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Complete ^{15}N and ^{1}H NMR assignments for the amino-terminal domain of the phage 434 repressor in the urea-unfolded form

(sequence-specific NMR assignments/protein denaturation/protein folding/isotope-labeled proteins)

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ABSTRACT **The amino-terminal domain of the phage 434 repressor consisting of residues 1–69 forms a globular structure of five tightly packed helices, with nearly identical molecular architectures in crystals and in solution. Upon addition of urea to an aqueous solution of this protein, the NMR spectrum of a second form of the protein appears in addition to the native form, and at a urea concentration of 7 M, this urea-unfolded form is the only species observed. At intermediate urea concentrations, the two forms of the protein interconvert at a rate that allows the observation of the exchange process by NMR. Starting from the previous assignments for the native protein, we obtained nearly complete sequence-specific ^{1}H and ^{15}N NMR assignments for the unfolded form of the protein. For most amino acid residues, the ^{1}H chemical shifts of the urea-unfolded protein are very similar to the random coil values, but some discrete regions of the polypeptide chain were identified that are likely to retain residual nonrandom spatial structure as evidenced by deviations of ^{1}H chemical shifts and amide proton exchange rates from the expected random coil values.**

Protein folding—that is, the relations between amino acid sequence, folding pathways and kinetics, and the functional three-dimensional arrangement of a polypeptide chain—is presently the least understood step in any attempts at relating storage of genetic information with its expression by protein functions (e.g., refs. 1–3). Although many hundred native protein structures have been solved by x-ray diffraction in crystals (4) or by NMR spectroscopy in solution (5) and over 100 such structures are currently added every year (6), reliable information on the structure of unfolded polypeptide chains or structures of folding intermediates is still scarce and mostly limited to model peptides (e.g., refs. 7–9). The experience available from studies of "denatured" proteins and from polypeptides that adopt flexible extended structures in their functional form indicates that NMR in solution is a promising technique for structural characterization of such species. A prerequisite for obtaining the desired structural information by NMR is the availability of sequence-specific resonance assignments for the individual spins. Obtaining resonance assignments for a protein under denaturing conditions is, however, a challenging task, since the chemical shift dispersion is greatly reduced once the globular structure is lost (5). The present paper describes how nearly complete ^{1}H and ^{15}N resonance assignments were obtained for an unfolded form of a globular protein.

We studied the amino-terminal DNA-binding domain consisting of residues 1–69 of the phage 434 repressor [434 repressor(1–69)] (10) in the urea-unfolded form. Although it does not contain any disulfide bonds, the native form of this small protein is very stable; it has a melting temperature of 67°C in water at pH 5.2. The denaturation of the 434 repressor(1–69) is a reversible process, which is a prerequisite for the experiments described here, and the urea-unfolded form is in equilibrium with the native protein over a wide range of urea concentrations. The structure of the protein has been determined both by x-ray diffraction in crystals (11) and by NMR in solution (12), and the two structures are very similar. An overexpression system for the protein is available (13), which allows us to label the protein with ^{15}N and/or ^{13}C (12, 14). The use of the ^{15}N-labeled protein was indispensable, since the dispersion of the ^{1}H NMR chemical shifts in the unfolded form would not have been sufficient to allow the presently described resonance assignments.

MATERIALS AND METHODS

The uniformly ^{15}N-labeled 434 repressor(1–69) was prepared as described elsewhere (12, 14). For the NMR experiments, we used 3 mM protein samples in a mixed solvent of 90% H_2O/10% $^{2}H_2O$ at pH 4.8, containing 20 mM $NaClO_4$ and variable urea concentrations in the range from 0 to 7 M. All the spectra were recorded at a proton resonance frequency of 600 MHz on a Bruker AM600 instrument.

