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Small angle X-ray scattering study of the yeast prion Ure2p☆

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Abstract

The GdmCl-induced equilibrium unfolding and dissociation of the dimeric yeast prion protein Ure2, and its prion domain deletion mutants Δ15-42Ure2 and 90Ure2, was studied by small angle X-ray scattering (SAXS) using synchrotron radiation and by chemical cross-linking with dithiobis(succinimidyl propionate) (DTSP). The native state is globular and predominantly dimeric prior to the onset of unfolding. R_g values of 32 and 45 Å were obtained for the native and 5 M GdmCl denatured states of Δ15-42Ure2, respectively; the corresponding values for 90Ure2 were 2–3 Å lower. SAXS suggests residual structure in the 4 M GdmCl denatured state and chemical cross-linking detects persistence of dimeric structure under these conditions. Hexamers consisting of globular subunits could be detected by SAXS at high protein concentration under partially denaturing conditions. The increased tendency of partially folded states to form small oligomers points to a mechanism for prion formation.

Keywords: Ure2; Small angle X-ray scattering; Dimer; Dissociation; Association; Protein folding; Aggregation; Amyloid; Cross-linking; Residual structure

Ure2 is a dimeric protein [1,2] involved in regulation of nitrogen metabolism in the yeast *Saccharomyces cerevisiae* [3,4]. Ure2 has been termed a yeast prion [5] because of its ability to propagate a heritable phenotype change at the protein level by undergoing a structural change into an aggregated form [5,6]. Structural and folding studies of Ure2 are therefore of utmost importance in order to understand the molecular mechanism of this prion behaviour.

The Ure2 protein consists of two structural regions with distinct functions. The unstructured N-terminal region of Ure2 [2,7] is required for prion function in vivo [6] and amyloid formation in vitro [1]. The globular C-terminal domain [2,7], for which the crystal structure has been solved [8,9], carries the regulatory function [6]. Removal of all or part of the N-terminal prion domain (PrD) has been shown to have no effect on the oligomeric state, thermodynamic stability, kinetics of folding or folding pathway of Ure2 over a wide range of experimental conditions [2,10–13]. Unfolding is reversible in Tris–HCl buffer, pH 8.4, at 25 °C and 1 μM protein concentration [2]. Under these conditions, the equilibrium denaturation transition appears two-state and dimer dissociation (detected by protein concentration dependence) is practically imperceptible [2,13]. Ure2 displays a strong tendency to aggregate or misfold, which is particularly marked at lower pH or in phosphate buffer, but greatly reduced for prion domain mutants with even small deletions, such as Δ15-42Ure2 [2,12,13].

Solution small angle X-ray scattering (SAXS) is a powerful tool for measurement of protein shape and compactness [14–17]. In particular, SAXS is able to provide information about the conformation of partially structured and denatured states. SAXS has been used not only for monitoring the conformational changes of

☆ *Abbreviations:* 90Ure2, Ure2 in which residues 1–89 have been deleted; Δ15-42Ure2, Ure2 in which residues 15–42 have been deleted; CD, circular dichroism; DTSP, dithiobis(succinimidyl propionate); GdmCl, guanidine hydrochloride; PrD, prion domain; SAXS, small angle X-ray scattering.

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globular proteins during folding, but also provides a useful means to study protein aggregation, which often accompanies the folding process [18].

"Snap-shot" fixation of reassociating/dissociating oligomers by chemical cross-linking and subsequent analysis by SDS–polyacrylamide electrophoresis is a powerful tool for the analysis of protein assembly/disassembly processes [19]. Dithiobis(succinimidyl propionate) (DTSP) is a bifunctional reagent that reacts with free amino groups on the protein molecule and forms intermolecular cross-links via a disulphide bond [20]. The particular advantage of DTSP for the purpose of this study is that the cross-linking reaction proceeds rapidly and to high yield even in the presence of high concentrations of GdmCl, and cross-linking can be reversed by reduction of the disulphide bonds [21,22].

Here we use SAXS, and chemical cross-linking with DTSP, to investigate the unfolding and dissociation of Ure2 under conditions where the mechanisms of folding and amyloid formation have been studied in detail [12,13]. SAXS experiments, which require mg/ml protein concentrations, were performed using Δ15-42Ure2 and 90Ure2, which lack part or all of the N-terminal PrD and are significantly more soluble than the wild-type protein [2]. Cross-linking experiments, which can be performed using low micromolar protein concentrations, were performed using full-length Ure2 as well as Δ15-42Ure2.

