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^1H NMR Studies of the Solution Conformations of an Analogue of the C-Peptide of Ribonuclease A[†]

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ABSTRACT: Two-dimensional NMR experiments have been performed on a peptide, succinyl-AE-TAAAKFLRAHA-NH₂, related to the amino-terminal sequence of ribonuclease A. This peptide contains 50-60% helix in 0.1 M NaCl solution, pH 5.2, 3 °C, as measured by circular dichroism. NOESY spectra of the peptide in aqueous solution at low temperatures show a number of NOE connectivities that are used to determine the highly populated conformations of the peptide in solution. Short-range $d_{\text{NN}}(i, i + 1)$ and $d_{\alpha\text{N}}(i, i + 1)$ connectivities and medium-range $d_{\alpha\beta}(i, i + 3)$ and $d_{\alpha\text{N}}(i, i + 3)$ connectivities are detected. The pattern of NOE connectivities unambiguously establishes the presence of helix in this peptide. The magnitudes of the $^3J_{\text{HN}\alpha}$ coupling constants and the intensities of the $d_{\text{NN}}(i, i + 1)$ and $d_{\alpha\text{N}}(i, i + 1)$ NOEs allow the evaluation of the position of the helix along the peptide backbone. These data indicate that the amino terminus of the peptide is less helical than the remainder of the peptide. The observation of several long-range NOEs that are atypical of helices indicates the presence of a high population of peptide molecules in which the first three residues are distorted out of the helical conformation. The absence of these NOEs in a related peptide, RN-31, in which Arg 10 has been changed to Ala, suggests that this distortion at the amino-terminal end of the peptide arises from the formation of a salt bridge between Glu 2 and Arg 10. We propose that the conformational ensemble of RN-24 in solution includes three principal conformations: a set of extended conformations, a set of largely helical conformations, and a set of conformations that contain a salt bridge between the side chains of Glu 2 and Arg 10.

According to the framework model of protein folding, elements of secondary structure form early in the folding process. These interact and become mutually stabilizing, and the

tertiary structure of the protein is built up from the sum of these interactions (Kim & Baldwin, 1982). The factors that affect the formation and localization of secondary structure are clearly important in directing the early stages of protein folding. Short- and medium-range interactions (along the peptide chain and within a given element of secondary structure) should predominate over long-range or tertiary interactions (between different elements of secondary structure) in these early stages. This prediction has made linear

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peptides attractive as model systems for the study of secondary structure formation in the early stages of protein folding.

Until recently linear peptides were believed to exhibit very little structure in water (Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969; Howard et al., 1975). This view was also supported by helix/coil transition theory (Zimm & Bragg, 1959; Shoemaker et al., 1985). However, the C-peptide of ribonuclease A (the amino-terminal 13 residues produced by cyanogen bromide cleavage, KETAAAKFERQH-homoserine lactone) is found to form a helix in water at low temperatures (Brown & Klee, 1971). More recently, β -turns and nascent helices have been detected in linear peptides in water by two-dimensional ^1H NMR¹ (Williamson et al., 1986; Dyson et al., 1985, 1988a,b).

Helix formation in analogues of the C-peptide of ribonuclease A has been studied extensively by circular dichroism (Kim & Baldwin, 1984; Shoemaker et al., 1985, 1987a,b). Amino acid substitution studies show that specific side-chain interactions including salt bridge formation (Shoemaker et al., 1987a) and an aromatic interaction (Shoemaker et al., 1987a; Rico et al., 1986) as well as interaction of terminal charged groups with the helix dipole (Shoemaker et al., 1987b) can stabilize the helical conformation. However, it is not possible by use of circular dichroism spectroscopy alone to determine the effect of these interactions on the location of the helix in the peptide chain.

Here we present results of two-dimensional ^1H NMR studies designed to characterize the conformations present in an analogue of the C-peptide of ribonuclease A, RN-24 (succinyl-AETAAAKFLRAHA-NH₂) in water at low temperatures. Three distinct types of conformations are revealed by the NMR data, unfolded extended-chain conformations, helical conformations, and conformations in which a Glu 2-Arg 10 salt bridge truncates the helix at the amino-terminal end of the peptide.