The assignments of the amide ^{15}N and ^{1}H resonances of the urea-denatured form of the 434 repressor(1–69) were obtained at 18°C and 4.2 M urea by using the exchange of heteronuclear longitudinal 2-spin order with the previously assigned native protein in a two-dimensional (2D) difference [^{15}N, ^{1}H] correlation experiment (15). At 7 M urea, sequential assignments were obtained with a nuclear Overhauser effect (NOE)-relayed [^{15}N, ^{1}H] 2D correlated spectroscopy (COSY) experiment (16) recorded with a mixing time of 300 ms, 160 t_1 values, 2048 t_2 values, t_{1max} = 57 ms, t_{2max} = 131 ms, 15-h measuring time. The assignments for the amide groups were further extended to the aliphatic ^{1}H resonances by using a 2D total correlated spectroscopy (TOCSY)-relayed [^{15}N, ^{1}H]-COSY experiment (16) recorded with a mixing time of 100 ms, 160 t_1 values, 2048 t_2 values, t_{1max} = 61 ms, t_{2max} = 132 ms, 18-h measuring time. For the assignments of the ring protons of Pro-42, Phe-44, Pro-46, and Trp-58, we used homonuclear [^{1}H, ^{1}H]-TOCSY spectra recorded at 500 MHz in a 7 M urea solution in $^{2}H_2O$, 512 t_1 values, 2048 t_2 values, t_{1max} = 39 ms, t_{2max} = 155 ms, 19-h measuring time. [^{15}N, ^{1}H]-COSY spectra were recorded by using a modified form (16) of the pulse sequence of Bodenhausen and Ruben (17) with 160 t_1 values, 2048 t_2 values, t_{1max} = 57 ms, t_{2max} = 131 ms, 35-min measuring time.

Exchange rates with the solvent for the amide protons of the urea-unfolded form of the 434 repressor(1–69), k_{ex}, were measured at 7.0 M urea, pH 4.8, and 18°C by dissolving a lyophilized protein sample into the urea-containing $^{2}H_2O$ solution and following the exchange process with a series of

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Abbreviations: 2D, two-dimensional; COSY, 2D correlated spectroscopy; NOE, nuclear Overhauser effect; NOESY, 2D NOE spectroscopy; TOCSY, 2D total correlated spectroscopy.

[^{15}N, ^{1}H]-COSY spectra. Sequence-dependent intrinsic exchange rate constants, k_c, were calculated by using the rules of Englander, Molday, and co-workers (18, 19). Slowed exchange in the urea-unfolded protein is described by protection factors k_c/k_{ex} (9).

RESULTS

Fig. 1*a* shows the [^{15}N, ^{1}H]-COSY spectrum of the 434 repressor(1–69) in the absence of urea. In this two-dimensional spectrum, the backbone amide group of each amino acid residue is represented by a cross peak. The cross peaks are widely spread over the [^{15}N, ^{1}H] plane, and with few exceptions are well separated. When urea is added to the protein solution (Fig. 1*b*), a second complete set of peaks appears, as is best seen by concentrating on the cross peaks of the Gly residues at the top of the three spectra. The spread of this second set of cross peaks in the [^{15}N, ^{1}H] plane is greatly reduced relative to that of the cross peaks corresponding to the native conformation. With few exceptions, in particular Asn-16, Thr-26, Arg-41, Phe-44, Glu-47, and Thr-63, the chemical shifts of the native form do not change substantially when urea is added [$\Delta\delta(^{1}H) < 0.1$ ppm; $\Delta\delta(^{15}N) < 0.5$ ppm)]. By further addition of urea, the cross peaks corresponding to the native-like form decrease in intensity, and those corresponding to the urea-unfolded form become more intense without showing a significant dependence of the chemical shifts on urea concentration [in all cases, $\Delta\delta(^{1}H) < 0.05$ ppm and $\Delta\delta(^{15}N) < 0.3$ ppm between 4 and 7 M urea]. Eventually, at 7 M urea all the protein is in the urea-unfolded form (Fig. 1*c*). This experiment has been repeated under different conditions, with temperature values ranging from 4°C to 34°C and the pH varying from 4.8 to 7.2, and results similar to those shown in Fig. 1 were obtained.