Materials and methods

Materials. Ultrapure GdmCl was obtained from ICN Biochemicals. Tris, DTSP, and the high molecular weight standard kit (SDS-6H) were from Sigma. All other reagents were local products of analytical grade.

Protein production and purification. Ure2 and N-terminal variants, 90Ure2 and Δ15-42Ure2, were produced in *Escherichia coli* and purified using nickel affinity chromatography as described previously [2,13]. 90Ure2 lacks the glutamine/asparagine-rich region from residues 1–89 and therefore lacks the entire PrD as defined either by biological activity [23] or inspection of the protein sequence [2]. Δ15-42Ure2 contains the entire glutamine/asparagine-rich region, but the small region of 'normal' amino acid sequence from residues 15–42 has been excised [2].

Synchrotron small angle X-ray scattering measurements. X-ray solution scattering measurements were performed on the beamline 15A small angle installation (BL-15A) of the Photon Factory, High Energy Accelerator Research Organization (KEK), Tsukuba, Japan. A stable beam of photons with a wavelength of 1.503 Å was provided by a bent-crystal horizontally focusing monochromator and a vertically focusing mirror [24]. X-ray patterns were recorded using a CCD detector [25,26]. The structural information in the scattering experiment is contained in the scattering intensity, $I(h)$, at momentum transfer, h, which is defined as $h = (4\pi\sin\theta)/\lambda$, where 2θ is the scattering angle and λ is the wavelength of the incident X-rays. Camera length, i.e., sample-to-detector distance, was 2359 mm corresponding to the scattering vector range: $0.00027202\,\text{Å}^{-1} < h < 0.1389\,\text{Å}^{-1}$. The background data for the buffer solution at different concentrations of GdmCl were collected before or after data collection for the protein solutions. The correction of the SAXS data for the difference in the electron density (i.e., contrast) between the protein and the solvent molecules as well as for X-ray absorption by the solution was made according to the standard data provided by G.V. Semisotnov (unpublished). In the small h region, the R_g value was estimated using the Guinier approximation, $I(h) = I(0)\exp(-R_g^2h^2/3)$, where h is the scattering vector and $I(0)$ is the scattering intensity at zero angle [14]. For the native globular protein, the R_g was calculated by fitting the curve $\ln I(h)$ against h to the Guinier equation in the region between 0.017 and 0.070 Å^{-1} in h. For denatured states, such as a random coil, the Guinier approximation cannot be used because the previous approximation is valid only for $R_g * h \leqslant 1$. In this case, the scattering profile can be described to a first approximation in the small h region ($R_g * h \leqslant 3$) by the Debye function, $I(h)/I(0) = (2/x^2)(x - 1 + e^{-x})$, where $x = h^2 * R_g^2$ [27]. Where there is aggregation present, the same methods can be used to analyse the scattering profiles. The fitting regions of Guinier plots for the GdmCl-denatured samples were smaller than that for native protein. All experiments were performed in duplicate, except where the protein concentration was 1 mg/ml or less, when each measurement was performed four times. Measurements were carried out at 25 °C in a thermostatted cuvette. The samples were allowed to pre-equilibrate in different concentrations of GdmCl in 50 mM Tris, pH 8.4, containing 0.2 M NaCl at 25 °C overnight before measurements were taken. The final concentrations of Δ15-42Ure2 and 90Ure2 in the unfolding experiments were 3 mg/ml (77 μM) and 2 mg/ml (62 μM), respectively. The protein concentration dependence of the values was measured between 0.5 and 10 mg/ml for the native state and between 0.5 and 6.0 mg/ml for the 5 M GdmCl denatured state.

