Interaction of urea with an unfolded protein
The DNA-binding domain of the 434-repressor

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Abstract Experimental techniques are presented for the observation of the solvation of the unfolded form of a globular protein, the N-terminal 63-residue polypeptide from the 434 repressor, in 7 M aqueous urea solution by both water and urea. With the use of ^{15}N-labelled urea it is demonstrated that the cross sections through two-dimensional nuclear Overhauser enhancement (NOE) spectra at the chemical shifts of H_2O and urea both contain direct NOEs with the protein, under conditions where exchange peaks are observed only in the water cross section. A preliminary analysis of the data showed that the residence times of urea molecules in solvation sites near the methyl groups of Val, Leu and Ile are significantly longer than those of water molecules in the same sites.

Key words: Protein folding; Urea denaturation; Protein solvation; NMR

1. Introduction

Urea is a frequently used denaturing agent, and thermodynamic and kinetic studies of urea-induced unfolding in aqueous solution have been reported for a large variety of proteins (e.g. [1–8]). For further improved understanding of these mixed-solvent systems it appears, however, that examination of protein–urea interactions on the molecular level will be needed. NMR spectroscopy is a promising technique for such investigations. A recent publication reported NMR studies of interactions between urea and the protein BPTI under conditions where the protein maintains its native three-dimensional structure, and it was found that at 4°C urea binds preferentially to "pockets and grooves on the protein surface" [9]. The present paper reports NMR studies of urea–protein interactions under conditions where the globular protein studied, 434 repressor-

(1–63), is nearly completely unfolded [10]. The observations on urea-binding are complemented by studies of protein hydration in the presence of urea.

The use of NMR spectroscopy for investigations of urea–protein interactions in solutions relies on the same principles as NMR studies of protein hydration [11–14]: close approach of urea to polypeptide hydrogen atoms on the protein surface is manifested by cross peaks in NOESY and ROESY spectra, and from the relative signs and intensities of the peaks in these two experiments one derives information on the life-times of the urea molecules in the solvation sites. As is generally encountered with solvent systems containing two or several protic components [15], complications may arise in aqueous urea solutions because apparent urea–protein NOEs might be mediated by proton exchange between water and urea. Therefore, much care was exercised in the selection of pH and temperature to minimize the rate of the urea–water proton exchange, which resulted in conditions where high resolution NMR spectra can be obtained only with special care.

2. Materials and methods

2.1. Expression and purification of the protein

The plasmid pT7-7/434A, which contains the 434-repressor(1–63) gene [16], the promoter of phage T7 [17] and an ampicillin resistance marker was used for the overexpression of the N-terminal 63-residue polypeptide of the 434-repressor. The protein was overexpressed in BL21/DE3 cells in Luria broth medium [17] containing 50 μg/ml of ampicillin. When the A_{600} of the cell suspension reached 1, over-expression was induced with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). Cell growth was continued for 5 h. Cells were harvested by centrifugation and the cell paste was resuspended in a minimal volume of lysis buffer containing 50 mM sodium acetate (pH 4.8), 3% glycerol, 30 mM NaCl, 0.5 mM EDTA, 1 mM β-mercaptoethanol, and 1 mM Pefabloc SC (Pentapharm AG) as a protease inhibitor. The cells were then disrupted in a French press at a pressure of about 15,000 psi. The suspension was centrifuged for 60 min at 17,000 rpm. The supernatant was loaded on a 30 ml SP-Sepharose column (Pharmacia) and the column was washed with lysis buffer until the OD_{280} returned to the baseline. The 434-repressor(1–63) was eluted from the column with a gradient from 30 to 500 mM NaCl in lysis buffer at a flow rate of 1.5 ml/min. All relevant fractions were analysed by 18% SDS-PAGE, and fractions which consisted of more than 95% 434-repressor(1–63) were pooled and dialysed against 6 l of pure water. During this initial dialysis a contaminating protein precipitated. The precipitate was removed by centrifugation and the supernatant adjusted to 50 mM potassium phosphate (pH 7.0). The protein was applied to a 10 ml phosphocellulose column (P11, Whatman) which had been equilibrated in 50 mM potassium phosphate and 50 mM NaCl. The protein was eluted with a linear gradient from 50 mM to 500 mM NaCl at a flow rate of 1 ml/min. Fractions containing 434-repressor(1–63) were pooled and dialysed against pure water. The final yield was approximately 60 mg per liter of culture.

