Salt-stabilized globular protein structure in 7 M aqueous urea solution

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Abstract A 7 M aqueous urea solution of the 63-residue N-terminal domain of the 434-repressor at pH 7.5 and 18°C contains a mixture of about 10% native, folded protein and 90% unfolded protein. Interconversion between the two conformations is slow on the NMR chemical shift time scale, so that observation of separate resonances can be used to monitor the equilibrium between folded and unfolded protein when changing the solution conditions. In this paper we describe the influence of various salts or non-ionic compounds on this conformational equilibrium. Solution conditions are described which contain a homogenous preparation of the folded protein in the presence of 6 to 7 M urea, providing a basis for an NMR structure determination in concentrated urea and for studies of the solvation of the folded protein in mixed water/urea/salt environments.

Key words: Protein folding; Urea denaturation; Salt-stabilized protein structure; NMR

1. Introduction

In traditional protein chemistry the monitoring of solubility and conformational equilibria by the addition of salts and non-ionic compounds to aqueous protein solutions had an important role in basic and applied research alike. In this paper we combined the use of the protein denaturant urea with the addition of a variety of different salts and non-ionic additives in search of conditions where folded forms of a protein would be stable in the presence of otherwise denaturing urea concentrations.

Although urea is widely used as a protein denaturant and the hypothesis has been popularly advanced that its destabilizing effect is largely due to binding to the hydrophobic amino acid sidechains of the unfolded protein [1–8], experimental data on specific protein–urea interactions are scarce [9–12]. In one such study, nuclear Overhauser effects (NOE) between urea and the

basic pancreatic trypsin inhibitor were measured under conditions where this protein is fully folded in the presence of urea, and specific interactions of urea molecules with the protein were detected for 'pockets and grooves on the protein surface' [11]. Similar experiments with unfolded 434-repressor(1–63) in 7 M urea indicated preferential urea solvation of methyl-bearing aliphatic side chains [12]. To pursue these studies on a more systematic level it will be desirable to study urea–protein interactions with folded and unfolded conformations of the same protein. The present work identifies solution conditions where folded and unfolded 434-repressor(1–63) can be studied at the same temperature in the presence of high, 'denaturing' urea concentrations.

2. Materials and methods

2.1. Expression and purification of 434-repressor(1–63)

The expression system used and the isolation and purification of 434-repressor(1–63) were previously described [12,13]. For the production of the uniformly ^{15}N-labelled protein, a slightly modified procedure was used. Minimal medium supplemented with 50 μg/ml of ampicillin and containing 0.5 g/l of [^{15}N]ammonium sulphate as the sole nitrogen source was inoculated with the cell paste of an overnight culture of BL21/DE3 cells containing the plasmid pt7-7/434A [12] grown on unlabelled minimal medium. Growth was continued to an A_{600} of about one, and the cells were harvested by centrifugation at 4500 × g for 10 minutes at room temperature. The cell paste was resuspended in fresh, pre-warmed minimal medium prepared in the same way as the medium used for cell growth. Thirty minutes after resuspension the cells were induced with 0.4 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and harvested after 6 h by centrifugation. The supernatant was saved and used a second time in an identical manner. The yield of the first round of growth was 50 mg/l, of the second one 15 mg/l.

2.2. NMR measurements

All NMR experiments were carried out with uniformly ^{15}N-labelled 434-repressor(1–63) at a concentration of 4 mM in a solvent of 90% H_2O/10% D_2O containing 6 M or 7 M urea (Fluka). The pH was adjusted to 7.6. ^{15}N,[^{1}H]-COSY spectra [14,15] were recorded on a Bruker AMX 500 spectrometer (spectral width in ω_1 = 2000 Hz and in ω_2 = 6900 Hz, t_{1max} = 72 ms, t_{2max} = 152 ms, time domain data size 200 × 2048 points, 4 scans per t_1 increment). The temperature was 291 K. The effect of salts and other chemicals on the folding equilibrium of the 434-repressor(1–63) in 7 M urea was tested by stepwise addition of concentrated solutions of these compounds in 7 M urea to the protein solution. Such titrations were carried out with sodium trifluoracetate (Fluka), sodium sulphate (Fluka), sodium chloride (Fluka) and glucose (Fluka).