Determination of oligomeric state by chemical cross-linking. Chemical cross-linking in the presence of a range of GdmCl concentrations was performed using dithiobis(succinimidyl propionate) (DTSP) as the cross-linking agent [20–22]. Samples containing a series of GdmCl concentrations between 0 and 6 M were prepared in 50 mM Tris–HCl, pH 8.4, or 50 mM Na_2HPO_4–KH_2PO_4, pH 8.4, each containing 0.2 M NaCl, and incubated overnight at 25 °C. The final protein concentration was 4 μM (0.16 mg/ml) for wild-type Ure2 and 4 or 20 μM (0.78 mg/ml) for Δ15-42Ure2. The equilibrated samples were mixed with a small volume of 20 mg/ml DTSP freshly dissolved in DMSO to give a final concentration of DTSP of 1 mg/ml. After incubation at 25 °C for 2 h, the reaction was quenched by adding 60 μl of 1 M ammonium acetate per ml of sample with rapid mixing. The quenching reaction was allowed to proceed for 30 min. The resulting solution was then treated to remove GdmCl before analysis by SDS–PAGE, either by thorough dialysis at 4 °C against the corresponding buffer or by acetone precipitation using the procedure given below. The recovery yield and molecular weight distribution of protein were the same whether dialysis or acetone precipitation was used. For acetone precipitation, the original sample was diluted with the corresponding buffer where necessary, so that the final GdmCl concentration did not exceed 2 M. A 5-fold sample volume of cold acetone was then added and the samples were placed at −70 °C for 1 h. After centrifuging at 16,000*g* for 10 min, the precipitate was washed twice with cold acetone and 30 μl of 8 M urea was added to dissolve the pellet, before proceeding to SDS–PAGE analysis.

The oligomeric state of the cross-linked protein was analysed by 10–12% SDS–PAGE after incubation of the samples at 37 °C for 30 min in loading buffer lacking β-mercaptoethanol. Alternatively, to reduce the cross-linking disulphide bonds, the samples were boiled at 100 °C for 5 min in loading buffer containing β-mercaptoethanol. Densitometry of SDS–PAGE bands was analysed using TotalLab 1.10 (Nonlinear Dynamics, UK).

Results

The R_g values of native and unfolded forms of Δ15-42 Ure2

The solution X-ray scattering of native Δ15-42Ure2 at different protein concentrations was measured (Fig. 1).

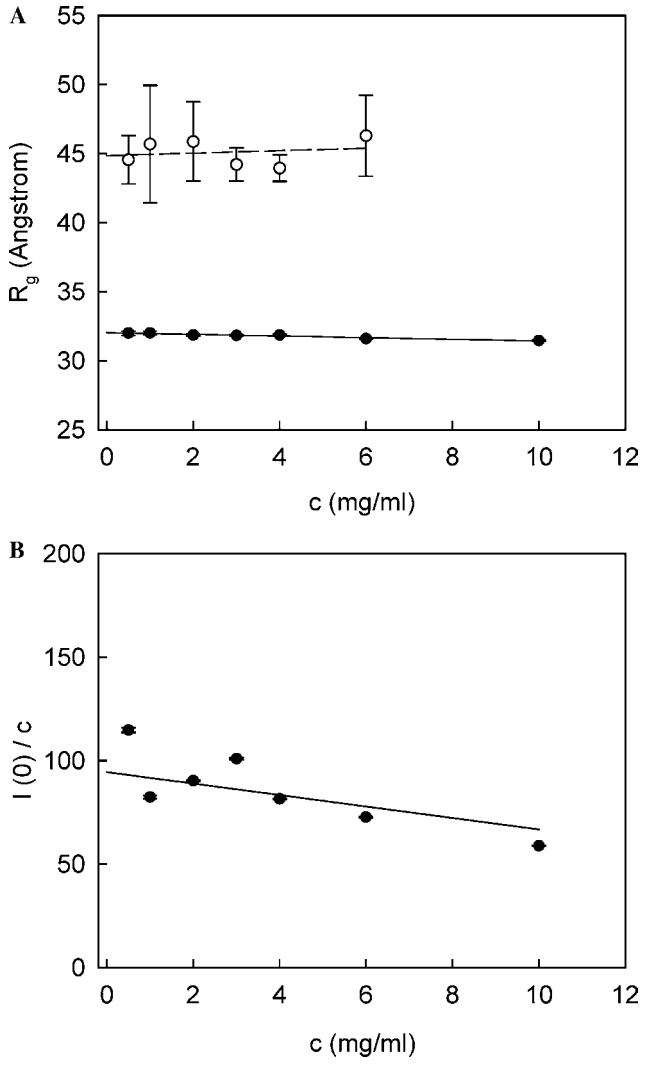

Fig. 1. Protein concentration dependence of the apparent R_g values (A) and $I(0)$ values (B) for native (●) and 5 M GdmCl denatured (○) Δ15-42Ure2. Error bars are derived from the fits of the Guinier plots.

