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Sequential Assignment of the ^{1}H Nuclear Magnetic Resonance Spectrum of Barnase†

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ABSTRACT: Two-dimensional nuclear magnetic resonance spectroscopy has been used to study the bacterial ribonuclease barnase (MW 12382). Resonance assignments have been made for protons in all of the 110 residues. Analysis of medium- and long-range contacts in NOESY spectra has demonstrated that the major elements of secondary structure in barnase in solution are essentially identical with those found in the crystal structure.

Barnase is a guanine-specific bacterial ribonuclease (MW 12382) containing 110 amino acids (Hartley, 1989). This protein is currently being studied in detail in this laboratory by using site-directed mutagenesis. A number of areas are being investigated including the factors affecting protein stability, the pathway of protein folding, and the mechanism of catalysis (Kellis et al., 1988, 1989; Sali et al., 1988; Matouschek et al., 1989; Mossakowska et al., 1989). NMR spectroscopy has the potential to provide useful information on all of the above areas (Jardetzky & Roberts, 1981). The availability of extensive assignments for barnase is, however, an essential prerequisite for any detailed study using NMR spectroscopy. In recent years, the development of two-dimensional NMR methods (Ernst et al., 1987) has enabled sequence-specific resonance assignments to be obtained for small proteins (Wüthrich, 1986). We report here the assignment of proton resonances in all of the 110 residues of barnase.

Experimental Procedures

Barnase was purified from cultures of *Escherichia coli* containing the plasmid pMT410 (Paddon & Hartley, 1987) as described previously (Mossakowska et al., 1989). Purified barnase was dialyzed exhaustively against distilled water and then lyophilized. NMR samples were prepared by dissolving lyophilized protein in 0.5 mL of 90% H_2O/10% D_2O or 100% D_2O and adjusting the pH or pD with DCl. Protein concentrations were between 4 and 6 mM. NMR spectra were

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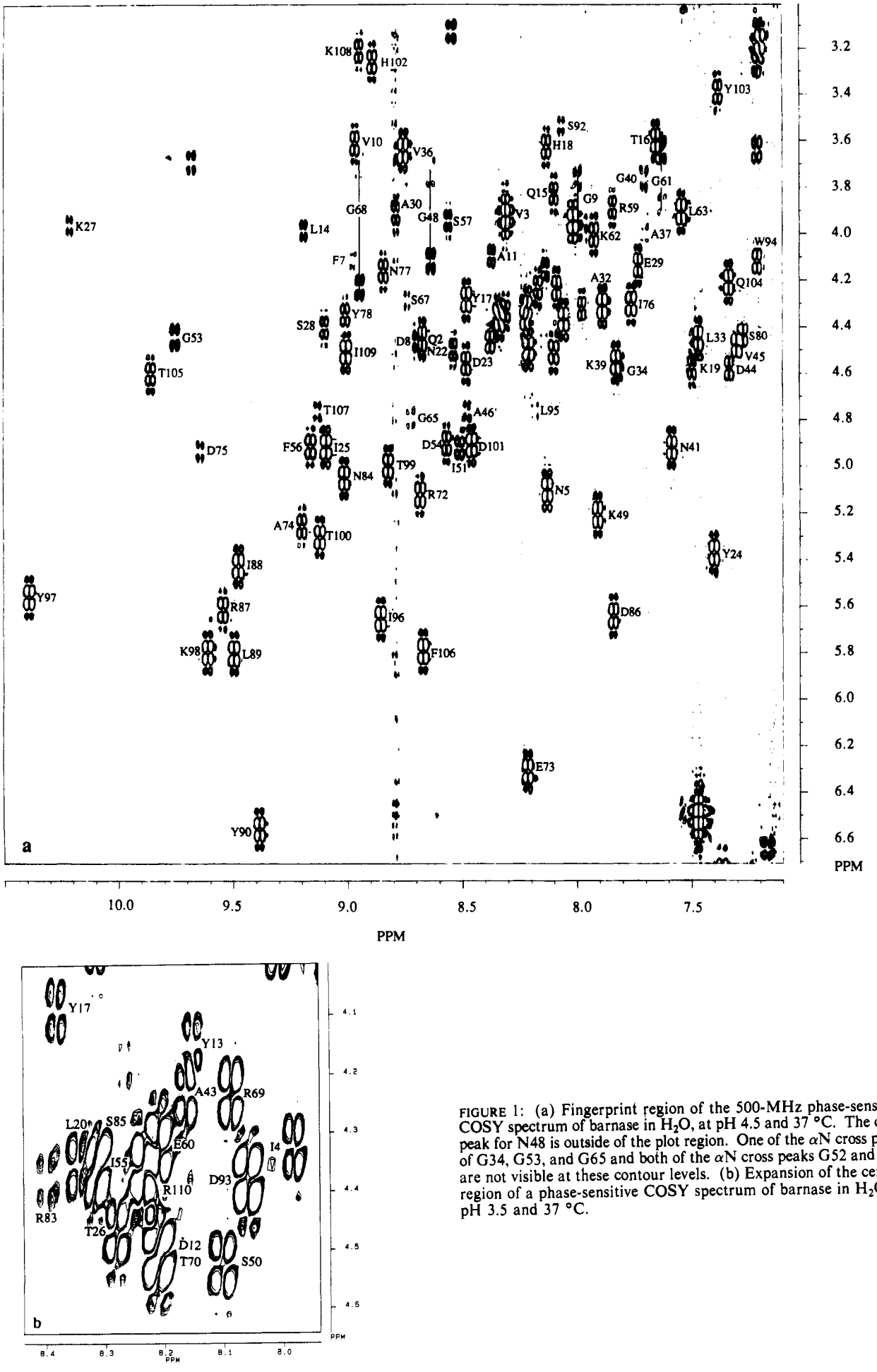

FIGURE 1: (a) Fingerprint region of the 500-MHz phase-sensitive COSY spectrum of barnase in H_2O, at pH 4.5 and 37 °C. The cross peak for N48 is outside of the plot region. One of the αN cross peaks of G34, G53, and G65 and both of the αN cross peaks G52 and G81 are not visible at these contour levels. (b) Expansion of the central region of a phase-sensitive COSY spectrum of barnase in H_2O, at pH 3.5 and 37 °C.

