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Proton NMR Assignments and Regular Backbone Structure of Bovine Pancreatic Ribonuclease A in Aqueous Solution†

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ABSTRACT: Proton NMR assignments have been made for 121 of the 124 residues of bovine pancreatic ribonuclease A (RNase A). During the first stage of assignment, COSY and relayed COSY data were used to identify 40 amino acid spin systems belonging to alanine, valine, threonine, isoleucine, and serine residues. Approximately 60 other NH-αCH-βCH systems were also identified but not assigned to specific amino acid type. NOESY data then were used to connect sequentially neighboring spin systems; approximately 475 of the possible 700 resonances in RNase A were assigned in this way. Our assignments agree with those for 20 residues assigned previously [Hahn, U., & Rüterjans, H. (1985) *Eur. J. Biochem. 152*, 481-491]. Additional NOESY correlations were used to identify regular backbone structure elements in RNase A, which are very similar to those observed in X-ray crystallographic studies [Wlodawer, A., Borkakoti, N., Moss, D. S., & Howlin, B. (1986) *Acta Crystallogr. B42*, 379-387].

Bovine pancreatic ribonuclease A (RNase A)[1] has played a pivotal role in studies of protein structure (Scheraga & Rupley, 1962; Richards & Wyckoff, 1971), folding (Kim & Baldwin, 1982), and enzyme catalysis (Blackburn & Moore, 1982). We propose extending these studies through the use of NMR spectroscopy, which recently has begun to yield detailed information about the solution structure of small proteins (Wüthrich, 1986).

NMR studies of RNase A date from 1957, with the publication of the first ^{1}H NMR spectrum for a protein (Saunders et al., 1957). Work since then has led to assignments for many of the aromatic protons (Bradbury & Scheraga, 1966; Meadows et al., 1968; Patel et al., 1975; Lenstra et al., 1979; Tanokura, 1983) as well as a number of carbon resonances (Walters & Allerhand, 1980; Howarth & Lian, 1984). Hahn and Rüterjans (1985) made sequence-specific ^{1}H NMR as-

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[1] Abbreviations: COSY, two-dimensional chemical shift correlation spectroscopy; NMR, nuclear magnetic resonance; DQF-COSY, double-quantum-filtered COSY; FID, free induction decay; NOE, nuclear Overhauser enhancement; NOESY, two-dimensional NOE spectroscopy; RELAY, relayed COSY; RNase A, bovine pancreatic ribonuclease A (EC 3.1.27); DSS, 2,2-dimethyl-2-silapentane-5-sulfonate. In discussions of NOESY data, $d_{AB}(i,j)$ designates the distance between proton types A and B located in amino acid residues i and j, respectively, where N, α, β, and γ denote the amide protons, αCH, βCH, and γCH, respectively. The $d_{AB}(i,j)$ notation is also used as an adjective; e.g., $d_{\alpha N}$-type NOE, referring to the NOE associated with the $d_{\alpha N}(i, i+1)$ distance.

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signments for 20 residues in RNase A using 2D NMR methods. These assignments were used to locate conformational changes associated with nucleotide binding. Additional assignments and use of NOE data will permit more extensive study of conformational perturbations resulting from substrate and inhibitor binding and chemical modification of RNase A.

Recent technical developments have made it possible to study the structure of folding intermediates with NMR spectroscopy (Adler & Scheraga, 1988). NMR spectroscopy also is being used to monitor indirectly the kinetics of regular backbone structure formation in RNase A during refolding (Udgaonkar & Baldwin, 1988). Rigorous interpretation of both types of experiments requires assignment of the resonances in the ^{1}H NMR spectrum of RNase A. The present work confirms the assignments of Hahn and Rüterjans (1985) and continues the sequential assignments of the backbone resonances of RNase A to near completion.

Materials and Methods

Sample Preparation. RNase A was purchased from Sigma Chemical Co. (type XII-A powder) and used without further purification. D_2O and DCl were obtained from Merck and Co. NMR samples were 0.4–0.5 mL of 5–7 mM RNase A. H_2O samples at several different pH readings, 3.2, 3.5, 3.7, and 4.0, were prepared. These samples had 10% D_2O to provide a signal for the lock. Fully exchanged RNase A was prepared by dissolving the protein in D_2O, adjusting the pH to 3.2 using DCl, and heating at 60 °C for 1 h. The sample was then lyophilized repeatedly from 99.9% D_2O. Partly exchanged RNase A was prepared by dissolving the protein in H_2O, adjusting the pH to 3.2, lyophilizing, and redissolving in 99.96% D_2O. All pH values are glass electrode readings with no correction for isotope effects.

NMR Spectroscopy. DQF-COSY (Piantini et al., 1982; Rance et al., 1983), NOESY (Kumar et al., 1980), and RELAY (Wagner, 1983; Bax & Drobny, 1985) data were acquired with quadrature detection in both time dimensions by using time-proportional phase incrementation in t_1 (Marion & Wüthrich, 1983). Phase cycling for the DQF-COSY was carried out as described by Piantini et al. (1982) and for the RELAY as described by Wagner (1983). The RELAY mixing time, 2τ, was 36 ms. The phase cycling for NOESY was that of Bodenhausen et al. (1984) as modified by Otting et al. (1986) to provide sine modulation in both t_1 and t_2. The NOESY mixing time was 200 ms with a random variation of ±20 ms to suppress scalar coupling effects (Macura et al., 1982).

The experiments were carried out on a Bruker WM 300 spectrometer at Cornell University, on Bruker AM 500 spectrometers at the Biotechnology Research Institute in Montreal and the National Magnetic Resonance Facility at Madison, and on a General Electric GN 500 spectrometer located at Stanford University. The carrier frequency was placed in the center of the spectrum on the H_2O or HOD resonance frequency. For the spectra presented here, the sweep width was 7042 Hz for spectra obtained in H_2O and 6024 Hz for D_2O. Each FID was composed of 2048 data points and was the sum of 64 scans for DQF-COSY and RELAY and 160 scans for NOESY. Four dummy scans were used for each FID, and a recycle delay of 1.8 s including acquisition was used. Solvent suppression was carried out by presaturation at all times except during acquisition. A total of 512 t_1 values were collected for RELAY and NOESY and 600 t_1 values for DQF-COSY. For a given sample, the three NMR experiments were carried out in one contiguous block of time without removing the sample from the spectrometer.