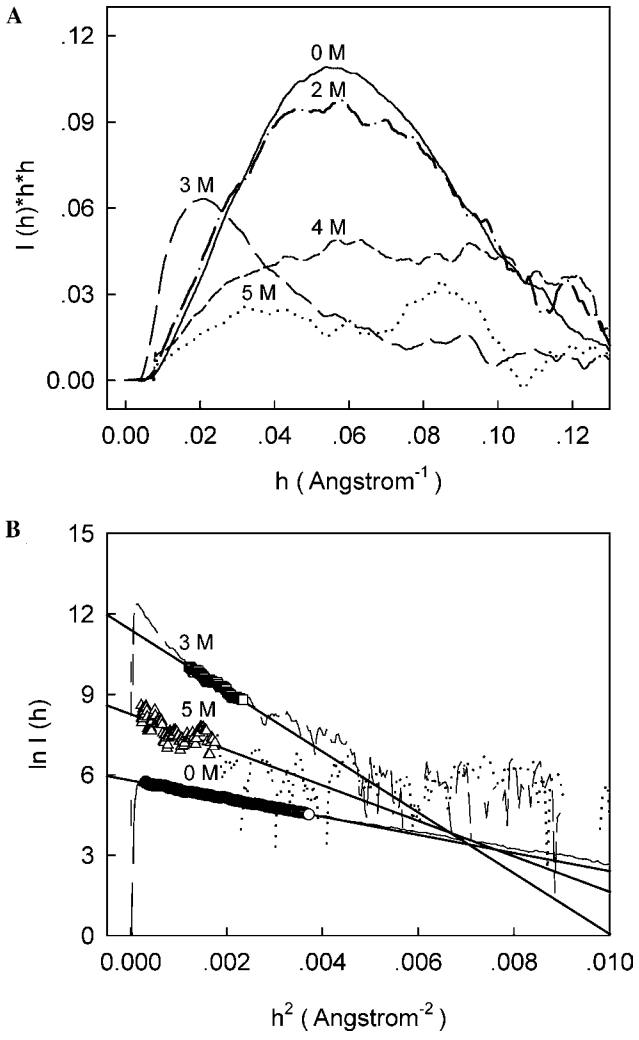

Fig. 2. Representative Kratky plots (A) and Guinier plots (B) for Δ15-42Ure2 or 90Ure2 at various concentrations of GdmCl, as indicated. The data shown are for Δ15-42Ure2. (A) Each curve was normalized using the $I(0)$ value obtained from the Guinier analysis. A mild smoothing function was applied for clarity of the plot. (B) The linear regions used in fitting to obtain R_g (see Materials and methods) are shown as symbols and the fit to a straight line is shown. For clarity, each curve is shifted along the ln $I(h)$ axis.

The conditions used for all SAXS experiments were 50 mM Tris, pH 8.4, 0.2 M NaCl at 25 °C. The apparent R_g values at different protein concentrations were calculated from a series of Guinier plots (see Materials and methods and Fig. 2). The R_g was then obtained by extrapolating linearly the apparent R_g values to zero protein concentration. No significant change in the R_g value with protein concentration was detected for native or 5 M GdmCl denatured Δ15-42Ure2 (Fig. 1A). The R_g values extrapolated to zero protein concentration were 32.03 ± 0.03 Å for the native state and 44.8 ± 0.8 Å for the 5 M GdmCl denatured state.

The function $I(0)$ is highly sensitive to changes in the aggregation state of the protein [18]. An increase in the term $I(0)/c$ (where c is the protein concentration) with increasing protein concentration would indicate an increase in the molecular weight of the scattering agent [28]. This is not observed for the native state of Δ15-42Ure2 (Fig. 1B), indicating the absence of aggregation under these conditions.