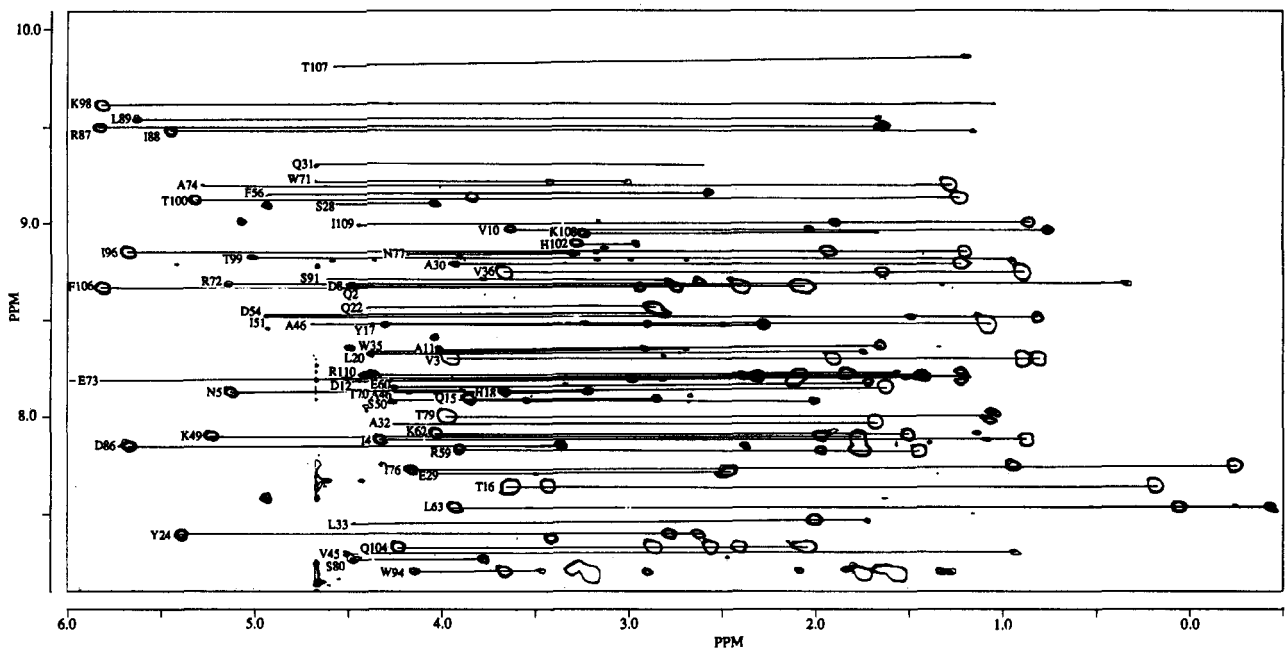

FIGURE 2: N-H–aliphatic region of the 500-MHz TOCSY spectrum of barnase in H_2O, with a 55-ms spin-lock time, at pH 4.5 and 37 °C.

acquired on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer. Spectra were recorded at 37 °C, pH 4.5 and pH 3.5, and 27 °C, pH 3.5. COSY (Aue et al., 1976; Bax & Freeman, 1981), DQF-COSY (Piantini et al., 1982; Rance et al., 1983), TOCSY (Braunschweiler & Ernst, 1983; Bax & Davies, 1985), RELAY (Eich et al., 1982; Bax & Drobny, 1985), and NOESY (Jeener et al., 1979; Anil Kumar et al., 1980) experiments were recorded with 2048 data points in t_2 and 512 t_1 increments with a spectral width of 8000 Hz in both dimensions. A relaxation delay of 1.5 s was used. Suppression of the solvent signal was achieved by presaturation of the water resonance during the relaxation delay. NOESY spectra were also acquired in which water suppression was achieved by replacing the last 90° pulse with a 90°–x–t–90°–x (1–1) pulse sequence (Plateau & Gueron, 1982; Sklener & Bax, 1987) with the modifications described by Driscoll et al. (1989). The carrier was placed on the solvent signal and a t value of 80 μs was used. Phase-sensitive two-dimensional spectra were obtained by using the time-proportional phase incrementation method (Marion & Wüthrich, 1983).

TOCSY experiments were carried out by using spin-lock times of 55, 79, and 100 ms. RELAY spectra were recorded with mixing periods of 36 ms in H_2O and 30 ms in D_2O. A 150-ms mixing time was used in NOESY experiments.

For COSY, TOCSY, and RELAY spectra, 64 transients per increment were collected, while for NOESY spectra and TOCSY spectra with 79- and 100-ms spin-lock times, 128 transients per increment were acquired.

NMR data were processed by using the Bruker DISNMR package. Spectra were zero-filled to give 2048 × 1024 real data points along f_2 and f_1, respectively.

COSY, DQF-COSY, and RELAY spectra were processed with an unshifted sine bell window function in both dimensions. NOESY data were processed with a sine bell window function shifted $\pi/9$ in f_1 and $\pi/12$ in f_2. TOCSY data were processed with a sine bell window function shifted $\pi/8$ in f_1 and a trapezoidal window function in f_2.

Results and Discussion

Sequence-specific assignments for barnase were obtained by using the approach described by Wüthrich (1986). Amino acid spin systems were first identified by using COSY, RELAY, and TOCSY experiments that give information on through-bond scalar coupling. Neighboring spin systems were then identified from sequential interresidue NOE effects in NOESY spectra. Finally, sequence-specific assignments were obtained by matching segments of adjacent spin systems to the amino acid sequence.