MATERIALS AND METHODS

Peptide synthesis and purification were performed as described previously (Stewart & Young, 1984; Shoemaker et al., 1985). This peptide exhibits a maximum in helix formation measured by CD of 50–60% at pH 5.25 in 0.1 M NaCl at 3 °C (Shoemaker et al., 1987b). Samples for NMR were prepared in 90% $^1\text{H}_2\text{O}$ /10% $^2\text{H}_2\text{O}$ and 0.02 M NaCl, pH 5.2 \pm 0.05. pH values correspond to uncorrected meter readings. Dioxane was included as an internal standard, but spectra are referenced to TSS [(trimethylsilyl)propanesulfonic acid] at 0 ppm.

Peptide concentration was 6.0 mM as determined by comparison with an external dioxane standard. The dioxane standard was calibrated against phenylalanine solutions, 0.1 M phosphate buffer in $^2\text{H}_2\text{O}$ pH 7.1, whose concentration was determined spectrophotometrically (molar extinction coefficient of 154.1 at 252.2 nm).

The two-dimensional NMR spectra were recorded on a Bruker AM500 equipped with digital phase-shifting hardware. Temperatures were calibrated by using a methanol standard as described by Van Geet (1979). Both COSY (Marion & Wüthrich, 1983) and NOESY (Jeener et al., 1979) experiments were performed in the phase-sensitive mode by using the time proportional phase incrementation method (TPPI)

(Drobny et al., 1979; Marion & Wüthrich, 1983). The water resonance was suppressed by gated irradiation during a 1.8-s delay and during the mixing time; 2048 complex points were collected for each of 512–600 t_1 values, and 64 scans per t_1 value were accumulated. The spectral width was 4237 Hz (8.475 ppm) in both dimensions. NOESY spectra were acquired with mixing times of 200, 300, 400, and 600 ms. The ω_2 dimension was processed with Lorentzian-to-Gaussian weighting, while the ω_1 dimension was processed with a $\pi/4$ or $\pi/2$ phase-shifted sine ball weighting function. The data were zero filled in ω_1 to yield a final matrix of 2048 real points in both dimensions. Data processing was performed on a Convex computer or on a SUN 3 data station by using software written by Dr. Dennis Hare and modified for the SUN by Dr. James Sayre. The quality of the NOESY spectra were improved by base-line correction before the ω_1 transform (Zuiderweg et al., 1985) and through t_1 ridge suppression (Otting et al., 1986). Cross-peak intensities were determined by volume integration. The $^3J_{\text{HN}\alpha}$ coupling constants were measured from one-dimensional spectra.

RESULTS

Since it is our intention to study peptide interactions that approximate the short- and medium-range interactions which are expected to direct the early stages of protein folding, it is important that the helix we observe in this peptide be stabilized intramolecularly and not be the result of aggregation or association. Brown and Klee (1971) were able to demonstrate by sedimentation equilibrium experiments that the C-peptide remains a monomer under helix-forming conditions. The amount of helix formed by derivatives of C-peptide with closely related sequences is not dependent upon peptide concentration over the concentration range used for circular dichroism measurements (10 μM –1.0 mM). To investigate the tendency of RN-24 to aggregate at the higher concentrations employed in the NMR experiments, a series of NMR spectra were obtained over a concentration range of 0.1–6 mM. There is no measurable change in the line width of the amide proton resonances over this concentration range. In addition, there are no significant changes (>0.01 ppm) in chemical shift. In contrast, for several peptides that are known to associate at high concentration, we have observed large concentration-dependent changes in both line width and chemical shift of amide proton resonances (J. P. Waltho and P. E. Wright, unpublished observations). We thus conclude that RN-24 is not significantly associated under the conditions of the NMR experiments and that the folded structures formed by it were stabilized by intramolecular interactions. This is in complete accordance with earlier CD studies of the C-peptide and its analogues (Brown & Klee, 1971; Bierzynski et al., 1982; Shoemaker et al., 1987).

Assignment of the proton spectrum of RN-24 was achieved by using standard two-dimensional methods. Spin system assignments were made from phase-sensitive COSY spectra; sequence-specific assignments were made by using phase-sensitive NOESY spectra (Figure 1A). Assignments for the proton resonances of the peptide are summarized in Table I.

