and 0% TFE, plotted vs residue number, are also shown in fig.3.

4. DISCUSSION

The CD spectrum of the CM-RNase 50–61 frag-

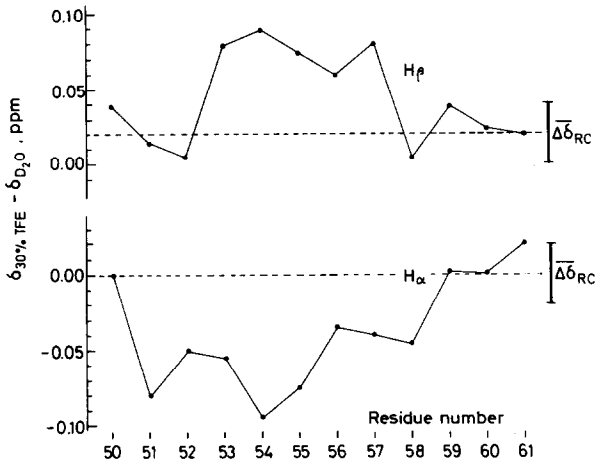

Fig.3. TFE-induced shifts in δ values of H_α and H_β signals of CM-RNase 50–61, as a function of residue number (22°C, pH 5.4). The dashed line indicates the mean value of TFE-induced shifts measured for a series [11] of random peptides ($\overline{\Delta\delta}_{RC}$) under the same conditions; the range of variation (denoted by $\pm 2\sigma$) is shown by vertical bars.

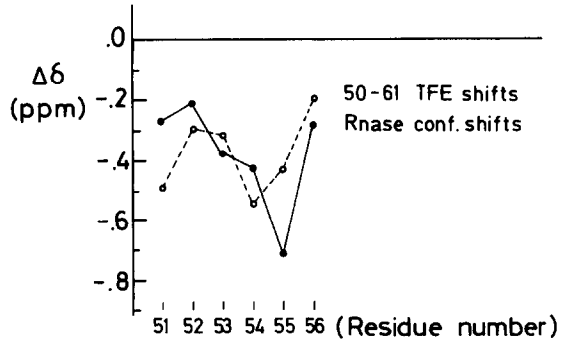

Fig.4. TFE-induced shifts in H_α signals of CM-RNase 50–61 (○) and conformational shifts ($\delta-\delta_{RC}$) obtained from published H_α δ values in the same region of the native protein [20] (●). For comparison purposes the observed TFE shifts (~18% helix) were scaled up to 100% helix.

ment in water at 25°C (fig.1) is indicative of an essentially unstructured peptide, although the CD curves show a very minor but still detectable negative band at 222 nm that may indicate the presence of a small population of a folded structure. The addition of 6 M urea appears to confirm the latter, since the CD curve shifts slightly towards less negative ellipticity values. The population of folded structure is significantly increased by both addition of TFE and decrease in temperature, as deduced from the remarkable shift observed in the corresponding CD curves.

The δ values of H_α NMR signals of CM-RNase 50–61 (table 1) do not differ greatly from those reported for model polypeptides, δ_{RC} (mean value of differences 0.06 ppm) and the $^3J_{HNCH}$ values are also within the range expected for flexible peptides. Amide protons are similarly exposed to solvent in the presence or absence of urea, the only exception being the D53 amide signal (table 1), the α value of which increases by 1.6 ppb/K upon urea addition. A similar behavior of some amide α values with urea addition has been ascribed to the partial formation of structure in other peptides [12,13]. These NMR results are in accordance with CD data, indicating the presence in solution of a mainly flexible peptide with a small amount of folded structure. Since such a structure is stabilized in TFE, as deduced from the CD spectra, an analysis of the TFE effects on NMR chemical shifts will provide useful information for its characterization.

A great deal of information relative to the ^1H δ values of protein residues in the known native state is now available. We have examined the conformational shifts ($\delta-\delta_{RC}$) of H_α and H_β protons of five representative proteins, viz. trypsin inhibitor [14], λ-CRO repressor [15], protein E-L30 [16], acyl carrier protein [17] and tendamistat [18], and have found interesting regularities. First, all the H_α signals of residues belonging to an α-helix are shifted upfield, with only 9 exceptions out of 104 (−0.35 ppm average); this trend was already known in homopolymers [19] and the S-peptide system [3], but has now been confirmed. Second, the H_α signals of residues involved in a β-sheet structure are shifted downfield, with only 18 exceptions out of 99 residues (0.40 ppm average). Third, H_β protons show smaller shift changes than H_α protons; in α-helices the average value is zero, but in β-sheets the signals shift upfield by −0.16 ppm on average.

It can be seen (fig.3) that TFE shifts on CM-RNase 50–61 resonances are not uniform along the polypeptide chain. The H_α signals corresponding to terminal or near-terminal residues (50 and 59–61) do not change appreciably as in random peptides. On the contrary H_α shifts of residues in the central region of the chain (51–58) are larger

and, without exception, towards high field, as expected for helical residues. These results clearly differ from those observed for an unspecific TFE solvent effect (fig.3), and are in strong support of a structure being formed in the central region of the chain. The signs of the observed TFE shifts permit one to discard the β-structure in favor of the α-helix, since some H_α signals should move downfield and some H_β upfield, the opposite being observed. The conformational shifts of H_β signals corresponding to helical residues (end plus central) average to zero as already mentioned, however, in our case we observed a systematic downfield shift for central residues 53–57.

As judged by the magnitude of the 50–61 H_α TFE shifts, the helix spans from residues 51 to 55 and most probably also from residues 56 to 58. Fortunately, we can compare the TFE shifts for some H_α protons in the fragment 50–61 with those found in the native structure of RNase A, since they are among the few which have been assigned [20]. With this purpose, it is convenient to make an approximate estimation of the peptide helix content. Assuming 9 helical residues, and taking as 0 and 100% helix reference values those of $[\theta]_{222}$ in 6 M urea and previous data [21], respectively, we found, from CD spectra, a value of 18% at pH 5.4, 22°C and 30% TFE. Once scaled to 100% the TFE shifts found in the peptide for residues 51–56 are compared in fig.4 with the conformational shifts ($\delta-\delta_{RC}$) observed in the native protein. A striking resemblance can be noted between the two profiles, illustrating how the structure of the isolated 50–61 fragment compares with the native one. This resemblance is especially good if one bears in mind that tertiary effects on chemical shifts are absent in the fragment.

The CM-RNase 50–61 helix is very unstable in pure water but a small population is still present as deduced from the CD spectra. One may ask to what extent this instability is due to the presence of a non-native residue in the sequence, CM-C or of a positive charge at the amino end of the helix (charged group unstabilizing effect [22]). Studies on analogues are needed to answer this question, but in any case it is clear that the propensity to form an α-helix is somehow contained in this very short sequence of amino acid residues, and that the helix is actually formed in solution, irrespective of the existence of such destabilizing effects.

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