Data Processing. The time domain data were transferred to a local area Vax cluster and processed by using FTNMR software (Hare Research, Inc.). RELAY data were multiplied by an unshifted sine-bell filter in both dimensions. DQF-COSY data in H_2O and D_2O were multiplied by a shifted sine bell with phase shifts of $\pi/32$ and $\pi/20$ in t_1 and t_2, respectively. NOESY data were multiplied by a shifted sine bell with phase shifts of $\pi/8$ and $\pi/16$ in t_1 and t_2, respectively. All the spectra were zero-filled to 1024 × 2048 real data points along f_1 and f_2, respectively. All spectra presented here were collected on samples adjusted to pH 3.2 and maintained at 30 °C during data acquisition.

Results and Discussion

Assignment Strategy. The strategy for carrying out the sequential assignments is as described by Wüthrich (1986). This involves identification of amino acid spin systems using DQF-COSY and RELAY, which are based on scalar or through-bond coupling, followed by assignment of the spin systems to specific residues in the amino acid sequence of the protein by analysis of NOESY (through-space coupling) data. The steps involved are (1) identification of the unique spin systems, i.e., Ala, Thr, Val, Gly, Ile, and Leu; (2) identification of the serine AMX spin system based on its characteristic downfield βCH resonance; (3) identification of the remaining NH–αCH–βCH spin systems from the RELAY data; (4) use of $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ NOEs to assign sequence-specifically unique short peptide sequences whose spin systems were identified in the first three steps; (5) extension of the sequential assignments from these anchor points to the remaining spin systems with the NOESY data.

In a few instances, where NH–βCH RELAY peaks were not observed, COSY and NOESY data were used to identify NH–αCH–βCH spin systems. All such spin systems are described in the following section.

Spin System Identification. The 124 amino acid residues of RNase A should give rise to 122 αCH–NH crosspeaks in the COSY spectrum in H_2O (124 residues − 4 prolines − N-terminal residue + 3 glycines). Up to 28 additional crosspeaks representing arginine and lysine side-chain resonances can be expected in this region of the COSY spectrum as well. Some of these crosspeaks, however, can be distinguished from backbone crosspeaks since they typically lie upfield from the αCH–NH signals. The αCH–NH, or fingerprint, region of the COSY spectrum of RNase A is shown in Figure 1; 131 crosspeaks are visible in this spectrum.

Of the three glycines in RNase A, only one can be identified at this stage by the characteristic pair of crosspeaks at approximately 7.9 ppm. A second glycine spin system was identified with RELAY data, where the second αCH–NH crosspeak is observed. The corresponding αCH–αCH crosspeak has the characteristic appearance of a glycine (later assigned to G112).

Methyl residues have the most unique and readily identifiable spin systems. For example, alanine αCH–βCH and threonine βCH–γCH crosspeaks fall in a characteristic region of the COSY spectrum. All 12 alanine residues were identified by using RELAY data, where intense NH–βCH crosspeaks permit unambiguous attachment of the side-chain methyl resonances to the NH resonances of the same residue (Figure 2). Nine of the 10 threonine βCH–γCH COSY crosspeaks then could be identified by elimination of the alanine αCH–βCH crosspeaks. Eight of the 10 threonine αCH were located through αCH–βCH correlations in the COSY spectrum and αCH–γCH crosspeaks in the RELAY experiment. Of these eight, five also showed NH–βCH crosspeaks in the RELAY