All of the expected $d_{\alpha\text{N}}(i, i + 1)$ NOEs were observed (Figure 1A). The observation of strong $d_{\alpha\text{N}}(i, i + 1)$ NOEs [in the terminology of Wüthrich et al. (1984)] indicates that the conformations adopted by RN-24 contain a significant proportion of extended-chain structures with backbone dihedral angles in the β -region of conformational space. Strong $d_{\alpha\text{N}}(i, i + 1)$ NOEs have been observed in the unfolded forms of many short peptides in water solution (Dyson et al., 1988a). In addition, $d_{\text{NN}}(i, i + 1)$ connectivities were observed (Figure

¹ Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; $d_{\alpha\text{N}}(i, j)$, $d_{\text{NN}}(i, j)$, etc., intramolecular distance between the protons C $^{\alpha}$ H and NH, NH and NH, etc., on residues i and j ; $^3J_{\text{HN}\alpha}$, NH-C $^{\alpha}$ H coupling constant; CD, circular dichroism spectroscopy.

Table I: Assignments for RN-24 at 3 °C, pH 5.2, 20 mM NaCl

	NH	C $^{\alpha}$ H	C $^{\beta}$ H	C $^{\gamma}$ H	other
Suc		2.55/2.56			
Ala 1	8.53	4.23	1.40		
Glu 2	8.63	4.34	2.02/2.09	2.33	
Thr 3	8.14	4.24	4.47	1.31	
Ala 4	8.57	4.13	1.47		
Ala 5	8.43	4.23	1.46		
Ala 6	8.04	4.19	1.51		
Lys 7	8.24	4.00	1.83	3.99	1.64 (C $^{\delta}$ H ₂), 2.93 (C $^{\epsilon}$ H ₂), 7.66 (NH ₃ ⁺)
Phe 8	8.18	4.40	3.17/3.23		7.24 (C $^{\delta}$ H ₂), 7.12 (C $^{\epsilon}$ H ₂), 7.23 (C $^{\epsilon}$ H)
Leu 9	8.13	4.17	1.68	1.80	0.96 (C $^{\delta}$ H ₃)
Arg 10	7.99	4.17	1.88	1.64/1.74	3.23 (C $^{\delta}$ H ₂), 7.68 (N $^{\epsilon}$ H)
Ala 11	7.95	4.19	1.30		
His 12	8.01	4.56	2.87/3.27		8.36 (C $^{\epsilon}$ H), 7.06 (C $^{\delta}$ H)
Ala 13	8.02	4.26	1.42		
terminal NH ₂	7.18/7.69				

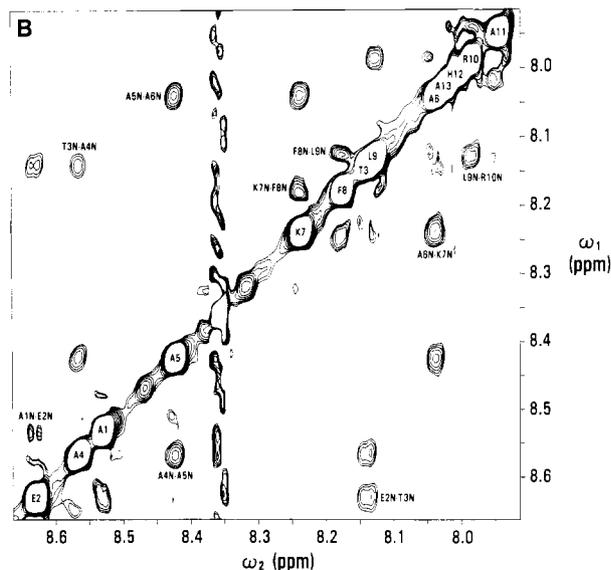
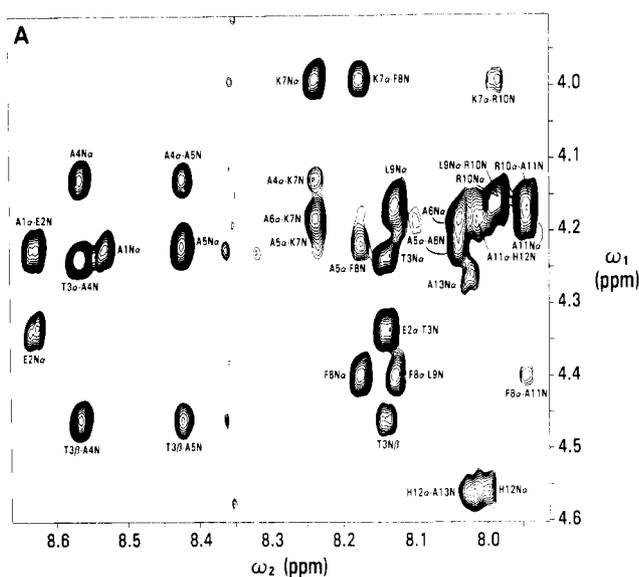