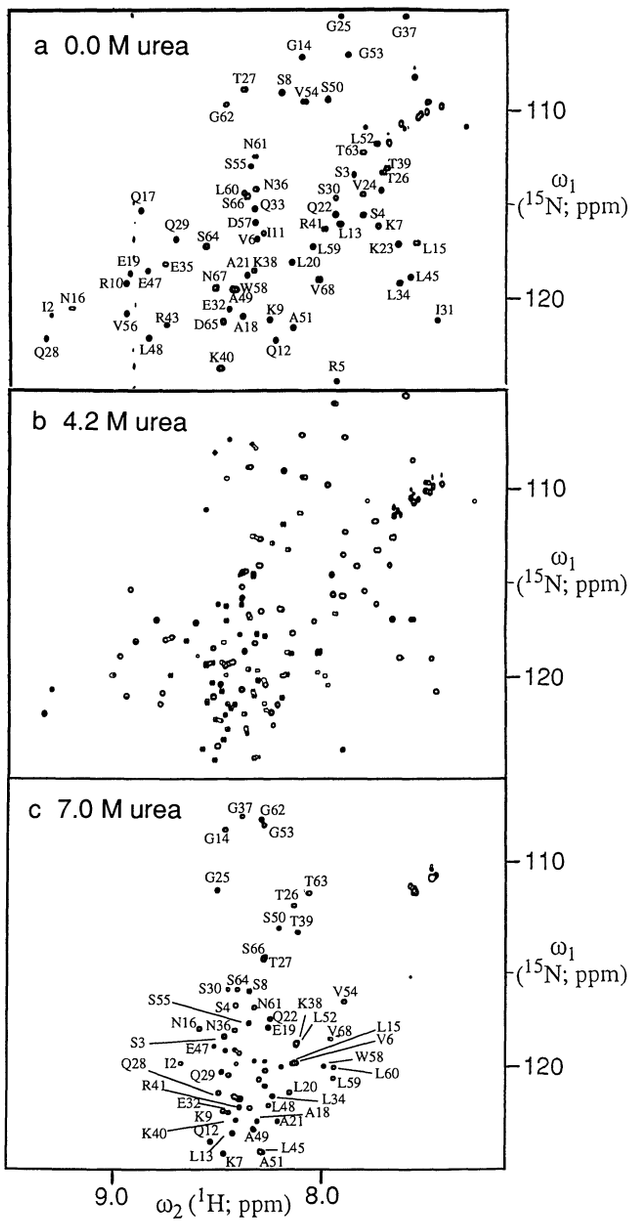

FIG. 1. (*a*) Two-dimensional [^{15}N, ^{1}H]-COSY spectrum of uniformly ^{15}N-labeled 434 repressor(1–69) (3 mM protein concentration, mixed solvent of 90% H_2O/10% $^{2}H_2O$ containing 20 mM $NaClO_4$ at pH 4.8, t = 18°C). The assignments are given with the one-letter amino acid code and the sequence positions. Phe-44 and Arg-69 lie outside of the plotted region. (*b*) Same as in *a*, but the sample contained 4.2 M urea. (*c*) Same as in *a*, but the sample contained 7.0 M urea. Only the urea-unfolded form is present under these conditions. Some sequence-specific resonance assignments are indicated as in *a*; for the other peaks, the assignments can be taken from Table 1.