Changes in the globularity of Ure2 during denaturation by GdmCl

The equilibrium unfolding transitions for Δ15-42Ure2 and 90Ure2 were measured by SAXS using GdmCl as a denaturant. Fig. 2A shows typical Kratky plots at various concentrations of GdmCl. Representation of the data as a Kratky plot provides information about the globularity of folded or partially folded states [14,29] and can also allow the degree of residual structure in different denatured states to be compared [18,30,31]. The Kratky plots of native Δ15-42Ure2 and 90Ure2 show a clear

peak at an h value of 0.058 and little change is observed at GdmCl concentrations up to 2.0 M. This indicates that the native protein is globular and undergoes little change in shape prior to the onset of the main unfolding transition observed by fluorescence or circular dichroism (CD) under the same buffer conditions [2]. Between 2.6 and 3.6 M GdmCl, the peak maximum shifts to a significantly lower value of h. This indicates that intermolecular association occurs within this GdmCl range, but that the subunits retain a globular shape within the aggregates [32]. Representative Guinier plots are shown in Fig. 2B. The upward curvature towards $h = 0$ in 3 M GdmCl is also indicative of the presence of aggregation under these conditions [28]. In 4 M GdmCl the shape of the Kratky plot is a raised plateau, without a clear peak, indicating that globular structure has been lost (Fig. 2A). Between 4 and 5 M GdmCl the Kratky plot becomes progressively lower and flatter in shape, suggesting a progressive loss in residual structure until the protein approaches a random coil in 5 M GdmCl.

Unfolding transition derived from the changes in the R_g and $I(0)$ values

From the SAXS spectra of Δ15-42Ure2 and 90Ure2 obtained at different concentrations of GdmCl, the apparent R_g values and $I(0)$ values were estimated (Fig. 3). (Plotting the change in integrated intensity of the SAXS signal with [GdmCl] produced similar curves to $I(0)$ and so is not shown.) Below 2.4 M GdmCl, the R_g and $I(0)$ values show little change, indicating the absence of any significant degree of association, dissociation or unfolding in this GdmCl concentration range. The marked increase and then decrease in both R_g and $I(0)$ between 2.6 and 3.6 M GdmCl indicates the presence of aggregation within the unfolding transition region, which is dispersed at higher GdmCl concentrations. The aggregated state appears to correspond to a hexamer.

The pre-transition baseline when measuring either R_g or $I(0)$ is linear and sloping (Fig. 3). The R_g value measured by SAXS includes the hydration layer [14,28]. The value of $I(0)$ is also affected by changes in the partial specific volume of the protein [33] and the size of the hydration shell [34]. The sloping baselines therefore indicate a steady increase in the size of the hydration shell and/or expansion of the protein molecules with increasing denaturant concentration. A similar result was obtained by hydrodynamic measurements using velocity sedimentation and size exclusion chromatography [2]. The denaturation curves corrected for the sloping baselines are also shown for comparison (Fig. 3, insets). Examination of the baseline-corrected $I(0)$ plot (Fig. 3B, inset) indicates that the molecular weight of the 6 M GdmCl denatured state approaches half that of the native dimer, as expected, suggesting that this is a reasonable treatment of the data (see also cross-linking