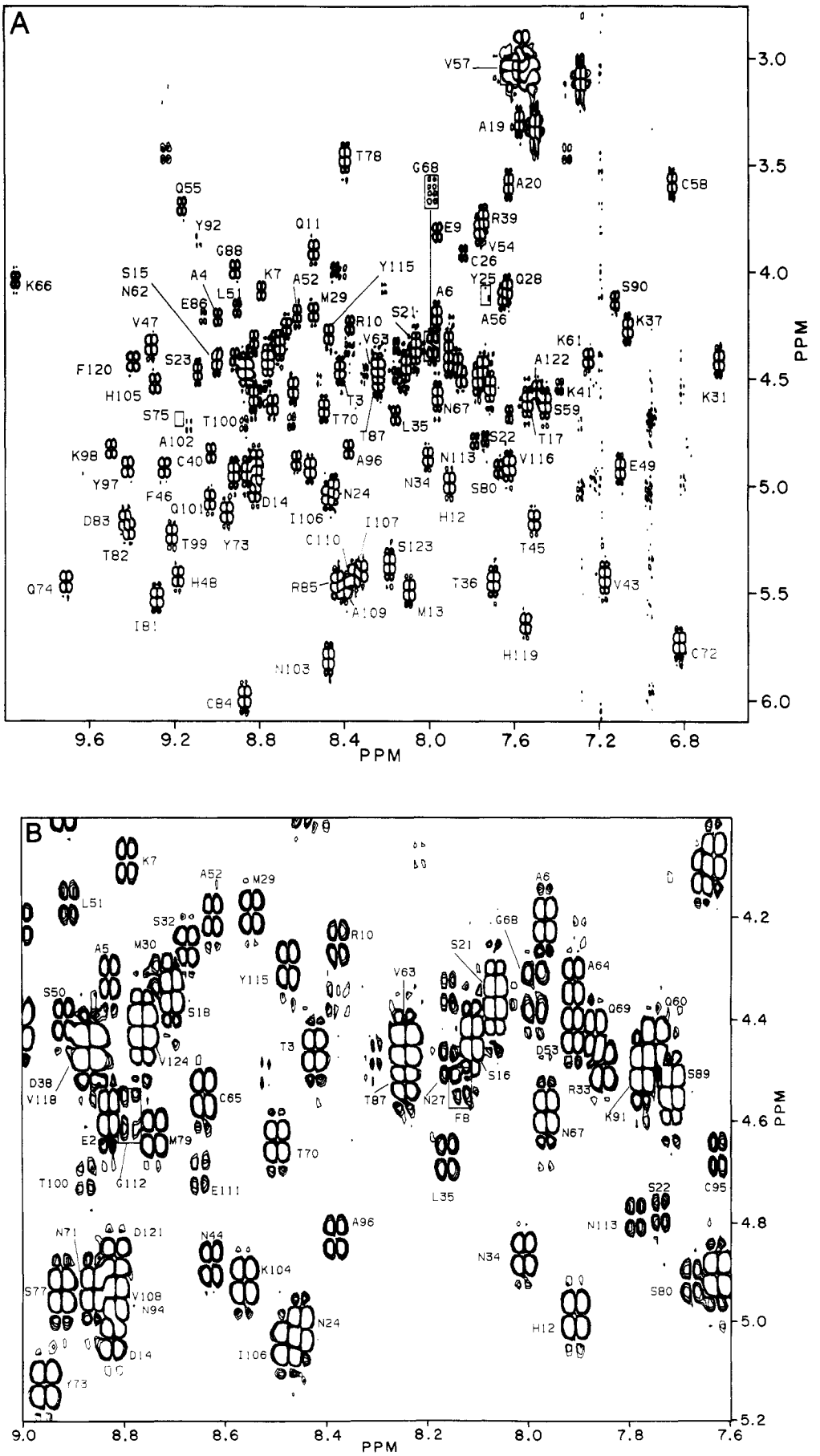

FIGURE 1: (A) Fingerprint region of the COSY spectrum of RNase A in H_2O. Most of the crosspeaks in this plot represent three-bond coupling between NH and αCH protons; additional crosspeaks for arginine and lysine side chains are also present. The labels indicate backbone crosspeak assignments. S75 is observed in the D_2O COSY spectra and in the H_2O RELAY spectra. (B) Expansion of the central part of the fingerprint region with additional crosspeak labels.

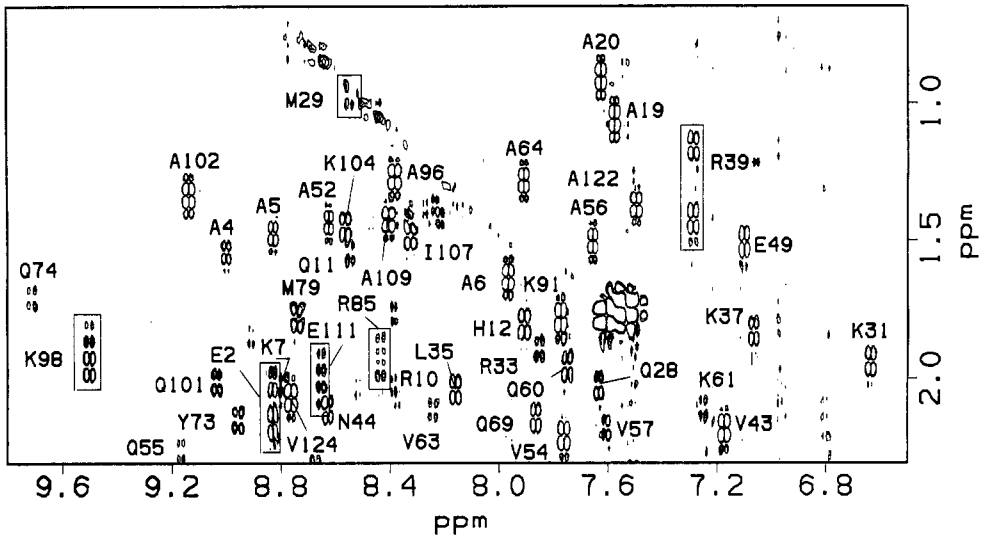

FIGURE 2: Expansion of the NH–βCH region of the RELAY spectrum of RNase A in H_2O. Most labels refer to NH–βCH RELAY crosspeaks, and boxes are to facilitate inspection. Asterisks indicate peaks arising from side-chain NH's.

spectrum, in addition to the αCH–NH COSY crosspeaks. A sixth threonine NH (subsequently assigned to T70) could be located by observation of a αCH–NH COSY crosspeak and NH–βCH and NH–γCH intraresidue NOEs in the NOESY spectrum. COSY fingerprint crosspeaks and NH–γCH NOEs provided tentative identification of two other threonine NH resonances (subsequently assigned to T3 and T87).

RNase A has nine valine residues. Seven complete spin systems, initially believed to be valines, could be identified unambiguously by observation of the NH–βCH and αCH–γCH crosspeaks in the RELAY spectrum. One of these valines (V57) has a fingerprint that overlaps with side-chain crosspeaks. An eighth valine spin system has αCH–γCH and γCH–γ′CH RELAY crosspeaks. A number of COSY αCH–NH crosspeaks were found at this αCH position, but only one of the NH's showed an NOE to the corresponding γCH. This gave an eighth complete valine spin system (subsequently assigned to V118). Two other valine βCH–γ,γ′CH spin systems (subsequently assigned to V108 and V116) could be identified from the COSY and RELAY γCH–γ′CH crosspeaks. This gives 10 spin systems when there should be only 9. One of these was later identified as a threonine (T45), as described below under Sequential Assignments.

The two leucine residues proved more elusive; only one showed a βCH–δCH RELAY, and the βCH resonance could not be aligned with any observable αCH–βCH COSY crosspeaks. We observed NOEs between two δCH resonances of one leucine (later assigned to L51) and a putative αCH, as did Hahn and Rüterjans (1985). A single COSY fingerprint peak could be aligned with this αCH resonance. δCH resonances from the other leucine residue showed NOEs to a single well-resolved NH resonance, but no further evidence could be obtained for its identification as an intraresidue NOE. All three isoleucine spin systems were at least partially identified with COSY and RELAY data, and the COSY fingerprint crosspeaks were located.

At this point, complete spin systems were identified for 2 glycines, all 12 alanines, 8 threonines, and 8 valines. Partial spin systems (NH, αCH, and part of the side-chain protons) were identified for all three isoleucines. Two fingerprint crosspeaks were connected tentatively to leucine side-chain spin systems.

Serine spin systems usually are not identified prior to sequential assignment, and yet the chemical shifts of this spin system are nearly unique (Wüthrich, 1986, see Table 2.3). Identification of serines in the COSY and RELAY spectra may be complicated by crosspeaks from glycines and threonine residues. At this stage, however, most of the threonine αCH–βCH and glycine αCH–αCH peaks had been located. With the COSY and RELAY data, we were able to identify fingerprint crosspeaks for 10 of the 15 serine residues in RNase A. These and the spin systems described above represent 44 of the 122 possible backbone crosspeaks in the fingerprint region of the COSY spectrum. Two other fingerprint crosspeaks are candidates for leucines.