FIGURE 1: Portions of a 500-MHz NOESY spectrum ($\tau = 400$ ms) of 6 mM RN-24, pH 5.2, and 20 mM NaCl at 3 °C. (A) NH-C $^{\alpha}$ H region. (B) NH-NH region. The level of the first contour in (A) is half that in (B). Additional medium-range $d_{\alpha N}$ NOE connectivities that are shown in Figure 2 are only seen at lower contour levels.

1B) between all amino acids except His 12 and Ala 13, the amide proton resonances of which are too close for the observation of an NOE. Five of a possible 10 $d_{\alpha N}(i, i + 3)$ NOE connectivities and 8 of a possible 10 $d_{\alpha\beta}(i, i + 3)$ NOE con-

Suc A E T A A A K F L R A H A-NH

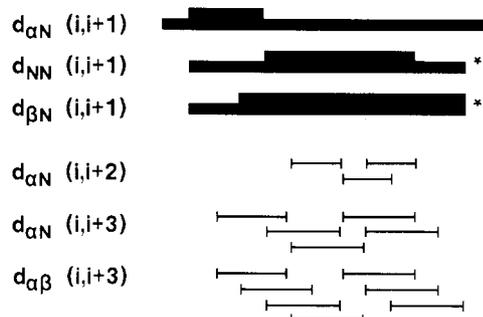


FIGURE 2: Summary of the NOE connectivities observed in the NOESY spectra of 6 mM RN-24, pH 5.2, and 20 mM NaCl at 3 °C. The asterisk denotes that an NOE is unobservable due to resonance overlap.

Table II: NOEs Observed for RN-24 but Not for RN-31 (Arg 10 \rightarrow Ala)

NOE ^a	intensity ^b	distance ^c (Å)
Thr3NH-Ala6C $^{\beta}$ H ₃	medium	3.0
Thr3C $^{\beta}$ H-Ala5NH	medium	2.4
Thr3C $^{\beta}$ H-Ala6NH	weak	3.2
Glu2C $^{\gamma}$ H ₂ -Lys7NH	weak	2.7, 4.1
Thr3NH-Ala6NH	medium	3.8

^a These NOEs would not be expected in a regular helix. ^b Relative intensity of NOE cross peaks. ^c Interproton distances calculated from the X-ray structure of ribonuclease (Wlodawer & Sjölin, 1983). Protons were added in standard geometries by using a program written by Dr. Keith Cross. Distances involving methyl protons are r^6 averaged.

nectivities are also observed. The interresidue NOE connectivities for RN-24 are summarized in Figure 2. The observation of sequential $d_{NN}(i, i + 1)$ NOEs and overlapping medium-range $d_{\alpha N}(i, i + 3)$ and $d_{\alpha\beta}(i, i + 3)$ NOE connectivities indicates the presence of helix and suggests that the helix includes, at least transiently, residues 2–13 of the peptide. We note that neither the present NMR experiments nor conventional CD measurements can distinguish between α -helix and 3_{10} helix. The very weak $d_{\alpha N}(i, i + 2)$ NOE connectivities observed between Ala 5 and Lys 7, between Lys 7 and Leu 9, and between Phe 8 and Arg 10 could arise from either 3_{10} helix (Wüthrich et al., 1984) or a low population of nascent helical turns (Dyson et al., 1988b).

All of the NOEs reported were measured at 400 ms or shorter mixing times. NOE buildup curves derived from a series of NOESY experiments performed sequentially with mixing times of 200, 300, and 400 ms suggest that contributions from spin diffusion are negligible at these mixing times.