Barnase contains 110 amino acids including three proline residues. In H_2O the COSY spectrum should, therefore, contain 107 αC-H–N-H fingerprint region cross peaks (or pairs of cross peaks for glycine residues). The COSY spectrum of barnase in H_2O, at 37 °C and pH 4.5, contains 98 fingerprint region cross peaks (Figure 1). In TOCSY spectra recorded under the same conditions, N–β peaks can be observed for four amino acids whose N–α cross peaks are obscured by the water resonance (Figure 2). Three additional fingerprint region cross peaks can be observed in COSY spectra recorded at pH 3.5 and 27 °C in D_2O. This accounts for 105 of the expected 107 fingerprint region COSY cross peaks.

Identification of Spin Systems. Eight of the ten glycine residues in barnase could be identified from the presence of two fingerprint region COSY cross peaks along the same NH chemical shift axis and from strong α–α peaks in DQF-COSY spectra (Figure 3). The other two glycine residues were identified during the sequential assignment procedure.

Fingerprint region cross peaks for the amino acids A, T, I, V, and L can be assigned uniquely if connectivities can be established between the N-H and methyl protons. Fingerprint region COSY peaks for seven out of the eight Ala residues could be identified from N–β cross peaks in RELAY and TOCSY spectra (Figure 2). The α–β COSY peak of the eighth alanine residue corresponds to an α proton resonance for which there is no fingerprint region COSY peak, and this accounts for one of the two missing fingerprint region peaks. Fingerprint region COSY peaks of eight of the nine threonine spin systems could be assigned from α–β and β–γ peaks in COSY spectra and from N–γ and α–γ peaks in TOCSY and RELAY spectra (Figures 2 and 3). The resonances of the remaining threonine residue were identified only during the sequential assignment process. No α–N COSY resonance was observed for this residue, and this accounts for the other absent

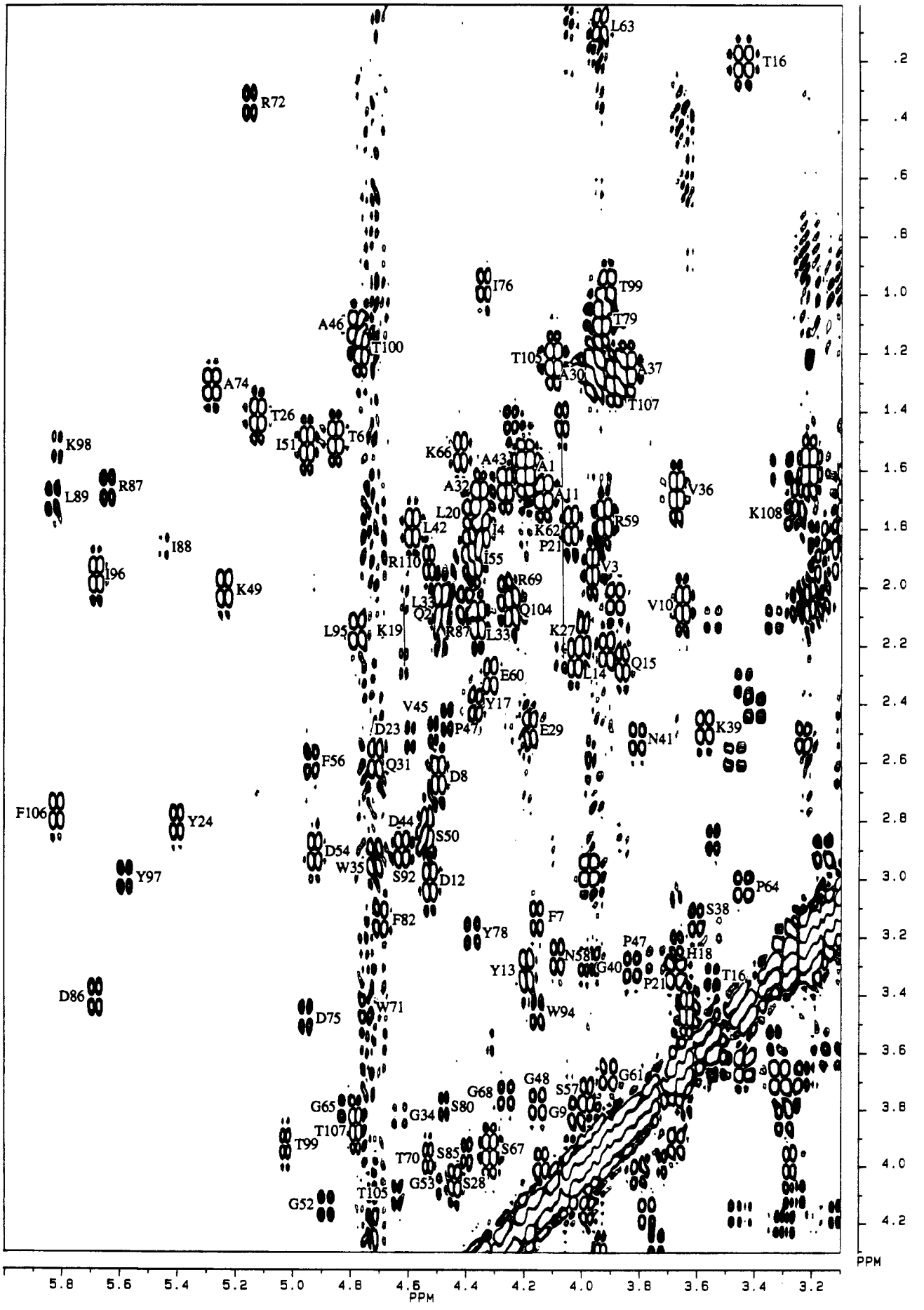

FIGURE 3: Part of the DQF-COSY spectrum of barnase in D_2O, at pH 4.5 and 37 °C. The cross peaks that are labeled are α–α for Gly, δ–δ for Pro, β–γ for threonine, and α–β for all other residues.