2.3. CD measurements

CD spectra were measured on a Jasco J710 CD spectrometer with a cuvette of 0.2 mm path length. Spectra were taken in the wavelength interval between 200 and 250 nm with a step size of 0.2 nm and a scan speed of 50 nm/min. The protein concentration was 60 μM and the solutions contained variable concentrations of urea. The pH was adjusted to 7.6, the temperature was 291 K. To study the effect of salts or glucose on the folding equilibrium, concentrated solutions of these chemicals containing the same concentrations of urea as the protein solutions were added stepwise to the protein.

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Abbreviations: 434-repressor(1–63), N-terminal 63-residue polypeptide fragment from the 434-repressor; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; 1D, one-dimensional; 2D, two-dimensional; ^{15}N,[^{1}H]-COSY, 2D heteronuclear [15]N-^{1}H correlation spectroscopy.
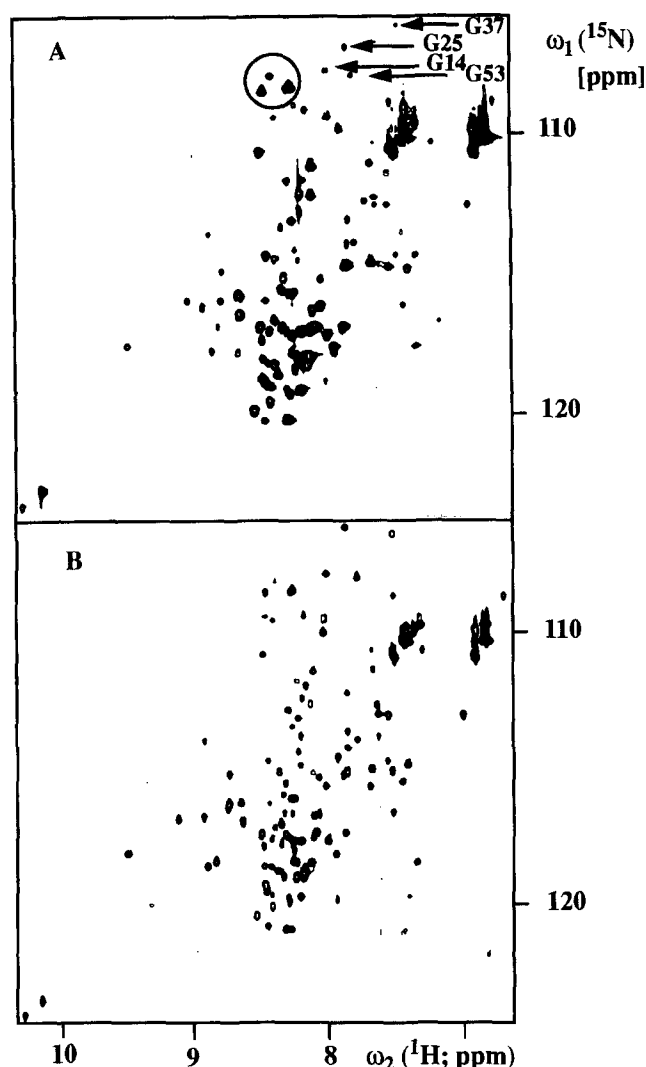
Fig. 1. [^{15}N,^{1}H]-COSY spectra of 434-repressor (1–63) (protein concentration 4 mM, solvent 90% H$_2$O/10% D$_2$O, urea concentration 7 M, pH 7.6, temperature 291 K, ^{1}H frequency 500 MHz). (A) Without addition of salt. Arrows indicate the positions of four glycyl resonances in the folded protein, the circle encloses the corresponding peaks of the unfolded conformation [17]. (B) After addition of 0.2 M sodium trifluoracetate.