In addition to the NOEs that indicate the presence of helical conformations, there are several NOEs at the amino terminus

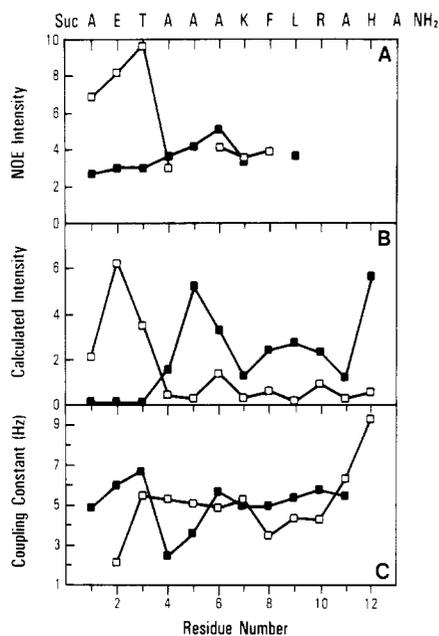


FIGURE 3: (A) Integrated intensities (arbitrary units) of the $d_{NN}(i, i + 1)$ (solid symbols) and $d_{\alpha N}(i, i + 1)$ (open symbols) NOE cross peaks observed for RN-24. (B) Theoretical relative intensities of the $d_{NN}(i, i + 1)$ (solid symbols) and $d_{\alpha N}(i, i + 1)$ (open symbols) NOE cross peaks calculated by using the two-spin approximation from the X-ray crystal structure of ribonuclease A (Wlodawer & Sjölin, 1983). (C) $^3J_{HN\alpha}$ coupling constants measured experimentally for RN-24 (solid symbols) and calculated from the X-ray structure of ribonuclease A (open symbols).

of the molecule which define a kink in the peptide backbone. These NOEs (Table II) are not expected in regular helices, and each represents a class of NOE that is unique in RN-24. The observation of these NOEs suggests a third significantly populated conformation of RN-24 in which the residues Ala 1–Thr 3 are twisted out of the helix.

These observations are supported by examination of the integrated intensities of the $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ NOEs and the values of the $^3J_{HN\alpha}$ coupling constants (Figure 3), which reflect the population of helix at different positions along the peptide backbone. The $d_{NN}(i, i + 1)$ NOE cross-peak intensities increase by approximately a factor of 2 from Ala 1 to Ala 6 (Figure 3A). This is consistent with a lowered population of helical conformations for residues 1–3. The $d_{\alpha N}(i, i + 1)$ NOEs are of 2–3-fold greater intensity than the $d_{NN}(i, i + 1)$ NOEs for positions 1–3 and then decrease abruptly at Ala 4. The Ala 4 C α H–Ala 5 NH NOE is smaller than the Ala 4 NH–Ala 5 NH NOE (Figure 3A). This drop in the intensity of the $d_{\alpha N}(i, i + 1)$ NOEs is paralleled in the behavior of the $^3J_{HN\alpha}$ coupling constants, which also show an abrupt decrease at position 4. Taken together, these data indicate an abrupt change in the conformation of the peptide chain at Ala 4. This conformation is apparently present in high population, since the value of the $^3J_{HN\alpha}$ coupling constant of Ala 4 is so small (2.5 Hz), indicating that relatively little conformational averaging is taking place at this position. An approximate ϕ angle of -42° can be calculated from the Karplus relationship (Wüthrich, 1986) from the observed $^3J_{HN\alpha}$ coupling constant for residue 4, but because there is conformational averaging with extended-chain forms, the actual value of ϕ must frequently be much smaller than this (up to $\sim -20^\circ$, where $^3J_{HN\alpha}$ is at a minimum).

The NOE connectivities at the N-terminal end of the peptide (Figure 3A and Table II) suggest the presence of a significant population of a conformation in which Ala 1, Glu 2, and Ala 3 adopt nonhelical conformations, while residues Ala 5–His

12 are in helix. The transition from helical to more extended backbone conformations appears to take place at Ala 4, for which the very small $^3J_{HN\alpha}$ coupling constant is observed. It appears that this conformational state, in which the amino-terminal residues are kinked out of helix, accompanies Glu 2 $^-$... Arg 10 $^+$ salt bridge formation.