2.2. NMR measurements

The protein was dissolved to a final concentration of 20 mM in a mixed solvent of 90% H_2O and 10% D_2O, containing a 7 M concentra-

tion of either unlabelled or ^{15}N-labelled urea (Fluka). The pH was adjusted to 7.6. All NMR spectra were recorded on a Bruker AMX600 spectrometer. Soft-NOESY experiments [18] were measured with a mixing time of 50 ms, using a modification of the pulse sequence of Otting et al. for water suppression [19] (spin locks $SL_{\phi4}$ = 0.5 ms and $SL_{\phi5}$ = 3 ms, delay τ = 120 μs, t_{1max} = 30 ms, t_{2max} = 152 ms, the spectra were folded once in ω_1, with spectral widths in ω_1 = 4,000 Hz and in ω_2 = 6,800 Hz, time domain data size 240 × 2,048 points, 128 scans per t_1 increment, sinc soft pulse length = 5 ms with the offset placed in the middle between urea and water). ROESY experiments [20,21] were recorded with a mixing time of 25 ms and water suppression as in [19] (spin locks $SL_{\phi4}$ = 0.5 ms and $SL_{\phi5}$ = 3 ms, delay τ = 120 μs, t_{1max} = 18 ms, t_{2max} = 152 ms, spectral width in ω_1 and ω_2 = 6,800 Hz, time domain data size 240 × 2,048 points, 128 scans per t_1 increment).

A NOESY-relayed [^{15}N,^{1}H]COSY experiment [22] was measured with a mixing time of 50 ms at a temperature of 265 K (t_{1max} = 192 ms, t_{2max} = 152 ms, spectral widths in ω_1 = 625 Hz and in ω_2 = 6,800 Hz). The urea signal was suppressed with two orthogonal spin lock pulses of duration SL_1 = 0.8 ms and SL_2 = 1.3 ms, separated by a delay τ = 120 μs. During the two INEPT steps the magnetization of protons which are not coupled to ^{15}N was destroyed with spin lock pulses of duration 2 ms. The size of the data matrix was 240 × 2048 points. 256 scans were accumulated per t_1 increment.

A NOESY experiment [23] using presaturation for water suppression [24] was recorded with a mixing time of 50 ms at a temperature of 265 K (t_{1max} = 24 ms, t_{2max} = 152 ms, the spectrum was folded once in ω_1, with spectral widths in ω_1 = 5,000 Hz and in ω_2 = 6,800 Hz). The size of the data matrix was 240 × 2,048 points, with 256 scans per t_1 increment.

3. Results and discussion

For the protein used in this study, 434-repressor(1–63), complete sequence-specific ^{1}H NMR assignments have previously been obtained both for a urea-unfolded form [16] as well as the native form (unpublished), and the three-dimensional structures have been characterized for both states ([10,25] as unpublished results). Although some local non-random structure is maintained in 7 M aqueous urea [10], the polypeptide chain has an extended, non-globular conformation under the conditions of the present experiments. As mentioned in section 1, the pH was adjusted for minimal exchange rate between urea and water protons. In earlier studies, pH_{min} had been reported to be near 7.3 at 25°C [26,27], and more recently it was found that the NMR line of urea in aqueous solution was narrowest at about pH 7.5 [9,28]. We therefore selected a pH value of 7.6, measured at room temperature, for the present experiments. In order to further lengthen the residence time of the solvent molecules on the protein surface, we measured NMR spectra at low temperatures, and to be able to observe surface solvation in spite of the intrinsic low sensitivity [12] we decided to work with 20 mM protein concentration. As an illustration of the data thus obtained, Fig. 1 shows a 1D ^{1}H NMR spectrum as well as cross sections along ω_2 through homonuclear ^{1}H NOESY and ^{1}H ROESY spectra taken at the ω_1 chemical shifts of urea, and H_2O, respectively.

The urea cross section from 0–4 ppm in the NOESY spectrum (Fig. 1C) shows positive NOE cross peaks to the aliphatic side chains of the protein, which corresponds to negative σ^{NOE} values. This part of the cross section contains all the peaks seen in the 1D ^{1}H NMR spectrum (Fig. 1I). The different relative intensities of the individual corresponding peaks in the spectra of Figs. 1C and 1I, in particular the reduced intensities for the methyl resonances near 0.9 ppm and the εCH_2 resonance of Lys near 3.1 ppm [29] in Fig. 1C, are largely due to the sine-shaped excitation profile along ω_2 caused by the water suppression method used in the NOESY experiment [19]. Only weak cross