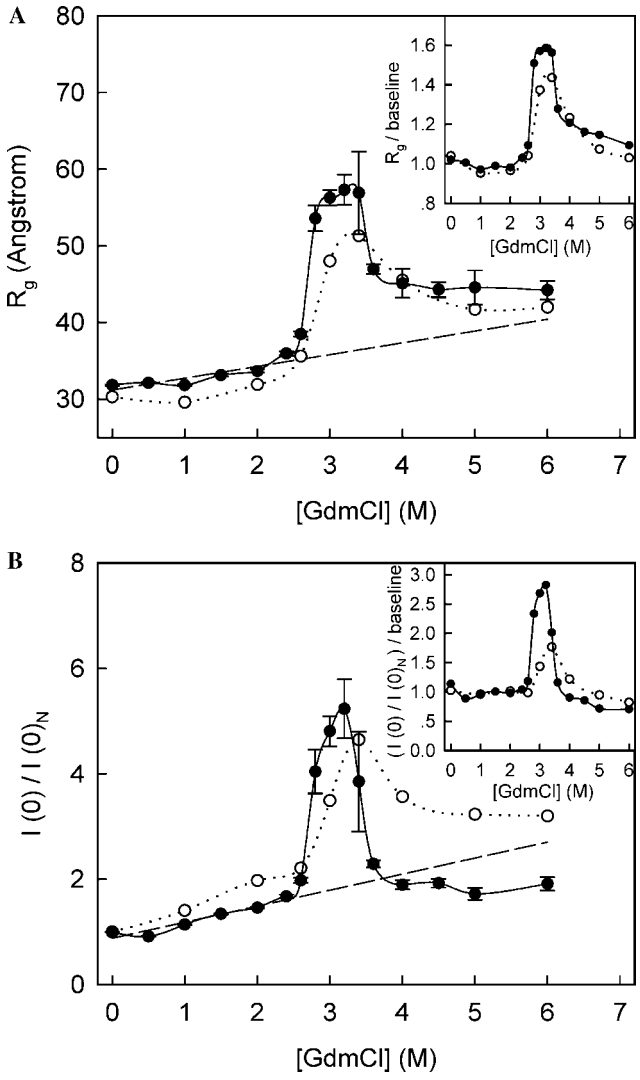

Fig. 3. GdmCl-induced equilibrium unfolding transitions for Δ15-42Ure2 (●) and 90Ure2 (○) derived from the R_g values (A) and $I(0)$ (B), estimated using the Guinier or Debye approximations (see Materials and methods). The $I(0)$ values were normalized using the value for the native state, $I(0)N$. The data were fitted to a smooth curve. The protein concentrations were 3 mg/ml for Δ15-42Ure2 and 2 mg/ml for 90Ure2. Insets: Plotting of the data after correction for the slope of the pre-transition baseline. (The slope of the Δ15-42Ure2 baselines is indicated in the main panels as a dashed line.)

data below). The slight decrease in the values between 3.6 and 5 M GdmCl suggests some structural change within this region, consistent with the changes in the Kratky plots (Fig. 2A).

Overall, the results obtained for the two proteins are extremely similar (Fig. 3). The lower height of the peak in R_g and $I(0)$ within the transition region for 90Ure2 is consistent with the lower protein concentration used (2 mg/ml for 90Ure2 vs. 3 mg/ml for Δ15-42Ure2) and is consistent with a tetramer or a mixture of hexamers and dimers in rapid exchange. It should be noted, however, that the data obtained within the region where there is aggregation are principally qualitative [28].

Dissociation from dimer to monomer detected by chemical cross-linking

We used chemical cross-linking as a complementary method to investigate changes in the oligomeric state of Ure2 during GdmCl-induced unfolding (Fig. 4). The agent used was DTSP, which has a linker length of