Support for this idea comes from preliminary analysis of the NOEs present in a second peptide, RN-31 (Suc-AE-TAAAKFLAAHA-NH $_2$), in which Arg 10 has been replaced by Ala. This peptide cannot, of course, form the Glu 2 $^-$... Arg 10 $^+$ salt bridge. The expectation is that the unusual NOEs which define the kinked terminus of RN-24 would be missing or reduced in RN-31, and this is confirmed experimentally. In addition, for those residues at the N-terminus for which both $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ NOEs can be observed without overlap, the intensity of the d_{NN} NOEs relative to the $d_{\alpha N}$ NOEs is greater for RN-31 than for RN-24. This is consistent with a higher population of helical conformations at the N-terminus of RN-31. Formation of a Glu 2 $^-$... Arg 10 $^+$ salt bridge has been proposed previously from CD studies of related synthetic peptides (Shoemaker et al., 1987a) and NMR investigations of the ribonuclease S peptide (Rico et al., 1984). We propose that the kinked amino terminus arises from the formation of this salt bridge and that the third major class of conformations of RN-24 are those in which the Glu 2 $^-$... Arg 10 $^+$ salt bridge forms and prevents extension of the helix in the amino-terminal direction.

A salt bridge between Glu 2 and Arg 10 is present in the X-ray structure of ribonuclease A (Wlodawer & Sjölin, 1983), so it is of interest to ask whether the conformation of the C-peptide region (the first 13 amino acids of the amino terminus of ribonuclease A) in the X-ray structure is similar to that observed for RN-24 in solution. Relative intensities of $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ NOEs to be expected for the C-peptide of RNase A were calculated from the X-ray coordinates. The hydrogen atom positions and interproton distances were calculated by assuming standard geometries for the amino acids, and the relative intensities of the expected NOEs were calculated by assuming a two-spin approximation. The resulting intensities were scaled arbitrarily and are presented in Figure 3B.

The theoretical NOE intensity pattern for the $d_{NN}(i, i + 1)$ and $d_{\alpha N}(i, i + 1)$ NOEs in the C-peptide in the ribonuclease crystal structure is similar to the pattern observed in RN-24 in solution (Figure 3B). The first three calculated $d_{NN}(i, i + 1)$ NOEs are small, as are the first three $d_{\alpha N}(i, i + 1)$ NOEs observed for RN-24. Conversely, the calculated $d_{\alpha N}(i, i + 1)$ NOEs are much larger for residues 1–3 than for the remainder of the peptide, as are the NOEs observed experimentally for the peptide. The interproton distances corresponding to the “unusual” NOEs were also calculated from the X-ray structure of the protein (Table II); most of these are within the range for which one might expect to observe NOE connectivities experimentally if the peptide adopts a similar conformation to that of the same sequence in the protein. None of these NOEs would be expected in helix or other regular secondary structures.

The results of these calculations imply that the conformation of the kinked form of RN-24 in solution is similar to the conformation of the amino terminus of ribonuclease A in the X-ray crystal structure. However, some differences are apparent. One difference can be inferred from comparison of the observed $^3J_{HN\alpha}$ coupling constants of the peptide in solution with the coupling constants calculated from the X-ray structure (Figure 3C). The calculated coupling constant at position 2

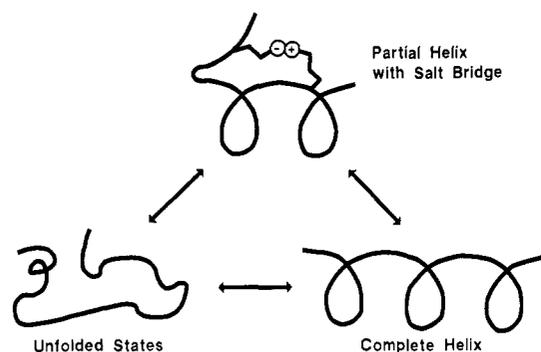


FIGURE 4: Schematic representation of the conformations adopted by RN-24 at 3 °C, pH 5.2, and 20 mM NaCl. The conformational ensemble of RN-24 contains three principal conformations that rapidly interconvert on the NMR time scale: (1) the set of extended forms, (2) the set of largely helical forms, and (3) the set of salt bridged (Glu 2⁻ ... Arg 10⁺) forms.

is small, and it then rises to 5–6 Hz for positions 3–7. In contrast, the experimentally measured $^3J_{\text{HN}\alpha}$ coupling constants of positions 1–3 in RN-24 are between 5 and 7 Hz with a drop to 2.5 Hz at position 4. In the crystal structure, Thr 3 has a ϕ angle within the helical range (as is indicated by the calculated coupling constant shown in Figure 3C) but the ψ angle (164°) places this residue well outside the helical region of ϕ, ψ space. The coupling constant data indicate the kink in the helix of the peptide RN-24 in solution begins at position 4, while in the X-ray crystal structure of ribonuclease A the kink begins at position 3.