3. Results and discussion

Previous experiments with 434-repressor(1–63) [13,16] and a closely related polypeptide, 434-repressor(1–69) [17], showed that conformational transitions of these proteins could readily be monitored by NMR spectroscopy. Aqueous solutions of these proteins containing 7 M urea at pH 4.8 were thus found to contain a uniform population of the unfolded protein [13,17], which includes a local non-random structure forming a hydrophobic cluster involving the residues 53–60 [16]. At lower urea concentrations the NMR spectrum contained the resonances of the folded protein in addition to those of this unfolded form [17]. In connection with recent NMR investigations of interactions of urea with the 434-repressor(1–63) [12] we explored a wider range of solution conditions and thus discovered that at pH 7.6 and temperatures around 291 K, a solution in 7 M urea

also contains a mixture of unfolded and folded protein. The percentage of the folded form is approximately 5 to 10% of the total protein concentration, and in [^{15}N,^{1}H]-COSY spectra (Fig. 1) this minor component can most readily be detected from the presence of two sets of glycyl resonances [17]. In Fig. 1A the peaks of the folded protein are indicated by arrows, the corresponding peaks of the unfolded protein lie within the circle. To increase the amount of folded conformation, sodium trifluoracetate, sodium sulphate, sodium chloride or glucose, which are all known to stabilize folded proteins [2,18,19], were added to the protein solution in 7 M urea. The NMR spectrum (Fig. 1B) of a solution containing 0.2 M sodium trifluoracetate shows the two sets of peaks in a ratio of approximately 50% of the unfolded form and 50% of the folded form. Titration with sodium sulphate yielded similar results, with 50% folded conformation at 0.35 M salt concentration. With sodium chloride, 50% folded protein was reached at 1.1 M salt concentration, and with glucose at a concentration of 0.6 M.

Using CD spectroscopy, these studies of folding equilibria of 434-repressor(1–63) at high urea concentrations were extended to search a wider range of solution conditions. First, salt concentrations of 2 M and higher were used to search for solution conditions where only the folded protein would be present even in 7 M urea. The folded state was indeed found to be 100% populated at 2 M concentration of either sodium trifluoracetate, sodium sulphate or glucose, or at 2.5 M sodium chloride. Next, we determined the minimal salt concentrations needed to obtain a homogenous solution of folded protein in 6 M urea. Titrations were carried out with sodium trifluoracetate and sodium chloride (Fig. 2). With sodium trifluoracetate the desired homogenous solution of folded protein is present at concentrations above 0.5 M, with sodium chloride at concentrations above 1.8 M. The results of the CD-titrations were also confirmed by [^{15}N,^{1}H]-COSY. Fig. 3A shows the spectrum of a sample containing 0.5 M sodium trifluoracetate, and Fig. 3B shows a spectrum measured in the presence of 1.8 M sodium chloride. Both spectra show only one set of peaks, and in each case the chemical shift dispersion is typical of a folded protein [20].

The NMR spectra of Fig. 3 provide some initial indications on the structure of folded 434-repressor(1–63) at high urea and
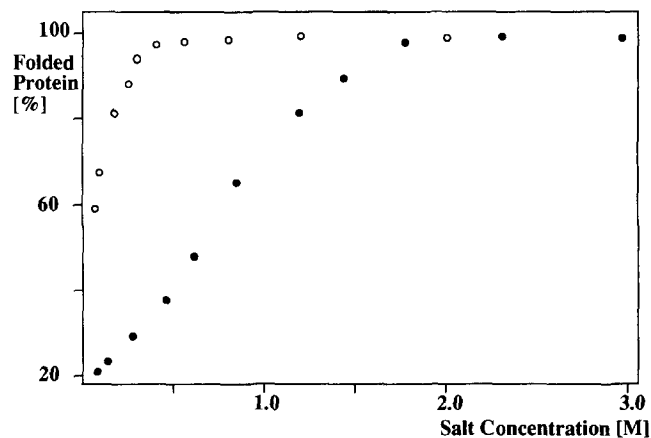