At intermediate urea concentrations (Fig. 1*b*), the native and urea-denatured forms of the 434 repressor(1–69) are in conformational equilibrium. The interconversion between the two forms was used to extend the sequence-specific assignments of the ^{15}N and amide proton resonances from the native (12) to the urea-unfolded form by following the exchange of heteronuclear longitudinal 2-spin order in a two-dimensional difference [^{15}N, ^{1}H] correlation experiment (15). In this experiment, the direct correlation peaks of the native and the unfolded form for a given residue are related to each other by a pair of symmetry-related exchange peaks having opposite sign (15). The results of this assignment procedure are listed in Table 1 and illustrated in Fig. 1*c*, where some of the cross peaks of the urea-unfolded form are identified. On the basis of these amide group assignments, additional assignments for nonlabile protons were obtained by means of a TOCSY-relayed [^{15}N, ^{1}H]-COSY experiment (16). In practice, since near the denaturation half-point the presence of the two species leads to doubling of the number of relayed cross peaks, this experiment was done at a 7.0 M urea concentration, where the protein is exclusively present in the urea-denatured form (Fig. 1*c*). The nonlabile proton resonances are closely clustered around the expected random coil positions for individual atom types in the different residues (5, 21). This is readily apparent in Fig. 2, where a region of the TOCSY-relayed [^{15}N, ^{1}H]-COSY spectrum is displayed. For example, in spite of the differences in ^{15}N chemical shifts for the individual amino acid residues, the γ-methylene proton resonances of the 10 Gln and Glu residues are all located between 2.3 and 2.4 ppm. Similarly, all four Ala methyl groups resonate around 1.4 ppm, and all the Lys ε-methylene proton resonances were found near 3.0 ppm. We therefore assigned the individual nonlabile proton resonances to individual atoms or groups of atoms within a given amino acid side chain on the basis of their chemical shift values. Furthermore, comparison of a TOCSY-relayed [^{15}N, ^{1}H]-COSY (16) with a ^{1}H-relayed [^{15}N, ^{1}H]-COSY experiment allowed an unambiguous distinction of the α-proton resonances from those of the other nonlabile protons and thus further confirmed the spin system assignments.

The results of the assignment procedure are summarized in Table 1. In many cases, these assignments were confirmed by observation of intraresidual and sequential NOE connectivities (5) in a 2D NOE spectroscopy (NOESY)-relayed [^{15}N, ^{1}H]-COSY experiment recorded in 7 M urea with a mixing time of 300 ms. For the Phe-44, Trp-58, Pro-42, and Pro-46 rings, the resonance assignments were obtained with standard homonuclear techniques (5). The Pro spin systems were

Table 1. Proton and ^{15}N chemical shifts of the 434 repressor(1–69) in the urea-unfolded state