Sixty of the remaining COSY fingerprint crosspeaks could be attached to at least one side-chain βCH resonance with COSY and RELAY data collected on RNase A dissolved in H_2O and D_2O. A summary of the COSY and RELAY crosspeaks observed at this stage of the assignments for RNase A is given in Figure 3.

Sequential Assignments. The sequence of RNase A contains a number of unique short peptide sequences containing amino acid types whose spin systems were identified in the first stage of assignment: A4-A5-A6, A19-A20, L35-T36, A52-D53-V54, V63-A64, I81-T82, T99-T100, I106-I107-V108-A109, and A122-S123-V124. These short peptides served as initiation sites for sequential connectivities. Using sequential $d_{\alpha N}$, d_{NN}, and $d_{\beta N}$ NOESY connectivities, sequence-specific assignment of the spin systems belonging to these peptides was carried out. The same connectivities were used to attach sequentially neighboring spin systems to these anchor points in the sequence, yielding sequential resonance assignments for nine partially overlapping stretches of peptide. These NOESY connectivities are summarized in Figure 4. The extensive d_{NN} connectivities, involving about half of the residues, proved to be particularly useful (Figure 5). In some cases, crosspeaks not resolved at pH 3.2 and 30 °C could be seen clearly at slightly higher pH (3.5–4.0) and temperature (35 °C). Breaks in the sequential connectivities occur at K41-P42, S75-Y76, Y92-P93, E111-G112, N113-Y115, V116-V118, and F120-D121. Some of the less straightforward aspects of the assignments are discussed below.

The P42 αCH resonance was located through a $d_{\alpha N}$ connectivity to V43. The remainder of this proline spin system was identified through observation of $d_{\gamma N}$ NOEs to V43 NH and αCH–βCH and γCH–δCH COSY crosspeaks.

The T45 spin system initially had been identified as a valine; the βCH resonance falls near 2 ppm, not the expected 4 ppm.

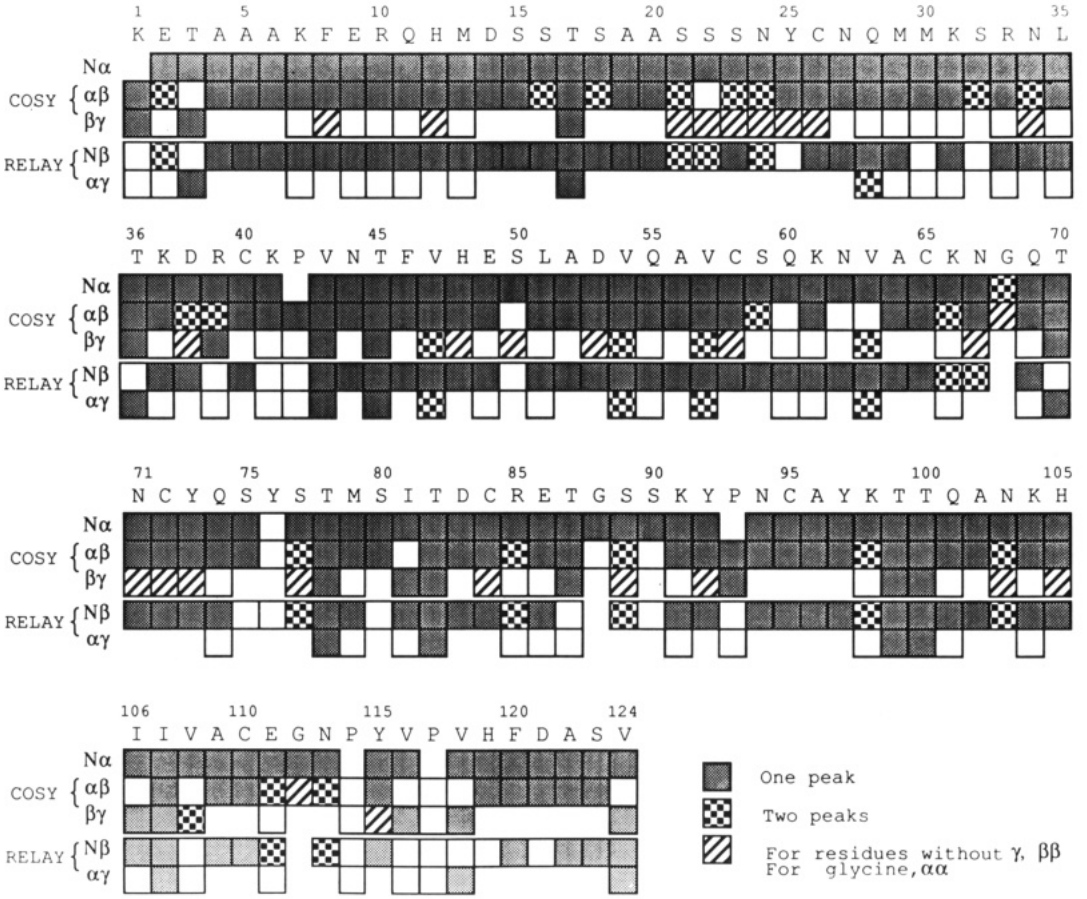

FIGURE 3: Summary of the observed COSY and RELAY correlations used to identify spin systems in the RNase A ^{1}H NMR spectrum. Under the one-letter code for each residue is a series of five boxes, each representing an intraresidue crosspeak in one of the two spectra. If a crosspeak is not observed, the box is blank. Shading indicates the presence of one crosspeak, and a checkered box indicates that two crosspeaks are evident. For residues that have no γCH, diagonal lines in the β–γ box indicate the presence of a crosspeak between the βCH resonances. Similar markings in the α–β box for glycine indicate the observed crosspeaks between αCH resonances. Crosspeak identifiers: N, backbone amide NH; α, αCH; β, βCH; γ, γCH.