Fig. 2. Salt-dependence of the equilibrium between folded and unfolded 434-repressor(1–63) in 6 M urea. Open circles: sodium trifluoracetate; filled circles: sodium chloride. The percentage of the folded protein was calculated from the CD-signal at 222 nm (protein concentration 60 μM, temperature 291 K, pH 7.6).
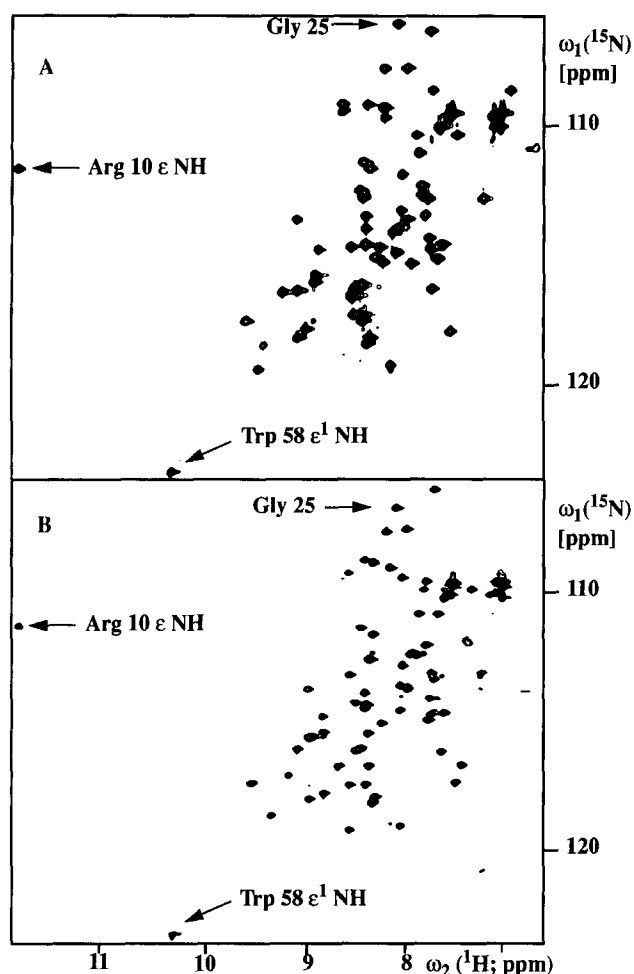
nally, the line broadening of the peaks in Fig. 3A when compared to Fig. 3B is indicative of aggregation. Sodium trifluoroacetate is well known to be a potent salting-out reagent, which not only stabilizes globular proteins but also tends to precipitate them from aqueous solution [2].

4. Conclusions

The data presented in this paper show that folded conformations of 434-repressor(1–63) can be stabilized by addition of salts to urea solutions in which the protein would otherwise be unfolded. This result opens new avenues for studies of the molecular mechanisms of protein unfolding by urea, including NMR structure determination of folded and unfolded forms of the same protein at identical urea concentration, and comparative investigations of solvation by water and urea for the folded and unfolded protein. There is further a good chance that such solvation studies [12,23,24] can be extended to trifluoroacetate, making use of $^{19}F-^{1}H$ NOEs [25].

Fig. 3. [^{15}N,^{1}H]-COSY spectra of 434-repressor(1–63) in 6 M urea after addition of salts. (protein concentration 4 mM, solvent 90% H$_2$O/10% D$_2$O, pH 7.6, temperature 291 K, ^{1}H-frequency 500 MHz). (A) 0.5 M sodium trifluoroacetate; (B) 1.8 M sodium chloride. The resonances of Arg-10 εNH, Gly-25 and Trp-58 ε^{1}NH are identified by arrows.

salt concentrations. Overall, the chemical shifts of the fingerprint peaks in Fig. 3B are closely similar but not strictly identical to those of the folded 434-repressor(1–69) in 4.2 M urea at pH 4.6 [17] and of the native protein in H2O solution [21]. Chemical shift differences can also readily be detected between the spectra of Fig. 3A and B. An illustration is Gly-25, which has similar chemical shift positions in the spectrum in urea and sodium chloride as in the native protein, but in the presence of sodium trifluoroacetate is shifted by 1 ppm in the ^{15}N-dimension relative to the native protein. The question whether these chemical shift differences are a direct consequence of the different solution conditions or reflect structural rearrangements will only be answered by complete structure determinations of 434-repressor(1–63) in the different solvent environments (work in progress). However, there are indications that the tertiary structure of the native protein is probably largely conserved. Thus, two ^{15}N-^{1}H cross peaks with outstanding chemical shifts (Arg-10 εNH and Trp-58 ε^{1}NH in Fig. 3) are in nearly identical positions as in native 434-repressor(1–63). In particular, the chemical shift of the resonance of the ε-proton of Arg-10 at 11.8 ppm, which reflects the formation of a salt bridge with Glu-35 [21,22], is a strong indicator of a native-like tertiary fold. Fi-

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