Residue	^{15}N	NH	αH	Other protons*
Ile-2	120.0	8.71	4.24	βH, 1.89; γH, 1.49, 1.19; γCH_3, 0.93; δCH_3, 0.89
Ser-3	118.6	8.50	4.53	βH, 3.91, 3.86
Ser-4	117.1	8.44	4.48	βH, 3.92, 3.86
Arg-5	121.7	8.42	4.34	δH, 3.18, 3.18; εNH, 7.18
Val-6	119.9	8.15	4.09	βH, 2.08; γCH_3, 0.94, 0.94
Lys-7	124.5	8.50	4.34	βH, 1.84, 1.74; γH, 1.48; δH, 1.68; εH, 2.99, 2.99
Ser-8	116.2	8.38	4.43	βH, 3.87
Lys-9	122.4	8.51	4.32	βH, 1.82, 1.72; γH, 1.45; δH, 1.68; εH, 2.98, 2.98
Arg-10	121.6	8.45	4.33	βH, 1.87, 1.75; γH, 1.63, 1.58; δH, 3.18, 3.18; εNH, 7.18
Ile-11	122.2	8.38	4.15	βH, 1.80; γH, 1.47, 1.17; γCH_3, 0.89; δCH_3, 0.86
Gln-12	123.9	8.57	4.40	βH, 2.07, 1.97; γH, 2.33, 2.33
Leu-13	123.5	8.46	4.37	βH and/or γH, 1.65, 1.57; δCH_3, 0.92, 0.86
Gly-14	108.1	8.50	4.00, 3.91	
Leu-15	120.0	8.17	4.32	βH and/or γH, 1.58; δCH_3, 0.90, 0.86
Asn-16	118.2	8.62	4.69†	βH, 2.87, 2.79
Gln-17	119.4	8.43	4.25	βH, 2.12, 2.04; γH, 2.36, 2.36
Ala-18	122.9	8.34	4.26	βCH_3, 1.40
Glu-19	118.1	8.29	4.29	βH, 2.07, 1.96; γH, 2.37, 2.37
Leu-20	121.4	8.18	4.35	βH and/or γH, 1.65, 1.56; δCH_3, 0.92, 0.87
Ala-21	122.9	8.24	4.28	βCH_3, 1.39
Gln-22	117.8	8.28	4.27	βH, 2.07, 1.98; γH, 2.39, 2.34
Lys-23	121.7	8.43	4.34	γH, 1.46, 1.39; εH, 2.98
Val-24	120.3	8.30	4.15	βH, 2.07; γCH_3, 0.96, 0.96
Gly-25	111.2	8.54	4.06, 4.02	
Thr-26	111.9	8.17	4.48	βH, 4.27; γCH_3, 1.22
Thr-27	114.6	8.30	4.40	βH, 4.25; γCH_3, 1.22
Gln-28	121.4	8.53	4.36	βH, 2.10, 1.99; γH, 2.37, 2.37
Gln-29	120.4	8.52	4.35	βH, 2.09, 1.98; γH, 2.37, 2.37
Ser-30	116.2	8.48	4.47	βH, 3.94, 3.86
Ile-31	121.1	8.31	4.16	βH, 1.87; γH, 1.47, 1.18; γCH_3, 0.91; δCH_3, 0.86
Glu-32	122.2	8.43	4.27	βH, 2.02, 1.96; γH, 2.36, 2.36
Gln-33	119.8	8.36	4.32	βH, 2.10, 2.01; γH, 2.37, 2.37
Leu-34	121.6	8.27	4.34	βH and/or γH, 1.65, 1.56; δCH_3, 0.91, 0.85
Glu-35	119.3	8.46	4.32	βH, 2.08, 1.97; γH, 2.39, 2.34
Asn-36	118.3	8.46	4.69†	βH, 2.83
Gly-37	107.4	8.42	3.97, 3.97	
Lys-38	119.0	8.14	4.39	βH, 1.87, 1.78; γH, 1.47, 1.39; δH, 1.68, 1.68; εH, 2.98, 2.98
Thr-39	113.3	8.14	4.33	βH, 4.19; γCH_3, 1.22
Lys-40	122.9	8.45	4.39	βH, 1.80, 1.71; γH, 1.46; δH, 1.67; εH, 2.97, 2.97
Arg-41	122.4	8.48	4.58	δH, 3.19, 3.19; εNH, 7.20
Pro-42			4.39	βH, 2.29, 1.78; γH, 2.00, 2.00; δH, 3.82, 3.60
Arg-43	120.0	8.45	4.33	βH, 1.85; δH, 3.13, 3.13; εNH, 7.15
Phe-44	119.8	8.30	4.67	βH, 3.29, 3.13; ring, 7.28, 7.30, 7.32
Leu-45	124.5	8.30	4.59	βH and/or γH, 1.55; δCH_3, 0.91, 0.88
Pro-46			4.33	βH, 2.25, 1.90; γH, 2.01; δH, 3.67, 3.59
Glu-47	119.0	8.55	4.25	βH, 2.04, 1.95; γH, 2.37, 2.37
Leu-48	122.0	8.28	4.35	βH and/or γH, 1.60; δCH_3, 0.93, 0.88
Ala-49	123.4	8.36	4.31	βCH_3, 1.41
Ser-50	113.1	8.24	4.40	βH, 3.91, 3.84
Ala-51	124.5	8.33	4.35	βCH_3, 1.40
Leu-52	119.0	8.16	4.32	βH and/or γH, 1.64, 1.59; δCH_3, 0.92, 0.87
Gly-53	107.8	8.31	3.98, 3.92	
Val-54	116.8	7.92	4.18	βH, 2.06; γCH_3, 0.89, 0.89
Ser-55	117.9	8.38	4.49	βH, 3.87, 3.81
Val-56	120.2	8.22	4.05	βH, 1.92; γCH_3, 0.81, 0.72
Asp-57	120.8	8.33	4.59	βH, 2.74, 2.61
Trp-58	120.2	7.97	4.59	βH, 3.30, 3.24; 2H, 7.24; 4H, 7.59; 5H, 7.14; 6H, 7.22; 7H, 7.47; NH, 10.13
Leu-59	120.8	7.97	4.22	βH and/or γH; 1.53, 1.47, 1.37; δCH_3, 0.85, 0.78
Leu-60	120.1	8.02	4.28	βH and/or γH, 1.62, 1.57; δCH_3, 0.92, 0.85
Asn-61	117.2	8.36	4.73†	βH, 2.85, 2.77
Gly-62	107.6	8.33	4.02, 3.95	
Thr-63	111.3	8.10	4.45	βH, 4.28; γCH_3, 1.20
Ser-64	116.3	8.44	4.51	βH, 3.93; 3.86
Asp-65	120.6	8.48	4.71†	βH, 2.80, 2.72
Ser-66	114.6	8.31	4.43	βH, 3.92, 3.84
Asn-67	119.3	8.50	4.74†	βH, 2.84, 2.76
Val-68	118.7	8.00	4.11	βH, 2.08; γCH_3, 0.93, 0.93
Arg-69	126.8	8.10	4.21	βH, 1.85, 1.72; γH, 1.59; δH, 3.17, 3.17; εNH, 7.22