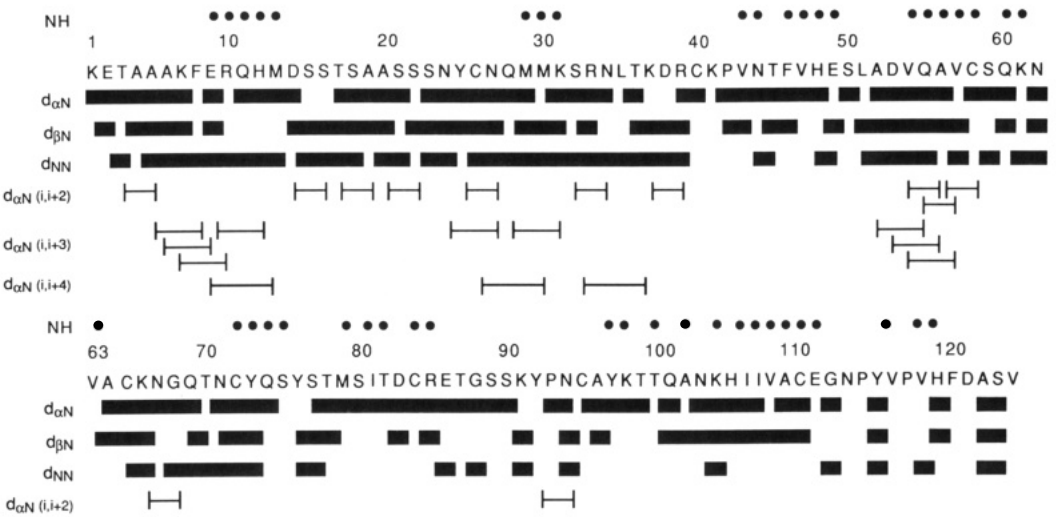

FIGURE 4: Summary of sequential and intermediate-range NOE data for RNase A. NOESY connectivities are indicated below the sequence. Solid circles above the sequence indicate slowly exchanging NH as defined by observation of a fingerprint crosspeak in the COSY spectrum of RNase A freshly dissolved in D_2O. Further details of sample preparation are provided under Materials and Methods.

The observation of the single γCH resonance, however, is consistent with its assignment to a threonine. This explains the missing threonine spin system and the extra valine spin system found in the first stage of spin system identification.

The S50 and L51 NH resonances nearly overlap at pH 3.2 and 30 °C. Hence, the potential $d_{\alpha N}$ NOE that is observed cannot be distinguished unambiguously from an intraresidue S50 αCH–NH NOE. At pH 4.0 and 35 °C, the NH resonances of S50 and L51 are well resolved and the $d_{\alpha N}$ connectivity can be made. Furthermore, these two amide protons both have NOEs to a proton with a chemical shift of about 4.1 ppm; this is likely to be one of the βCH resonances of S50. A putative βCH–β′CH COSY crosspeak is observed, but no αCH–βCH COSY crosspeaks are found. One of the βCH resonances is close in chemical shift (4.36 ppm) to the αCH resonance (4.40 ppm). As a result, αCH–βCH COSY

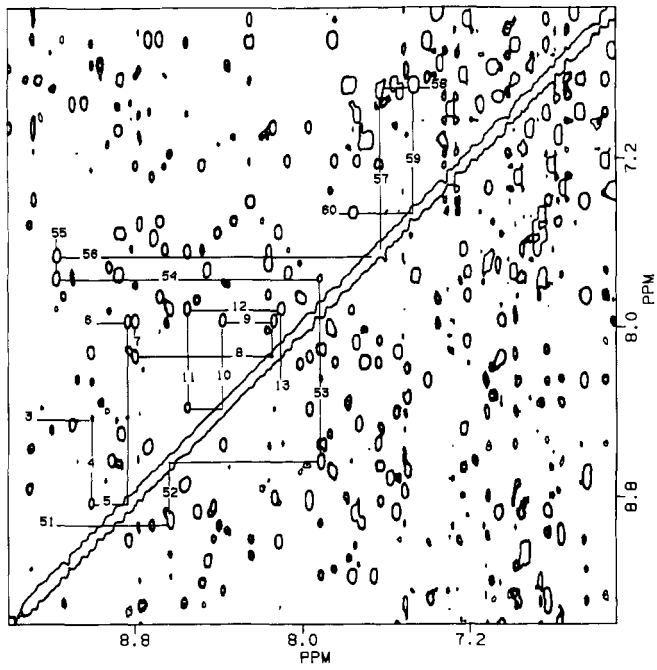

FIGURE 5: Expanded region of the NOESY spectrum of RNase A in H_2O. Sequential $d_{NN}(i, i + 1)$ NOEs for two helical stretches are shown.

crosspeaks would be difficult to observe since one would be close to the diagonal and the other would overlap with the βCH–β'CH crosspeak.

The N62 fingerprint was found to overlap with S15 through the observation of an NH–βCH crosspeak in the RELAY spectrum that clearly does not belong to S15. This βCH appears to show an NOE to V63 NH. Moreover, NOESY crosspeaks were observed connecting the αCH and βCH resonances of N62 to a pair of side-chain NH resonances, presumably the δNHs of N62. These same side-chain NH resonances showed strong NOEs to the backbone NH of V63.

The break in sequential connectivities at Y92-P93 is bridged by $d_{\alpha\alpha}$ and $d_{N\alpha}$ NOEs between these residues. In addition to permitting extension of the assignments to C95, these NOEs strongly suggest a cis peptide bond conformation between Y92 and P93 (Wüthrich et al., 1984), as was observed in X-ray studies (Wlodawer et al., 1986).

V108 was one of the valine spin systems for which only the βCH and γCH resonances could be identified in the first stage of spin system identification. The V108 fingerprint was assigned on the basis of $d_{\alpha N}$ and $d_{\beta N}$ connectivities from I107, and through observation of a $d_{\alpha N}$ connectivity to an alanine, presumably A109, all other alanines having been assigned. This putative V108 NH had NOEs to a pair of unassigned valine γCHs, which were not attributed to V108. These same γCHs showed NOEs to the NH of A109. The V108 fingerprint overlaps with that of N94 at pH 3.2 and 30 °C, but is resolved under other conditions.

After assignment of G68 and G88, the remaining glycine spin system could be assigned to G112, and two $d_{\alpha N}$ connectivities led to the assignment of N113. The remaining breaks in the sequential NOEs involved residues 114–120.