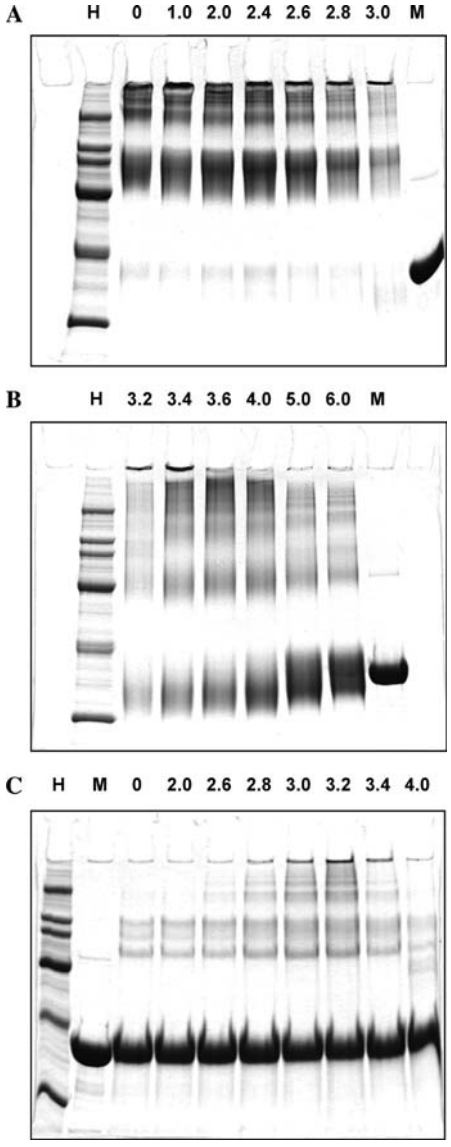

Fig. 4. SDS–PAGE of cross-linked Ure2 or Δ15-42Ure2 after denaturation in different concentrations of GdmCl in 50 mM Tris–HCl, pH 8.4, 0.2 M NaCl. The results shown are for 20 μM Δ15-42Ure2. Similar results were obtained in 50 mM Na_2HPO_4–KH_2PO_4, pH 8.4, 0.2 M NaCl (see Fig. 5). Results obtained for full-length Ure2 and Δ15-42Ure2 were identical. The high molecular weight standards (H) correspond to 205, 116, 97, 66, 45, and 29 kDa. Samples were first equilibrated in different GdmCl concentrations (as indicated) and then cross-linked with DTSP. Native protein without GdmCl or cross-linker (M) appears as a band corresponding to the monomeric molecular weight (39.0 kDa). (A,B) SDS–PAGE using loading buffer containing no β-mercaptoethanol. (C) SDS–PAGE of equivalent samples after reduction of the disulphide cross-links.

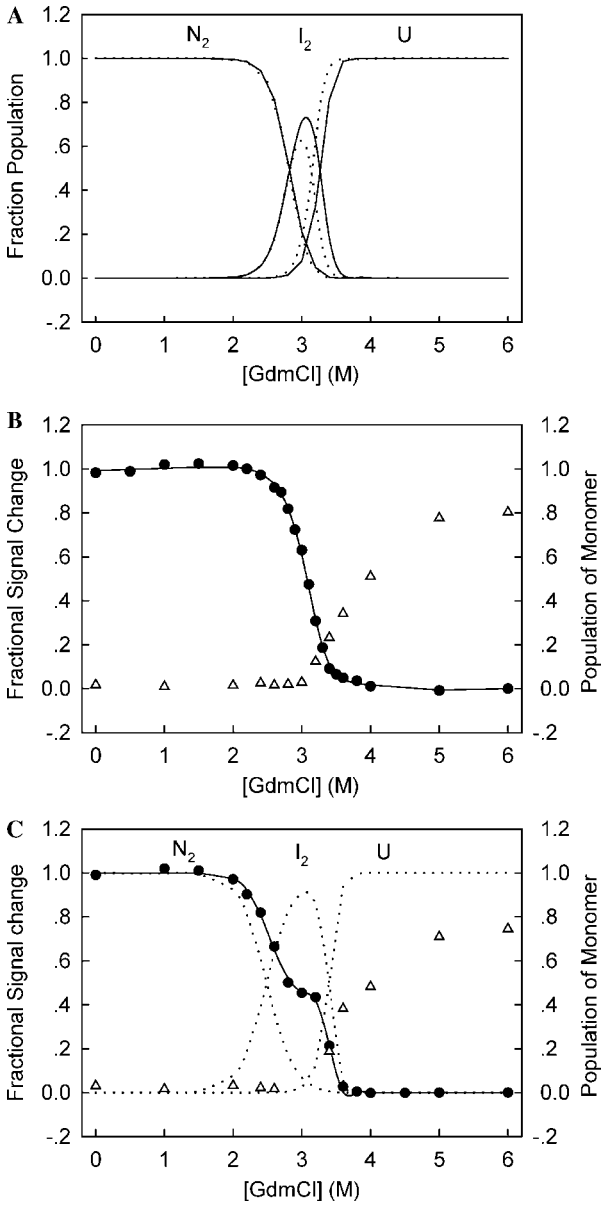

Fig. 5. Observation of Ure2 dimer dissociation during GdmCl denaturation by appearance of the monomer under strongly cross-linking conditions. Results are compared to the denaturation curves observed by intrinsic protein fluorescence, far- or near-UV CD [2,13]. (A) Fractions of native dimer (N_2), dimeric intermediate (I_2), and denatured monomer (U) calculated as described [13] from the thermodynamic data, extrapolated to the conditions of the SAXS experiments (77 μM protein concentration, solid lines) or the cross-linking experiments (20 μM protein concentration, dotted lines), in 50 mM Tris–HCl buffer, pH 8.4, 0.2 M NaCl, at 25 °C. The values used were: $[GdmCl]_{1/2,1} = 2.82$ M; $[GdmCl]_{1/2,2} = 3.15$ and 3.27 M, for 20 and 77 μM protein concentration, respectively; $m_1 = 4.0$ and $m_2 = 9$ kcal mol^{-1} M^{-1}. (B,C) Comparison between fraction fluorescence change (●) and fraction population of monomer detected in cross-linking experiments (△). The fraction population of monomer was derived from densitometry of the SDS–PAGE bands, as displayed in Fig. 4. Data are shown for 20 μM Δ15-42Ure2 incubated in 50 mM buffer, pH 8.4, 0.2 M NaCl at 25 °C. (B) Tris–HCl buffer. (C) Na/K phosphate buffer. The dotted line represents the fraction populations of N_2, I_2, and U, calculated as in (A) using the following values: $[GdmCl]_{1/2,1} = 2.49$ M, $[GdmCl]_{1/2,2} = 3.40$ M; $m_1 = 3.0$ and $m_2 = 10$ kcal mol^{-1} M^{-1}.