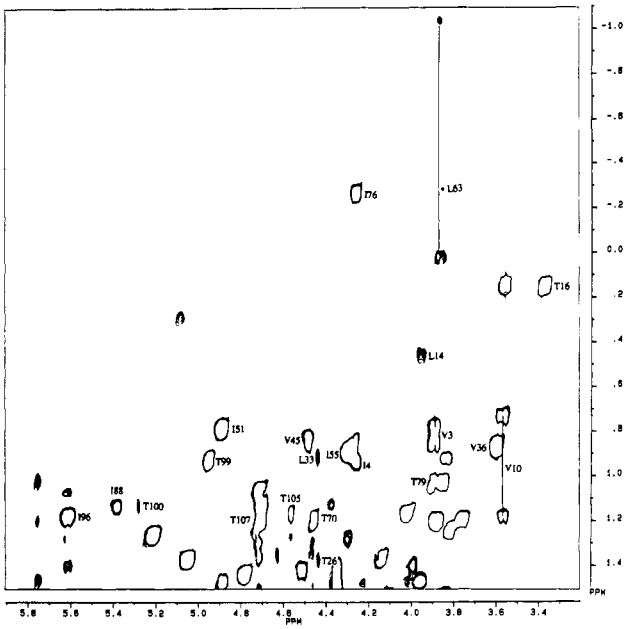

FIGURE 4: Cα-H–aliphatic region of the 500-MHz TOCSY spectrum of barnase in D_2O, with a 79-ms spin-lock time. The α–γ connectivities for Thr, Val, and Ile residues are labeled, together with α–δ connectivities for Leu residues.

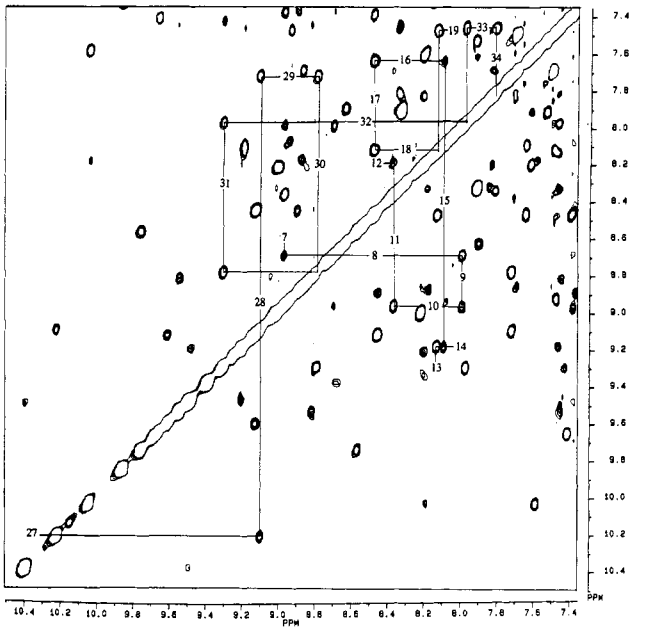

FIGURE 5: Expanded region of the phase-sensitive 500-MHz NOESY spectrum of barnase (in H_2O, at pH 4.5 and 37 °C), illustrating the assignment of residues 7–11, 13–18, and 27–34 via d_{NN} connectivities.

fingerprint region cross peak. Connectivities between the N-H and side-chain γ methyl protons for all of the valine and isoleucine residues were established from N–γ and α–γ cross peaks in TOCSY and RELAY spectra (Figures 2 and 3). Assignments for the γCH2 and δCH3 protons of four of the isoleucine residues could be obtained by following connectivities in COSY spectra, from α–γ cross peaks in TOCSY spectra and from α–δ intraresidue NOE contacts. Fingerprint region COSY peaks for four of the seven leucine residues could be assigned on the basis of intraresidue NOE effects between α and δ protons and from the observation of weak N–δ and α–γ cross peaks in TOCSY spectra.

For 63 of the remaining fingerprint region peaks, β-proton resonances could be identified via α–β COSY, N–β RELAY, and N–β TOCSY connectivities. The aromatic proton spin systems of the two histidine, three tryptophan, three of the four phenylalanine, and six of the seven tyrosine residues in barnase were identified by using DQF-COSY and TOCSY spectra. The aromatic resonances of these residues were linked to the rest of the amino acid spin systems by using NOE effects between ring protons and N, α, and β protons (Wüthrich, 1986). The sequential assignments obtained for the histidine C2-H protons are in agreement with those obtained previously by examination of the spectra of mutant proteins (Sali et al., 1988).

Sequential Assignment. Sequence-specific assignments for barnase were obtained by identifying neighboring amino acids from d_{NN}, $d_{\alpha N}$, and $d_{\beta N}$ connectivities in NOESY spectra (Figures 5, 6, and 7). Sequentially assigned peptide segments containing unique amino acid sequences were then matched to the primary sequence. Ninety-eight of the residues in barnase were assigned in a straightforward manner from five large (≥12 amino acid) peptide segments that contained the unique amino acid sequences G9-V10-A11 (residues 7–20), A30-Q31-A32 (residues 22–37), G52-G53 (residues 47–63), A74-D75-I76 (residues 64–91), and T99-T100 (residues 92–110). Once these amino acids had been assigned, the remaining residues were assigned from small peptide fragments. Residues 43–46 were assigned to a peptide fragment containing the sequence V-A, and residues 1–6 were assigned to a stretch of connectivities containing the sequence V-I. The remaining peptide fragments were assigned to residues 38–40 and 41–42. Assignments were thereby obtained for protons in all of the 110 residues in barnase. The details of the sequential assignment procedure are summarized in Figure 8, and the assignments are listed in Table I.