The chemical shift values are relative to internal 3-trimethylsilylpropionate. All chemical shifts were measured in aqueous solution containing 7 M urea and 20 mM sodium perchlorate at pH 4.8 and t = 18°C. The underlined chemical shifts deviate from the random coil values (5, 20) by more than 0.1 ppm for nonlabile protons and more than 0.3 ppm for amide protons.

*The side chain proton resonances were individually assigned within their spin systems on the basis of the close coincidence with the random coil chemical shift values (see text).

†The assignments for these protons were obtained from a NOESY-relayed [^{15}N, ^{1}H]-COSY spectrum recorded at 4°C, since at 18°C their resonances coincide with the residual water signal.

sequentially assigned by using a [^{1}H, ^{1}H]-NOESY spectrum with a mixing time of 500 ms and a NOESY-relayed [^{15}N, ^{1}H]-COSY spectrum. The δ-protons of Pro-42 showed NOEs to the amide proton and the α-protons of Arg-41; the δ-protons of Pro-46 showed NOEs to the α-proton and the δ-methyl groups of Leu-45.

DISCUSSION

With the experiments described in the preceding sections, complete assignments were obtained for the polypeptide backbone ^{15}N and ^{1}H resonances in the urea-unfolded 434 repressor(1–69). In addition, extensive assignments resulted for nonlabile amino acid side chain proton resonances. The presently used approach was successful because of the relatively large ^{15}N chemical shift dispersion of the protein resonances in the unfolded form. This is clearly seen in Fig. 2, where, for example, the 10 Glu and Gln residues have virtually identical proton shifts but are well separated along the ω_1 (^{15}N) axis. Although we are not aware of any systematic investigations on this point, it thus appears that the sequence dependence of the ^{15}N chemical shifts is sufficiently large so that the concept of "random coil shifts" used quite successfully for protons (5) cannot be applied. It will be interesting to see whether the ^{13}C chemical shift dispersion in ^{13}C-labeled proteins can further contribute along similar lines