The last two valine spin systems to be assigned belong to V116 and V118. The αCH resonance for one of these spin systems was identified in the first stage of assignment. The fingerprint crosspeak was located by searching for intraresidue NOEs and found to overlap with the D38 crosspeak at pH 3.2 and 30 °C. These crosspeaks are resolved under other conditions. The presence of d_{NN} and $d_{\alpha N}$ sequential connectivities to another spin system suggested that this fingerprint was V118; V116 is followed by P117, and no sequential NOEs involving NH resonances are possible. The remaining valine thus was assigned to V116 by elimination. Connection of the V116 fingerprint crosspeak to its side chain was made through the observation of an intraresidue NH–γCH NOE to a single resolved side-chain methyl resonance. The Y115 fingerprint crosspeak then was assigned by a $d_{\alpha N}$ connectivity to V116.

Residues for which no assignments have been made are Y76, P114, and P117. NH and two βCH resonances for Y76 were identified tentatively through d_{NN} and $d_{\beta N}$ NOEs to S77. In addition, possible intraresidue NOEs between the unassigned NH and both βCH are observed. No fingerprint crosspeak is visible in the COSY spectrum at this NH resonance; hence, definitive assignment of the Y76 spin system is not possible. Table I contains a list of the resonance assignments obtained. The supplementary material contains detailed figures documenting the assignments for RNase A.

Our assignments for RNase A agree with the 20 previously made by Hahn and Rüterjans (1985). In addition, their tentative assignment of V43 (Val F in their paper) is confirmed here, but their Val E has been assigned to T45. Chemical shifts of the T45 side-chain protons show large changes when nucleotides are bound to RNase A [see Figure 5 of Hahn and Rüterjans (1985)]. This is consistent with the suggested role of the T45 side-chain hydroxyl group in nucleotide binding.

Regular Backbone Structure. NOEs between residues that are distant in amino acid sequence can be used to identify helix, β-sheet, and turns (Wüthrich et al., 1984). These intermediate- and long-range connectivities observed in the NOESY spectrum of RNase A are summarized in Figures 4 and 6. Three helical segments were located through stretches of d_{NN}, $d_{\alpha N}(i, i + 3)$, and $d_{\alpha N}(i, i + 4)$ NOESY connectivities (Figure 4). Residues 5–12, 24–36, and 52–58 are in helical conformations. The third helix may also include L51, on the basis of a $d_{NN}(i, i + 2)$ connectivity to D53. Residues 14–22 show a number of $d_{\alpha N}(i, i + 2)$ connectivities, suggestive of a series of turns or 3_{10} helix. In the X-ray structure, these residues are involved in a series of turns.

β-Strands were identified by runs of $d_{\alpha N}$ connectivities and a lack of d_{NN} NOEs. Residues 44–50, 78–85, 95–112, and 119–122 were assigned extended chain conformations in this way. At this stage, residues for which no regular backbone structure has been assigned are residues 1–4, 37–43, 59–77, 86–94, and 113–118.

An NOE between the NH of T3 and the βCH of A5 and A6 suggests that residues 1–4 are not in a helical conformation. These NOEs have been observed in the isolated C-peptide and are thought to be evidence for a salt bridge between the side-chain groups of E2 and R10 (J. J. Osterhout, personal communication). Such an interaction is seen in X-ray-derived structures of RNase A (Wlodawer et al., 1986). A type I β-turn involving residues 36–39 is suggested by a $d_{\alpha N}(i, i + 2)$ connectivity between K37 and R39 and a d_{NN} connectivity between K37 and D38. The d_{NN} NOE is not expected in the case of a type II turn (Wüthrich et al., 1984). This is in agreement with the type I turn observed in the X-ray structure. A possible turn is also indicated at residues 65–68 by a $d_{\alpha N}(i, i + 2)$ NOE between K66 and G68. The N67 and G68 NH resonances are too close for a d_{NN} NOE to be observed. These NOE connectivities are consistent with the type I turn involving these residues that is observed in the X-ray structure. A $d_{\alpha N}(i, i + 2)$ NOE between Y92 and N94 and the cis peptide bond between Y92 and P93 are strong evidence for a type VI turn from K91 to N94 (Lewis et al., 1973). The absence of assignments for P114 and P117 limits determination

Table I: ^{1}H Chemical Shifts for Bovine Pancreatic Ribonuclease A at 30 °C and pH 3.2[a]