The proline residues in barnase were identified only during the second phase of the assignment process. The δ proton resonances could be identified from $d_{\alpha\delta}$ NOESY connectivities to preceding residues, and the α proton resonances were identified from $d_{\alpha N}$ connectivities to the succeeding residues. The remaining proton resonances in these amino acids were assigned by using COSY and TOCSY data.

Breaks in the assignment occur at eight positions in the sequence: T6-F7, L20-P21, A37-S38, G40-N41, L42-A43, A46-P47, L63-P64, and S91-S92. Three of these breaks occur at prolines, since it was not possible to unambiguously connect the α and δ proton resonances of these residues. The break at T6-F7 results from the absence of a fingerprint region cross peak for T6.

Structural Correlations. Secondary structure elements have distinctive short- and medium-range NOE contacts (Wüthrich, 1986). α-Helices are characterized by extended regions of d_{NN} connectivities and $d_{\alpha N}(i,i+3)$ and $d_{\alpha N}(i,i+4)$ contacts. In barnase, d_{NN} connectivities are observed for residues F7–H18 and for residues T26–W35. The assignment of helical character to these regions is confirmed by the observation of a number of $d_{\alpha N}(i,i+3)$ and $d_{\alpha N}(i,i+4)$ connectivities (Figure 8).

Regions of extended β-strand secondary structure are characterized by runs of $d_{\alpha N}$ connectivities connected by long-range interstrand NOE contacts. Examination of the NOESY spectrum of barnase reveals a number of long-range $d_{\alpha N}$, d_{NN}, and $d_{\alpha\alpha}$ NOE connectivities that indicate that residues 50–55, 72–76, 85–90, 95–101, and 106–108 form a five-stranded antiparallel β-sheet (Figure 9). These contacts are summarized in Figure 10. The interstrand contacts from the residues in the peripheral strand of the β-sheet formed by S50–I55 deviate from the pattern found in regular secondary structure, and it appears that residues 53–55 form a β-bulge.

Further evidence for the presence of secondary structural

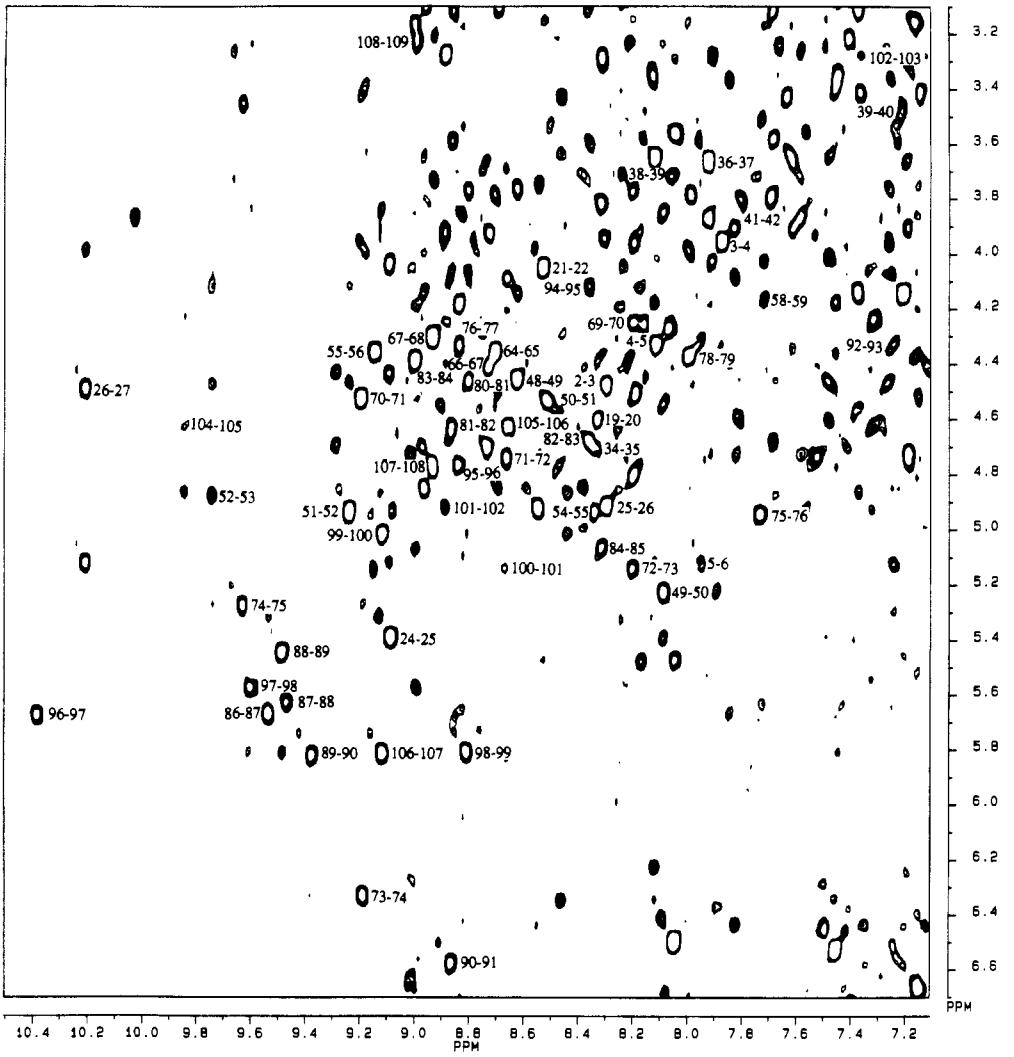

FIGURE 6: Fingerprint region of the phase-sensitive 500-MHz NOESY spectrum of barnase (in H_2O, at pH 4.5 and 37 °C). Sequential $d_{\alpha N}$ connectivities are labeled.