A second difference concerns the observation of an NOE of medium intensity between Thr 3 NH and Ala 6 NH in the peptide. The distance between these protons as calculated from the X-ray structure is 3.8 Å, which is rather long for the observation of an NOE of this intensity in the peptide in solution. These two protons must therefore be closer in RN-24 in solution. Thus, the conformation of the salt-bridged form of RN-24 is similar to but not identical with the conformation of the amino terminus of ribonuclease A from the X-ray crystal structure.

DISCUSSION

We propose a model for the conformational equilibria of RN-24 that contains three sets of conformations: (1) a set of largely extended conformations that sample the β -region of ϕ, ψ space; (2) a set of helical conformations; and (3) a set of conformations in which a salt bridge is formed between the side chains of Glu 2 and Arg 10, which results in a kink at the amino terminus (Figure 4).

RN-24 is 50–60% helical under the experimental conditions employed, as deduced from CD measurements. This implies the presence of a significant fraction of nonhelical conformations. Conformational energy calculations on small peptides (Brant et al., 1967; Zimmerman et al., 1977) show that the largest potential energy well is in the β -region of ϕ, ψ space. In the absence of specific side-chain interactions that modify the conformational properties of the peptide, the β -region of ϕ, ψ space should be preferred by short peptides in solution (Wright et al., 1988). Strong $d_{\alpha\text{N}}(i, i + 1)$ NOE connectivities are expected from β -structures because of the close approach of the involved nuclei (2.2 Å). $d_{\alpha\text{N}}(i, i + 1)$ NOEs are observed in largely unstructured peptides in water (Dyson et al., 1988a). The presence of a complete set of strong $d_{\alpha\text{N}}(i, i + 1)$ NOEs in RN-24 is evidence for significant population of the extended conformation.

The NMR identification of helix in RN-24 relies on techniques developed for the identification of secondary structure

in proteins (Wüthrich, 1986; Wüthrich et al., 1984) and extended to peptides (Dyson et al., 1988a,b; Wright et al., 1988). The NMR criteria include the observation of a series of $d_{\text{NN}}(i, i + 1)$ NOE connectivities, the observation of medium-range $d_{\alpha\beta}(i, i + 3)$ and $d_{\alpha\text{N}}(i, i + 3)$ NOE connectivities, and small $^3J_{\text{HN}\alpha}$ coupling constants. In RN-24 a nearly complete set of $d_{\text{NN}}(i, i + 1)$ NOE connectivities is observed (Figure 1A) together with a large number of the medium-range NOE connectivities (Figure 2) which clearly demonstrate the presence of a helical conformation. In addition, the average value for the $^3J_{\text{HN}\alpha}$ coupling constant in this peptide is near 6 Hz. The value of the $^3J_{\text{HN}\alpha}$ coupling constant is subject to conformational averaging. Since extended peptide conformations have $^3J_{\text{HN}\alpha}$ coupling constants in the range of 8–10 Hz, the low values of $^3J_{\text{HN}\alpha}$ observed for RN-24 are consistent with an extensive population of helical conformations in the peptide.

The third highly populated set of conformations sampled by RN-24 apparently contains a salt bridge between Glu 2 and Arg 10. Several lines of evidence arise from the NMR studies that support this conclusion. The intensities of the $d_{\text{NN}}(i, i + 1)$ and $d_{\alpha\text{N}}(i, i + 1)$ NOE connectivities show that the first four amino-terminal residues differ significantly in conformation from the rest of the peptide (Figure 2A). The $^3J_{\text{HN}\alpha}$ coupling constants also indicate an abrupt change in conformation at position 4 of the peptide. Several NOEs atypical of helix have been observed (Table II), and these define the kinked form of the amino terminus. These NOEs are not found in the NOESY spectrum of RN-31 (Arg 10 → Ala), a peptide in which the absence of Arg 10 prevents the formation of the salt bridge. These data strongly suggest that the conformation of RN-24 that contains a kinked amino terminus is highly populated and arises from the formation of a salt bridge between Glu 2 and Arg 10.