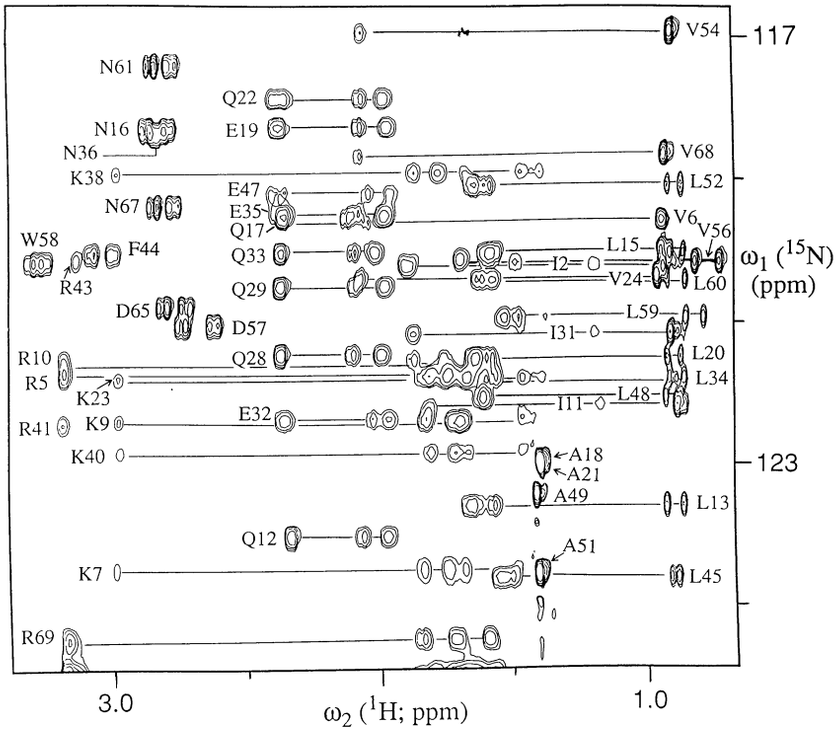

FIG. 2. Spectral region (ω_1 = 116.8–125.9 ppm; ω_2 = 0.7–3.4 ppm) of a TOCSY-relayed [^{15}N, ^{1}H]-COSY spectrum (16) recorded with uniformly ^{15}N-labeled 434 repressor(1–69) (3 mM protein concentration, mixed solvent of 90% H_2O/10% ^2H_2O containing 7.0 M urea and 20 mM $NaClO_4$ at pH 4.8, t = 18°C). Relayed peaks corresponding to protons that belong to the same spin system are connected by horizontal lines, and the amino acid residue is indicated with the one-letter amino acid code and the sequence position. To afford an impression of the different typical peak patterns present in the spectrum, the labels for the different amino acid types are placed near the relayed peaks with the most characteristic proton chemical shift—for example, the β-CH_3 resonance of Ala, the γ-CH_2 resonance of Glu and Gln, or the ε-CH_2 resonance of Lys.

to assignments of unfolded proteins, for example with HCCH experiments recorded with variable mixing times for spin system identification (21).

Another important factor for the successful assignment of the urea-unfolded form of 434 repressor(1–69) was that the native and unfolded forms of the protein exchanged on a time scale comparable to the spin relaxation times, which allowed us to extend the ^{15}N and amide proton resonance assignments from the native to the unfolded form by means of exchange-relayed [^{15}N, ^{1}H]-COSY (15). From the available literature, it appears that for many globular proteins one can find combinations of temperature, denaturant concentration, and pH such that the relative concentrations of the native and denatured forms are similar and the interconversion rates are suitable for exchange studies by NMR (22–26). Furthermore, in the case of the 434 repressor(1–69), the native form and the urea-denatured form coexisted at 4.2 M urea with only little overlap of peaks in the [^{15}N, ^{1}H]-COSY spectrum at 600 MHz (Fig. 1*b*). Overall, these observations indicate that the approach used here should be applicable for a wide range of proteins, including proteins larger than 434 repressor(1–69). In the situation where the chemical shifts of either the native or the unfolded form depend more strongly on the denaturant concentration than is the case with 434 repressor(1–69) in urea, additional measurements with variable denaturant concentrations may be needed to establish unambiguous correlations with the resonance assignments in the native form. As an alternative, it appears worthwhile to investigate whether proteins can be assigned under fully denaturing conditions with multidimensional heteronuclear experiments (21, 27) without making use of the chemical exchange between native and unfolded form, since part of the assignments for the unfolded 434 repressor(1–69) could also be established by the sequential approach (see preceding section). Yet another recently proposed approach for assignments in denatured proteins, state-correlated two-dimensional NMR (28), may extend such assignments to nonlabeled proteins, but seems otherwise more limited.