peaks between urea and amide protons in the region 8–9 ppm are observed in Fig. 1C. The negative sign of the corresponding cross peaks in the ROESY spectrum (Fig. 1D) indicates that the magnetization transfer between urea and all polypeptide protons is dominated by the NOE and not by chemical exchange [12,20]. These observations contrast in two features with the corresponding hydration data (Fig. 1A,B). Firstly, the strong water–polypeptide proton cross peaks in the chemical shift region from 6–9 ppm, which involve backbone amide protons and labile side chain protons of Asn, Gln, Arg and Lys [29], are due predominantly to chemical exchange between water and these protons (the differences between the NOESY and ROESY cross sections from 7.5 to 9 ppm indicate that at this temperature magnetization transfer by dipolar interaction may also contribute to some cross peak intensities). Secondly, comparison of the water and urea cross-sections in the NOESY spectrum shows pronounced differences in the aliphatic region (Fig. 1A,C). In the urea cross section around 0.9 ppm, positive cross peaks can be seen, while there are no corresponding peaks in the water cross section. In the ROESY spectrum, however, the corresponding peaks are present in both cross sections (Fig. 1B,D). This indicates that the methyl groups of Val, Leu and Ile are solvated by water as well as by urea, and that the residence times of urea molecules associated with these methyls are longer than for water molecules [12]. Otherwise the NOESY and ROESY water cross sections in the aliphatic region are dominated by some very strong peaks (note the different scales for the traces A and B, and C and D, respectively) near 1.2, 1.6 and 3.1 ppm, which are probably due to NOEs with labile amino acid side chain protons [30] (see below). In the NOESY and ROESY spectra measured at 277 K (Fig. 1E–H), the urea as well as the water cross sections show reduced intensity of the peaks attributed to NOEs when compared to the cross sections measured at 265 K. In the water cross section of the NOESY spectrum there are small peaks with negative sign at around 0.9 ppm, which shows that at this temperature the contact time between water molecules and the methyl protons of Val, Leu and Ile is shorter than 300 ps [12,31]. As expected, the intensity of the exchange cross peaks in the water cross sections has increased relative to Fig. 1A,B, due to faster chemical exchange at the higher temperature. The close similarity of the low field part of the NOESY and ROESY water cross sections (Fig. 1E,F) shows that at 277 K, magnetization transfer by chemical exchange is much more effective than transfer by dipolar interactions. At even higher temperatures, the intensity of the NOEs in NOESY and ROESY further decreases, while the intensity of the exchange cross peaks continue to increase. At 293 K the cross peaks in the high field part of the urea cross sections were hardly observable either in NOESY or in ROESY. In the water cross sections, positive cross peaks in the aliphatic region were detected even at this temperature, but cross peaks with negative sign at 293 K were observed only at 0.9 ppm (see Fig. 1E).