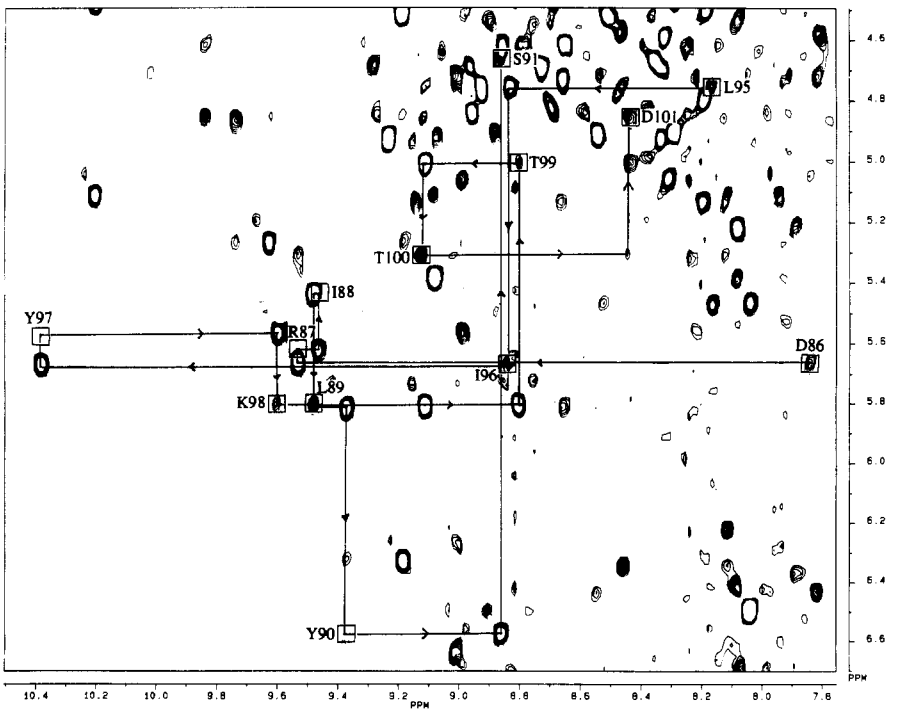

FIGURE 7: Region of the phase-sensitive 500-MHz NOESY spectrum of barnase (in H_2O, at pH 4.5 and 37 °C) illustrating the assignment of residues 86–91 and 95–101 via $d_{\alpha N}$ connectivities. Boxes indicate the positions of αN COSY peaks.

Table I: ^{1}H Chemical Shifts in Barnase[a]