residue	NH	αCH	βCH	γCH	others	residue	NH	αCH	βCH	γCH	others
K, 1		4.12	1.97	1.50	δCH 1.78; εCH 3.05; ζNH 7.61	N, 62	9.00	4.40	2.65		
						V, 63	8.24	4.45	2.12	0.75, 0.44	
E, 2	8.83	4.58	2.17, 2.01			A, 64	7.91	4.32	1.29		
T, 3	8.43	4.46	4.79	1.48		C, 65	8.65	4.55	2.80		
A, 4	9.00	4.21	1.55			K, 66	9.95	4.04	1.90, 1.70	1.81, 1.54	
A, 5	8.83	4.32	1.49			N, 67	7.96	4.58	3.26, 2.77		
A, 6	7.97	4.21	1.65			G, 68	7.99	4.34, 3.62			
K, 7	8.80	4.09	2.03			Q, 69	7.87	4.43	2.15		
F, 8	8.13	4.53	3.44, 3.07		δCH 7.05	T, 70	8.50	4.64	4.77	1.19	
E, 9	7.96	3.81	2.46			N, 71	8.86	4.94	3.69, 2.79		
R, 10	8.38	4.25	2.08			C, 72	6.82	5.74	2.80, 2.52		
Q, 11	8.55	3.90	1.55			Y, 73	8.96	5.13	2.89, 2.16		δCH 6.76
H, 12	7.91	4.99	2.66, 1.81		δCH 6.98; εCH 8.77	Q, 74	9.72	5.45	1.71		
M, 13	8.10	5.49	2.80			S, 75	9.20	4.69	4.12		
D, 14	8.82	5.03	2.19			Y, 76	8.72[b]		3.17, 2.99[b]		δCH 7.29; εCH 6.97
S, 15	9.01	4.41	3.90			S, 77	8.93	4.94	4.02, 3.86		
S, 16	8.11	4.44	4.01			T, 78	8.40	3.46	3.67	0.71	
T, 17	7.54	4.61	4.22	1.17		M, 79	8.74	4.62	1.79		
S, 18	8.71	4.34	3.87			S, 80	7.68	4.92	4.15		
A, 19	7.58	3.29	1.07			I, 81	9.29	5.53	1.78	0.91	
A, 20	7.63	3.59	0.92			T, 82	9.42	5.21	3.93	1.38	
S, 21	8.07	4.36	4.01, 3.84			D, 83	9.44	5.17	2.77, 2.71		
S, 22	7.74	4.78	4.19, 3.99			C, 84	8.87	5.99	2.97, 2.64		
S, 23	9.10	4.47	4.19, 4.10			R, 85	8.44	5.45	1.96, 1.87		
N, 24	8.45	5.02	2.98, 2.78			E, 86	9.07	4.21	2.11		
Y, 25	7.73	4.10	3.48, 2.97		δCH 7.21; εCH 6.55	T, 87	8.24	4.52	4.57	1.13	
C, 26	7.84	3.91	3.26			G, 88	8.92	3.99			
N, 27	8.16	4.49	2.93			S, 89	7.72	4.53	3.95, 3.84		
Q, 28	7.63	4.08	2.03	2.44, 2.29		S, 90	7.13	4.13	3.96		
M, 29	8.55	4.19	0.98			K, 91	7.77	4.50	1.79		
M, 30	8.73	4.31	1.77			Y, 92	9.09	3.85	3.43, 2.84		δCH 6.95
K, 31	6.64	4.41	1.95			P, 93		3.29	1.17	1.95	
S, 32	8.68	4.26	4.03, 3.96			N, 94	8.82	4.95	2.79		
R, 33	7.85	4.49	1.90			C, 95	7.63	4.66	2.99		
N, 34	8.01	4.87	3.21, 2.99			A, 96	8.38	4.83	1.28		
L, 35	8.16	4.67	2.05	1.62	δCH 0.94, 0.82	Y, 97	9.43	4.91	2.40		
T, 36	7.70	5.45	4.88	1.24		K, 98	9.50	4.83	1.96, 1.84		
K, 37	7.07	4.26	1.84			T, 99	9.22	5.23	4.28	1.34	
D, 38	8.87	4.45	2.89			T, 100	8.88	4.71	4.16	1.29	
R, 39	7.75	3.75	1.81, 1.59	1.43, 1.16	δCH 3.10; εNH 7.29	Q, 101	9.04	5.07	2.02		
C, 40	9.03	4.85	2.97, 2.81			A, 102	9.14	4.72	1.35		
K, 41	7.39	4.54	1.68			N, 103	8.48	5.81	2.64, 2.39		
P, 42		4.54	2.51	2.30, 2.17	δCH 4.14, 3.96	K, 104	8.56	4.92	1.45		
V, 43	7.17	5.43	2.19	1.04		H, 105	9.30	4.52	3.36, 3.21		δCH 7.51; εCH 8.77
N, 44	8.63	4.88	2.12			I, 106	8.48	5.05	1.85	1.09	
T, 45	7.51	5.16	2.35	0.76		I, 107	8.33	5.40	1.49	0.60	
F, 46	9.26	4.92	2.66		δCH 6.83; εCH 6.97; ζCH 6.67	V, 108	8.82	4.95	2.07	0.82, 0.68	
						A, 109	8.40	5.48	1.43		
V, 47	9.32	4.34	2.52	1.05, 1.00		C, 110	8.36	5.42	2.66		
H, 48	9.19	5.43	3.45, 3.18		δCH 6.86; εCH 8.24	E, 111	8.65	4.70	2.07, 1.94		
E, 49	7.10	4.92	1.51	2.58		G, 112	8.79	4.58, 3.81			
S, 50	8.92	4.40	4.36, 4.14			N, 113	7.79	4.79	2.93, 2.65		
L, 51	8.91	4.17	1.85, 1.75	1.56	δCH 1.14, 0.99	P, 114					
A, 52	8.63	4.20	1.45			Y, 115	8.48	4.29	2.97, 2.84		δCH 7.23; εCH 6.81
D, 53	7.91	4.42	3.17, 2.75			V, 116	7.63	4.91	2.20	0.85	
V, 54	7.76	3.80	2.21	1.14, 1.12		P, 117					
Q, 55	9.17	3.69	2.27			V, 118	8.87	4.45	2.01	0.81, 0.57	
A, 56	7.66	4.11	1.51			H, 119	7.55	5.64	3.35, 3.23		δCH 7.12; εCH 8.77
V, 57	7.61	3.06	2.20	1.11, 0.75		F, 120	9.40	4.42	2.76		δCH 6.77; εCH 7.09
C, 58	6.86	3.58	2.77, 2.66			D, 121	8.82	4.87			
S, 59	7.45	4.61	4.06, 3.96			A, 122	7.49	4.55	1.38		
Q, 60	7.75	4.45	1.96			S, 123	8.19	5.37	3.89		
K, 61	7.25	4.40	2.11			V, 124	8.76	4.41	2.07	0.84	

[a] Chemical shifts are in parts per million referenced to DSS. [b] Tentative assignments based solely on NOE data (see text).

of the conformation from residue 113 to residue 118. A loop or turn is suggested by an NH–NH NOE between E111 and V116.

The antiparallel β-strands can be oriented with respect to one another with interstrand NOEs; some of these are diagrammed in Figure 6. All of the NOESY data discussed here are consistent with a solution structure for RNase A that is similar to that seen in X-ray crystallographic studies (Wlodawer et al., 1986).