In contrast to the highly populated helix observed in RN-24, less stable structures were detected in a recent NMR study of a synthetic immunogenic peptide derived from the C-helix of myohemerythrin (Dyson et al., 1988b). A series of $d_{\text{NN}}(i, i + 1)$ NOE connectivities are observed in the C-terminal half of the peptide, indicating a preference for backbone conformations in the α -helical region of ϕ, ψ space. No $d_{\alpha\beta}(i, i + 3)$ or $d_{\alpha\text{N}}(i, i + 3)$ NOE connectivities are observed. Instead, two $d_{\alpha\text{N}}(i, i + 2)$ NOE connectivities are observed, suggesting that turnlike conformations are populated in this portion of the peptide. This structure has been termed "nascent helix" and is thought to consist of an ensemble of turnlike structures that interconvert via extended-chain forms (Dyson et al., 1988b). The absence of the medium-range NOE connectivities suggests that any one conformer does not form a complete turn of helix. The medium-range $d_{\alpha\text{N}}(i, i + 3)$ and $d_{\alpha\beta}(i, i + 3)$ NOE connectivities are observed when the experiments are run in moderate concentrations of TFE (Dyson et al., 1988b), suggesting that the nascent helix regions need only a small increase in stability to form complete helical turns.

The operational distinction between nascent helix and true helix must rest upon the observation of medium-range $d_{\alpha\beta}(i, i + 3)$ and $d_{\alpha\text{N}}(i, i + 3)$ NOE connectivities. Regions of nascent helix can be viewed as sequences that have significant populations of backbone conformations in the α -region of ϕ, ψ space. Formation of the helix from the nascent helix sequences during protein folding presumably depends upon tertiary stabilization by more distant regions of the protein. However, the observation that TFE seems to stabilize helix only from the nascent helix portions of the peptide (Dyson et al., 1988b) suggests either that the nascent helix portions of the sequence

have higher propensities to become helical than the surrounding sequences or that other portions of the sequence serve to limit helix extension. A limit to helix extension has been observed in the S-peptide of ribonuclease A (Kim & Baldwin, 1984) and postulated for the C-peptide (Shoemaker et al., 1987a). The internal helix-limiting properties of nascent helix sequences and the relative importance of the tertiary stabilization of these sequences during protein folding have only begun to be explored.

Problems associated with interpretation of NOE data for a linear peptide that adopts multiple conformations in water solution have been discussed elsewhere (Wright et al., 1988). We have restricted our analysis to the qualitative description of a limited number of conformational sets (extended forms, helical forms, salt-bridged forms). The multiple conformations adopted by peptides in solution complicate quantitative analysis since the relative intensities of the NOEs depend upon a population-weighted and $1/r^6$ -weighted average of the interproton distances in all of the conformations. Current methods for determining solution structures from NOE data such as distance geometry (Havel & Wüthrich, 1984) and constrained molecular dynamics (Clare et al., 1985) have been designed to yield the single structure that best fits the input data. As we show here, the solution conformations of flexible peptides can contain multiple forms. Uncritical application of distance geometry or constrained molecular dynamics to the analysis of NMR data in flexible peptide systems can yield structures that do not accurately reflect the situation in solution.

The analysis of the conformations of flexible peptides by NMR at the level presented here can be valuable for the study of peptides as models for the early stages of protein folding (Wright et al., 1988) or as development tools for the definition of determinants of conformation that can be used later in the design of de novo systems (Marqusee & Baldwin, 1987). The data presented here indicate the Glu 2⁻ ... Arg 10⁺ salt bridge limits helix extension in the amino-terminal direction in RN-24. Earlier work has indicated that the helix of the S-peptide of ribonuclease A is limited in solution by a helix stop signal (Kim & Baldwin, 1984). The present NMR study supports the notion that the S-peptide region of the ribonuclease A is an autonomous folding unit (Shoemaker et al., 1987a) and that the S-peptide sequence codes in large part for its own folding and can adopt in solution a conformation closely related to its conformation in the native protein in the crystalline state without the need of stabilizing tertiary interactions.

Considerable evidence has accumulated in recent years which shows that many short linear peptide fragments of proteins are able to adopt folded conformations in aqueous solutions [see Wright et al. (1988) for a review]. The factors that stabilize elements of secondary structure in short linear peptides are now accessible to experimental investigation. Such structures are likely to play a fundamental role in initiation of protein folding. Detailed studies of the conformational propensities of linear peptides will increase our understanding of the factors that determine and stabilize protein structures in solution and will provide new insights into the earliest events in protein folding.

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