residue	HN	αCH	βCH	γCH and others	residue	HN	αCH	βCH	γCH and others
Ala-1[b]		4.07	1.61		Ser-57	8.56	3.96	3.72, 3.54	
Gln-2	8.67	4.45	2.38, 2.05		Asn-58	6.38	4.05	3.24	
Val-3	8.30	3.94	1.91	γCH$_3$ 0.90, 0.76	Arg-59	7.85	3.90	1.72, 1.53	
Ile-4	7.89	4.31	1.77	γCH$_3$ 0.82	Glu-60	8.20	4.31	2.30, 2.09	
Asn-5	8.12	5.09	3.88, 3.43		Gly-61	7.63	3.89, 3.68		
Thr-6[b]	7.96	4.66	4.82	γCH$_3$ 1.40	Lys-62	7.91	4.00	1.76, 1.91	
Phe-7	8.97	4.13	3.09, 3.77		Leu-63[c]	7.54	3.92	0.05, −0.45	γCH −0.25, δCH$_3$ −0.98, −1.01
Asp-8	8.71	4.46	2.79, 2.61		Pro-64		4.35	2.30, 2.36	γCH$_2$ 2.34, δCH 3.00, 3.40
Gly-9	8.00	3.98, 3.78			Gly-65	8.73	4.82, 3.78		
Val-10[c]	8.96	3.63	2.00	γCH$_3$ 1.20, 0.76	Lys-66	8.21	4.37	1.50, 1.39	
Ala-11[c]	8.37	4.09	1.63		Ser-67	8.75	4.29	3.92	
Asp-12[c]	8.20	4.50	2.80, 2.96		Gly-68	8.93	4.24, 3.73		
Tyr-13[c]	8.14	4.15	3.29, 3.19	δCH 7.48, εCH 6.50	Arg-69	8.08	4.23	1.85, 1.48	
Leu-14[c]	9.18	4.00	2.21, 2.28	γCH 1.62, δ CH$_3$ 0.44, 0.20	Thr-70	8.21	4.48	3.90	γCH$_3$ 1.20
Gln-15[c]	8.09	3.84	2.23, 2.15		Trp-71	9.18	4.74	3.39, 2.98	N1H 8.93, C2H 7.15, C4H 7.48, C5H 7.00, C6H 7.16, C7H 6.98
Thr-16[c]	7.65	3.61	3.41	γCH$_3$ 0.16					
Tyr-17[c]	8.47	4.28	2.88, 2.23	δCH 6.32, εCH 6.58					
His-18[c]	8.12	3.64	3.20, 3.81	C2H 8.18, C4H, 6.19	Arg-72[c]	8.68	5.12	1.30, 0.32	
Lys-19[c]	7.50	4.54	2.30, 2.03		Glu-73[c]	8.21	6.29	2.10, 2.28	
Leu-20	8.34	4.35	1.73, 1.35	γCH 1.54, δCH$_3$ 0.64, 0.18	Ala-74[c]	9.19	5.25	1.27	
Pro-21		4.05	2.21, 2.33	γCH$_2$ 1.33, δCH 3.60, 3.40	Asp-75[c]	9.63	4.96	3.46, 2.76	
Asp-22	8.56	4.54	2.23		Ile-76[c]	7.74	4.30	0.95	γCH$_3$ −0.25, γCH$_2$ 1.07, 1.50, δCH$_3$ 0.38
Asn-23	8.49	4.56	2.47, 3.24						
Tyr-24	7.40	5.36	2.78, 2.60	δCH 6.86, εCH 7.25	Asn-77	8.83	4.15	3.26, 3.17	
Ile-25[c]	9.08	4.92	1.95	γCH$_3$ 0.82, γCH$_2$ 1.42, 0.93, δCH$_3$ 0.55	Tyr-78	8.99	4.34	3.15	δCH 7.17, εCH 6.65
					Thr-79	8.00	3.96	3.98	γCH$_3$ 1.05
Thr-26	8.32	4.46	5.10	γCH$_3$ 1.40	Ser-80	7.27	4.44	4.00, 3.77	
Lys-27	10.20	3.98	2.11, 2.02		Gly-81	8.80	4.65, 4.06		
Ser-28	9.09	4.40	3.99, 3.60		Phe-82	8.85	4.63	3.10, 3.58	δCH 7.70, εCH 7.51, ζCH 7.30
Glu-29	7.73	4.14	2.45, 2.55		Arg-83	8.40	4.38	2.04	
Ala-30[c]	8.79	3.93	1.21		Asn-84	9.00	5.04	3.80, 3.27	
Gln-31[c]	9.29	4.67	2.55, 2.36		Ser-85	8.31	4.32	3.93, 3.26	
Ala-32[c]	7.97	4.32	1.68		Asp-86	7.88	5.64	3.31, 2.37	
Leu-33[c]	7.47	4.45	2.03, 1.92	γCH 1.69, δCH$_3$ 0.99, 1.11	Arg-87[c]	9.53	5.61	1.68, 1.87	
Gly-34[c]	7.81	4.54, 3.80			Ile-88[c]	9.47	5.41	1.82	γCH$_3$ 1.16
Trp-35[c]	8.34	4.71	2.90, 2.68	N1H 10.02, C2H 7.20, C4H 7.59, C5H 7.27, C6H 7.10, C7H 7.44	Leu-89[c]	9.49	5.81	1.64, 1.72	γCH 1.54, δCH$_3$ 0.71, 0.48
					Tyr-90[c]	9.37	6.54	3.00, 2.90	
					Ser-91[c]	8.85	4.63	2.95	
Val-36[c]	8.77	3.66	1.66	γCH$_3$ 0.90, 0.84	Ser-92	8.08	3.55	3.71, 3.97	
Ala-37	7.95	3.81	1.25		Asp-93[c]	8.08	4.37	3.05, 2.48	
Ser-38	7.23	4.72	3.78		Trp-94[c]	7.21	4.13	3.42	N1H 9.65, C2H 6.89, C4H 7.42, C5H 6.93, C6H 6.82, C7H 7.22
Lys-39	7.84	4.55	1.77, 1.47						
Gly-40	7.70	3.77, 2.45							
Asn-41	7.58	4.90	3.55, 2.45		Leu-95[c]	8.17	4.76	2.12, 1.70	
Leu-42	8.21	3.98	2.19, 2.09		Ile-96[c]	8.84	5.65	1.94	γCH$_3$ 1.19, γCH$_2$ 1.82, 1.41, δCH$_3$ 1.07
Ala-43	8.18	4.22	1.61						
Asp-44[c]	7.34	4.55	2.87, 2.55		Tyr-97[c]	10.38	5.55	2.96, 2.81	δCH 7.02, εCH 6.92
Val-45[c]	7.30	4.49	2.45	γCH$_3$ 0.92, 0.95	Lys-98[c]	9.60	5.79	1.48, 1.69	
Ala-46[c]	8.48	4.75	1.08		Thr-99[c]	8.80	5.00	3.90	γCH$_3$ 0.95
Pro-47		4.43	2.40	γCH$_2$ 1.94, 2.09, δCH 3.53, 3.22	Thr-100[c]	9.12	5.28	4.77	γCH$_3$ 1.15
Gly-48	8.64	4.13, 3.76			Asp-101	8.45	4.92	3.01	
Lys-49[c]	7.90	5.20	1.99, 1.52		His-102	8.87	3.26	3.88, 2.97	C2H 8.78, C4H 7.12
Ser-50[c]	8.10	4.51	2.85, 2.64		Tyr-103	7.39	3.39	3.60	δCH 6.54, εCH 6.76
Ile-51[c]	8.50	4.94	1.48	γCH$_3$ 0.17, γCH$_2$ 0.25, 0.78, δCH3 −0.38	Gln-104	7.33	4.20	2.03	
					Thr-105[c]	9.84	4.62	4.12	γCH$_3$ 1.29
Gly-52	9.23	4.88, 4.12			Phe-106	8.67	5.80	2.74, 2.97	δCH 6.96, εCH 6.84, ζCH 7.22
Gly-53[c]	9.73	4.46, 4.08			Thr-107[c]	9.13	4.74	3.81	γCH$_3$ 1.20
Asp-54	8.57	4.92	2.88, 3.05		Lys-108	8.94	3.22	1.67, 2.04	
Ile-55[c]	8.31	4.31	1.88	γCH$_3$ 0.92	Ile-109[c]	8.99	4.50	1.91	γCH$_3$ 0.82
Phe-56[c]	9.14	4.93	2.55, 2.74	δCH 6.87, εCH 6.76, ζCH 7.36	Arg-110	8.22	4.36	1.90, 1.51	

[a] Values at pH 4.5 and 37 °C. Chemical shifts are referenced to DSS. [b] No fingerprint cross peak observed for this residue. [c] Indicates that the fingerprint region COSY peak is observed in spectra recorded in D_2O, at pH 3.5 and 27 °C.

elements can be obtained from amide exchange data, as the hydrogen bonding in α-helix and β-sheet structures often protects amide protons from exchange (Wüthrich, 1986). The COSY spectra of a barnase sample freshly dissolved in D_2O contains cross peaks from 45 amide protons (Table I). The majority of these slowly exchanging amide protons are located within the predicted regions of α-helix and β-sheet. A more detailed study of the factors affecting the amide proton exchange rates in barnase is presently being undertaken.