The identification of regular backbone structural elements can sometimes be aided by $^3J_{HN\alpha}$ coupling constant data. We have measured $^3J_{HN\alpha}$ from the COSY data, and none were found to be less than 6 Hz. The mean value for our helical residues is about 8.5 Hz, while that for nonhelical residues is about 9.3 Hz. The high values for the helical residues may perhaps be due to a combination of low digital resolution (approximately 3 Hz/point in the original data) and the line widths for a protein of this size. The measured *apparent*

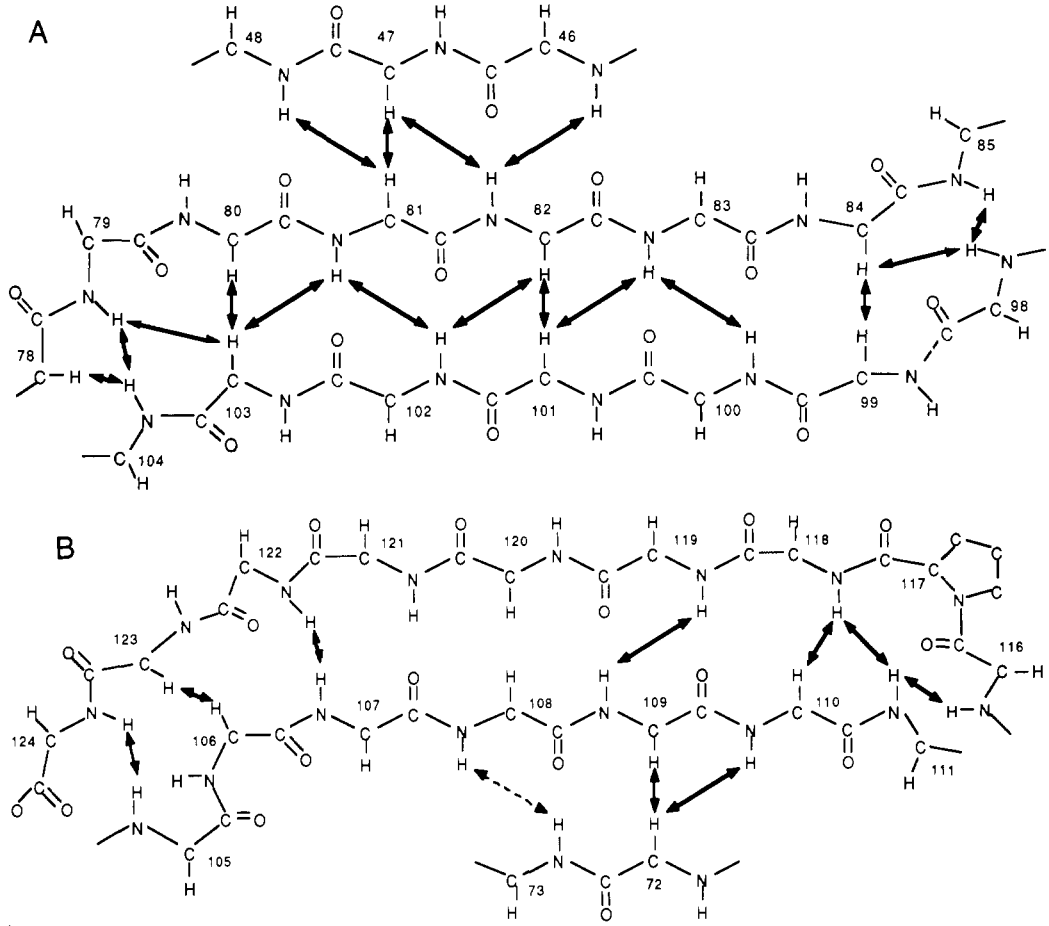

FIGURE 6: NOESY connectivities used to establish the relative orientation of β-sheet strands in RNase A. The dashed line in (B) indicates a NOESY connectivity that was obscured in H_2O but observed in D_2O.

coupling constants are therefore not very informative with regard to the regular backbone structures.

Amide Proton Exchange. Forty-five amide protons can be observed in the fingerprint region of a freshly prepared D_2O solution of RNase A. It is worthwhile to compare these with those observed by Wlodawer and Sjölin (1983) in their neutron diffraction studies of RNase A where they observe 26 slowly exchanging amides. Since the conditions of their experiment were different from ours, quantitative comparison is not possible. The deuterium exchange on their crystal was carried out at pH 5.5 in 55% deuterated *tert*-butyl alcohol and 45% D_2O over a period of 6 months. Our freshly prepared solution had a pH of 3.2 in 99.9% D_2O.

In the neutron diffraction data, the slowly exchanging amides occur at residues 11–13, 18, 30, 46–47, 54, 57, 58, 63, 65, 73–75, 79, 82, 89, 98, 100, 106–109, 116, and 118. The slowly exchanging amides observed in the COSY spectrum occur in the same stretches of residues but are more extensive: residues 9–13, 29–31, 43–44, 46–49, 54–58, 60–61, 63, 72–75, 79, 81–82, 84–85, 97–98, 100, 102, 104, 106–111, 116, and 118–119. All the slowly exchanging amides observed in the crystal are also observed in solution except for three: S18, S89, and C65. S18 and S89 are neither hydrogen-bonded nor buried in the crystal structure and yet are partially protected from amide exchange in neutron diffraction studies. Wlodawer and Sjölin (1983) attribute this apparent discrepancy to errors in the refinement procedure. Alternatively, these amides may be partially protected by intermolecular interactions in the crystal structure. In solution, these amides become fully exposed, resulting in rapid exchange. C65, which is partially protected in neutron diffraction studies, is both hydrogen-bonded and buried in the crystal structure and yet is not observed in the COSY spectrum of RNase freshly dissolved in D_2O. This may be an artifact of the lyophilization step in the sample preparation. The D_2O sample was prepared by redissolving a sample lyophilized from H_2O. The structure of lyophilized RNase A may be different from that in solution; addition of solvent may find C65 temporarily exposed, resulting in exchange at that position. This interpretation is supported by the observation that if the D_2O sample of RNase A is prepared by dilution of a sample in H_2O, then a weak C65 fingerprint crosspeak is observed in the COSY spectrum (A. D. Robertson and J. B. Udgaonkar, unpublished results). Aside from C65, fingerprints for residues 4, 7–8, 14, 33–34, and 59 are observed in the COSY spectrum of the D_2O sample prepared by dilution of H_2O.

Concluding Remarks. The sequential resonance assignments for RNase A have been carried out to near completion. The availability of these assignments, with additional side-chain assignments now being made, will make possible the determination of the *solution* structure of RNase A and will allow more detailed interpretation of folding experiments on RNase A.

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Herranz. Their work, describing assignments for 95 residues of RNase A, was presented in a poster at the XIII International Conference on Magnetic Resonance in Biological Systems, August 1988, at Madison, WI.

Supplementary Material Available

Figures showing COSY, NOESY, and RELAY connectivities used for the sequential proton resonance assignments for bovine pancreatic ribonuclease A (45 pages). Ordering information is given on any current masthead page.

Registry No. RNase, 9001-99-4.

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