The experiments of Fig. 1 were complemented with additional measurements to further check on possible short-circuiting of urea–protein coherence transfer via the solvent water. Using ^{15}N-labelled urea, a NOESY-relayed [^{15}N,^{1}H]COSY experiment [22] was recorded, where the magnetization of all protons that are not coupled to ^{15}N is destroyed with spin lock pulses during the INEPT steps. With this ^{15}N-filter before the mixing time, only NOESY cross peaks between urea and the protein are expected, while all intramolecular cross peaks of the
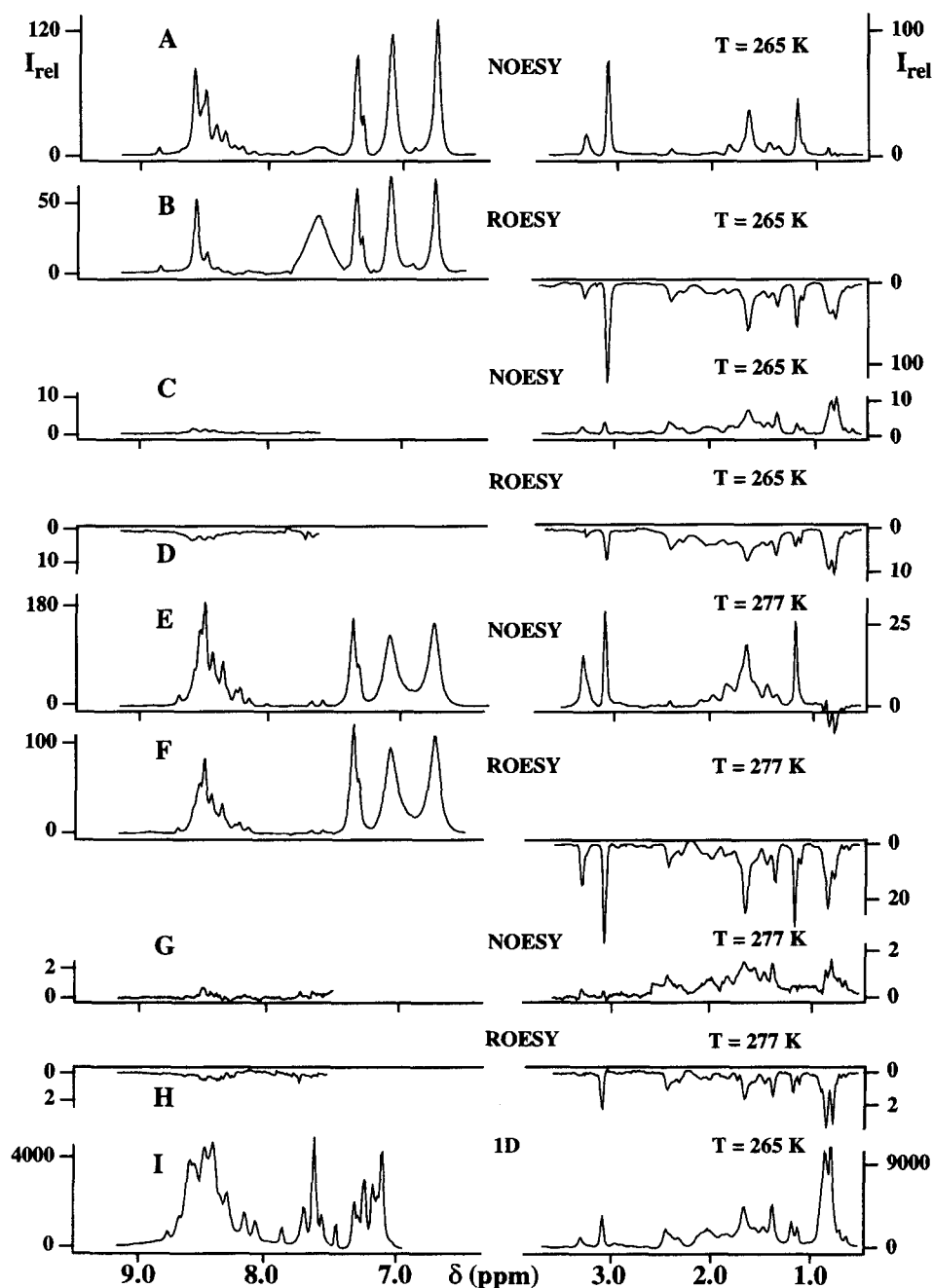
Fig. 1. ^{1}H NMR spectra recorded in a solution of unfolded 434 repressor(1-63) (protein concentration 20 mM, solvent 90% H_2O/10% D_2O, urea concentration = 7 M, pH = 7.6, ^{1}H frequency = 600 MHz). (A) Cross section along ω_2 taken at the ω_1 chemical shift of water through a 2D soft-NOESY spectrum measured at 265 K. (B) Corresponding cross section from a ROESY spectrum recorded with identical conditions. (C) and (D) Cross sections along ω_2 through the same spectra as in (A) and (B), respectively, taken at the ω_1 frequency of urea. (E–H) Same as (A–D) from spectra measured at 277 K. (I) 1D ^{1}H NMR spectrum measured at 265 K. The experimental scheme used to record the traces A, B, G and H was a soft-NOESY [18] with a mixing time τ_m = 50 ms and the water suppression scheme of [19]. The experimental scheme used to record B, D, F and H was ROESY with τ_m = 25 ms, and again using spin locks for water suppression [19]. Since this water suppression leads to a sign inversion of the signals upfield and downfield of the water line, the low-field regions of the NOESY and ROESY cross sections were inverted for improved readability. Different scaling of the individual cross sections is indicated on the left and the right of the figure.

protein should have very small intensity. Comparison of the cross section through the NOESY-relayed [^{15}N,^{1}H]COSY spectrum taken at the ω_1(^{15}N) chemical shift of urea (Fig. 2A) with Fig. 1C shows that the intensity distribution in the high field parts of the two cross sections is very similar, which supports