In barnase, only strands of the antiparallel β-sheet formed by residues 85–90 and 94–100 are linked by a tight hairpin turn. The other major strands of β-sheet are linked by large loops containing 10 and 15 amino acids. The α-helix formed by residues 26–34 and the β-sheet formed by residue 50–55 are also linked by a 16-residue loop. The loops that link the regions of secondary structure were the most problematic portions of barnase to assign, as the sequential NOESY connectivities in these areas are often weak and two of the breaks in the assignment are located in the loop formed by residues 35–49. Similar problems in assigning residues in the loops of a homologous ribonuclease have been reported previously (Hoffmann & Rütterjans, 1988).

Examination of the crystal structure of barnase (Mauguen et al., 1982) shows that the two helical regions predicted from the NMR data correspond to the two helices in the X-ray structure. There is also a good general agreement between

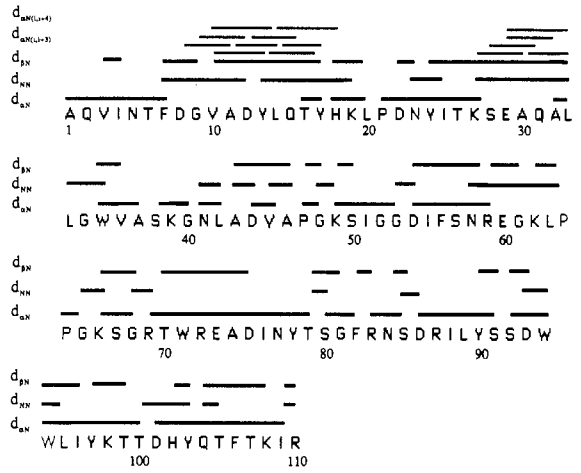

FIGURE 8: Summary of the sequential NOE contacts used in the assignment of barnase. Note that in order to show sequential residues residue L33 is repeated at the end of the top line and the beginning of the second, P64 at the end of the second and the beginning of the third, and W94 at the end of the third and the beginning of the fourth.

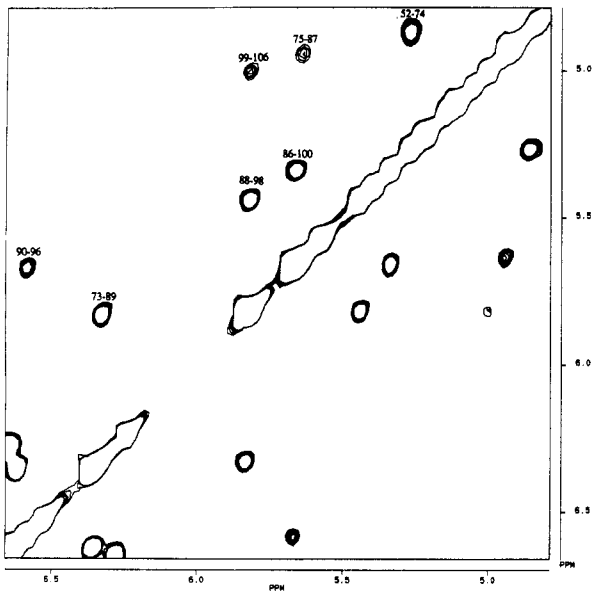

FIGURE 9: The region of the phase-sensitive 500-MHz NOESY spectrum of barnase (in D_2O, at pH 4.5 and 37 °C) containing interstrand $d_{\alpha\alpha}$ connectivities.

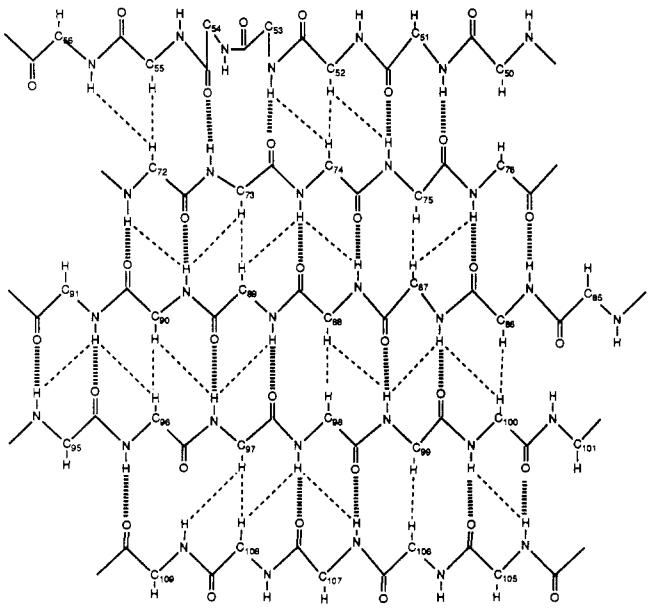

FIGURE 10: Schematic illustration of the long-range interstrand NOESY connectivities used to identify the regions of β-sheet in barnase.

the regions of β-sheet identified from the NMR data and those observed in the X-ray structure. It appears, therefore, that the major components of the secondary structure of barnase in solution are very similar to those found in the crystal structure. A comprehensive analysis of the interproton distance data obtained from NOESY spectra is underway to provide more detailed information on the solution conformation of barnase.

The availability of the assignments described herein will provide the basis for a detailed study to be made of the structure and dynamics of native barnase and of site-directed mutants by using NMR spectroscopy, and a variety of experiments are currently in progress in this laboratory.

Registry No. Barnase, 37300-74-6.

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