In Table 1, the chemical shifts that deviate from the random coil values (5, 20) by more than 0.3 ppm for amide protons and by more than 0.1 ppm for nonlabile protons are underlined. Overall, very few deviations exceeding these limits were observed. The largest deviations are in the polypeptide segments -Arg^{41}-Pro-Arg-Phe-Leu-Pro^{46}- and -Val^{54}-Ser-Val-Asp-Trp-Leu-Leu^{60}-. Both of these segments contain a central aromatic residue and several hydrophobic residues with which the aromatic side chain could interact. A visual impression of the magnitude and distribution of the chemical shift deviations from the random coil values is afforded by Fig. 3, where the deviations of the chemical shift values for the amide protons and α-protons of the urea-denatured form from the expected random coil positions (5, 20) are plotted. Similar chemical shift deviations from the random coil values have recently been reported for temperature-denatured lysozyme (26). Fig. 3 also identifies the amide protons in the urea-denatured protein, which are protected from fast exchange with the solvent (see *Materials and Methods*). The measured protection factors, k_c/k_{ex} (9), at 7.0 M urea, pH 4.8, and 18°C were 15 for Leu-20, 20 for Ala-21, 40 for Ala-51, 50 for Leu-52, 180 for Val-54, 20 for Trp-58, 50 for Leu-59, and 180 for Leu-60. Interestingly, the largest protection factors were observed for residues Ala-51, Leu-52, Val-54, Leu-59, and Leu-60. The coincidence with the location of large chemical shift deviations suggests that residual structure is maintained in the polypeptide segment 54–60 in the urea-denatured form. Evidence that some residual structure is present in the urea-unfolded form of the 434 repressor(1–69) also comes from observations made on the chemical shifts of

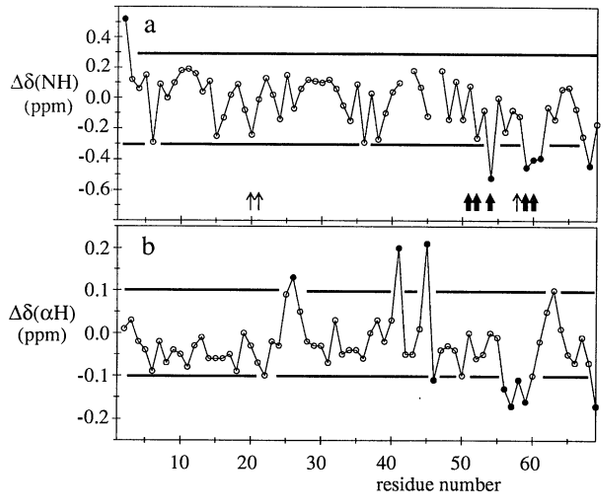

FIG. 3. Plots of the residues numbers versus the deviations of the chemical shifts in the urea-unfolded 434 repressor(1–69) (see Table 1 for the experimental conditions used) from the corresponding random coil chemical shift values (5, 28). (*a*) Backbone amide protons. The chemical shifts that deviate by more than 0.3 ppm from the random coil values are represented with solid circles; the others are represented with open circles. In addition to the chemical shift data, those residues with slowed amide proton exchange corresponding to a protection factor of 10 or larger (see *Materials and Methods*) are identified by thin arrows, and thick arrows indicate the residues with the slowest exchange rates. (*b*) α-Protons. The chemical shifts that deviate by more than 0.1 ppm from the random coil values are represented with solid circles; the others are represented with open circles.

the resonances of Leu-20 and Leu-48 (Table 1). These two residues are contained in identical tripeptide sequences Glu-Leu-Ala. If the protein were completely unfolded, one would expect a coincidence of the resonance frequencies for these two residues. In reality, one observes differences in the chemical shift values of the amide and methylene resonances of the two residues in the urea-unfolded form (Table 1). The resonance assignments described in this paper should thus enable a more complete structural characterization of the unfolded state than has hitherto been possible.

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