the interpretation that the observed cross peaks are caused by direct dipolar magnetization transfer between urea molecules and the protein rather than by other magnetization transfer pathways. In the low field part of the cross section in Fig. 2A the small cross peaks seen in Fig. 1C could not be detected,
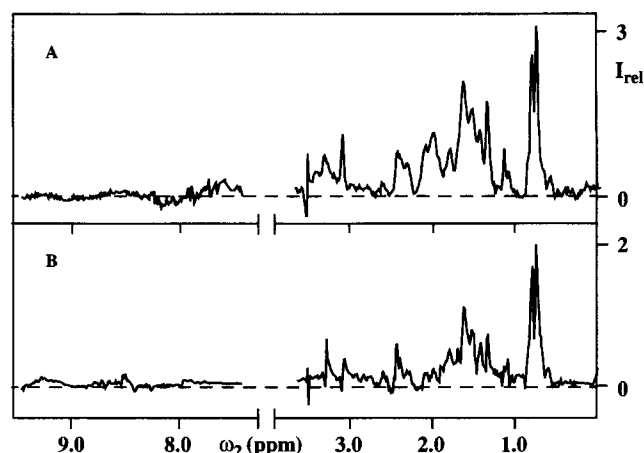
Fig. 2. (A) Cross section along $\omega_2(^1H)$ from a NOESY-relayed [$^{15}N,^1H$]COSY spectrum of unfolded 434 repressor(1–63) taken at the $\omega_1(^{15}N)$ chemical shift of urea (protein concentration 20 mM, solvent 90% H_2O/10% D_2O, [^{15}N]urea concentration = 7 M, pH = 7.6, temperature = 265 K, ^1H frequency = 600 MHz). The experimental scheme was NOESY-relayed [$^{15}N,^1H$]COSY [22] with τ_m = 50 ms. During the two INEPT steps the magnetization of protons which are not coupled to ^{15}N was destroyed with spin lock purge pulses of 2 ms duration. (B) Cross section along ω_2 from a [$^1H,^1H$]NOESY spectrum of unfolded 434 repressor(1–63) with water suppression by presaturation during the relaxation delay and during the mixing time τ_m = 50 ms (protein concentration 20 mM, solvent 90% H_2O/10% D_2O, urea concentration = 7 M, pH = 7.6, temperature = 265 K, ^1H frequency = 600 MHz). The peak intensities (I_{rel}) in this spectrum cannot be directly compared with Fig. 1C or Fig. 2A, since this experiment was recorded with a different signal-to-noise ratio.

which can be explained by the reduced signal-to-noise ratio achieved in the ^{15}N-filtered NOESY spectrum. (Note that the urea-associated ^{15}N-filter can also be used to extract a plane from 3D ^{15}N-resolved [$^1H,^1H$]NOESY, so that all NOEs with [^{15}N]urea can be observed in a two-dimensional experiment.)

In the experiment of Fig. 2B we measured a 2D [$^1H,^1H$]NOESY experiment with water suppression by presaturation during the relaxation delay and the mixing time [24]. Comparison of the cross section along ω_2 taken at the ω_1 chemical shift of urea with Figs. 1C and 2A shows that all three cross sections contain peaks in similar positions, although the relative intensities of the cross peaks in Fig. 2B differ from those in the other two cross sections. This is a direct consequence of the different water suppression methods used in the individual experiments. The two orthogonal spin lock pulses used for the spectra of Figs. 1C and 2A lead to a sine-shaped excitation profile along ω_2 [19], whereas the presaturation used for the spectrum of Fig. 2B leads to uniform excitation along ω_2 [24]. The close coincidence of the peak positions in the urea cross section of Fig. 2B with Figs. 1C and 2A further supports that the observed cross peaks are direct NOEs between urea and the protein and cannot be due to exchange-relay of NOEs via the solvent water.

4. Conclusion

With the data presented in this paper we have shown that experimental conditions are available for simultaneous observation of urea–polypeptide and water–polypeptide NOEs in situations where the protein is unfolded by the urea. This com-

plements observations made on polypeptide–urea NOEs under conditions where the protein retains its native globular conformation [9]. The data of Fig. 1 show clear-cut differences between solvation of the polypeptide by 7 M urea or by the nearly 10-fold more abundant solvent water. In particular, the solvation of the methyl groups of Val, Leu and Ile near 0.9 ppm by urea is characterized by a significantly longer residence time with respect to exchange from the solvation sites than the solvation by solvent water. However, a simplistic hypothesis that stabilization of the unfolded state of the protein is attributable to kinetically stable urea-binding could not be supported by these preliminary data. Rather, it is intriguing that the protein remains unfolded at higher temperatures where the life times of urea in the polypeptide solvation sites are in the subnanosecond time range. More detailed insights into the intermolecular interactions leading to protein unfolding in aqueous urea, including that polypeptide hydration might be significantly affected by the presence of the urea, should result from future experiments that will include assignments of the NOEs to individual sites of the polypeptide chain. This approach will enable unambiguous distinctions between peaks in the water cross sections that arise either from direct NOEs with the solvent or from NOEs with labile side-chain protons that are exchange-averaged with the water in the ^1H NMR spectra [11,12,30]. On this basis one may find an avenue for assessing the role of competitive polypeptide solvation by the two components in aqueous urea solutions [32].

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