around 11 Å [20–22,35]. Cross-linking was performed at pH 8.4 at 25 °C, as for the SAXS experiments, in either Tris–HCl or Na/K-phosphate buffer containing 0.2 M NaCl, using a protein concentration of 4–20 μM. Δ15-42 Ure2 (Fig. 4) and full-length Ure2 (not shown) showed identical results at the same protein concentration.

As shown by SDS–PAGE (Figs. 4A and B), the cross-linking reaction proceeds rapidly and with high efficiency under the conditions used, so that monomeric protein is detectable only at or above 3.0 M GdmCl, i.e., after the onset of unfolding (Fig. 5) as detected by fluorescence or CD [2,13]. This is consistent with the SAXS results presented above, which indicate that there is no dissociation prior to the onset of unfolding. Using phosphate buffer showed very similar results to Tris–HCl buffer (compare Figs. 5B and C). A significant band corresponding to the cross-linked dimer is observed even in 4.0 M GdmCl in both phosphate (not shown) and Tris–HCl buffers (Fig. 4B), even though the protein appears to be fully unfolded (Fig. 5) by multiple structural probes [2,13]. Higher-order aggregates are also observed, including some that are too large to enter the gel matrix (Fig. 4). This is slightly more marked within the transition region, consistent with the SAXS results. SDS–PAGE on the same samples using reducing sample buffer shows a single band of similar intensity at all GdmCl concentrations (Fig. 4C), confirming that the aggregates are soluble and that protein is not lost from solution, consistent with the ability of Ure2 to form high molecular weight soluble oligomers [2].

It should be noted that the cross-linking reaction is irreversible under the conditions of the experiment. Therefore, unlike SAXS experiments, a non-equilibrium distribution of species is represented. Cross-linking of a small proportion of tetramers, hexamers or larger aggregates present at equilibrium will continuously drive the equilibrium in the direction of the oligomer. Small oligomers can become further cross-linked to form larger oligomers. Thus, the ability to observe the dimer under native-like conditions indicates that this is the predominant species in solution and/or that larger oligomers are populated only very transiently, consistent with the SAXS results. Similarly, under conditions where the monomer is observed, dimers and other oligomers must represent a very small fraction of the protein molecules and/or form extremely transiently.

Discussion

Molecular size and shape of native and unfolded Ure2

SAXS provides a powerful tool to follow changes in protein conformation and oligomeric state during the denaturation process [14–18,28,29,34]. The R_g values for native and 5 M GdmCl denatured Δ15-42Ure2 were found to be 32.0 and 45 Å, respectively. No significant degree of aggregation in the native state of Δ15-42Ure2 was detected under the conditions used (Tris–HCl, pH 8.4, 25 °C and up to 10 mg/ml of native protein). The R_g values obtained are very close to 29.0 and 46 Å, which are the equivalent values measured for creatine kinase, another dimeric protein of similar molecular weight [36]. The R_g values for 90Ure2, measured under identical conditions to Δ15-42Ure2, are 2–3 Å lower for both the native and denatured states (Fig. 3A). This suggests that the N-terminal PrD is similarly unstructured in the native and denatured states, as indicated by GdmCl denaturation experiments, where wild-type Ure2 and a series of Ure2 mutants with deletions in the PrD all showed the same change in solvent accessible area (*m*-value) on unfolding [2].

The presence of residual structure in 4 M GdmCl is suggested by both SAXS and cross-linking experiments. Experiments under high hydrostatic pressure also suggest a tendency for Ure2 to form residual structure in the presence of high concentrations of GdmCl [10]. Residual β-structure has been shown to affect the shape of the Kratky plot, whereas helical structure without any globular or collapsed structure is not detected by SAXS [17,30]. As the native structure of Ure2 is predominantly helical [8,9], the residual structure may involve non-native interactions that are present in a partially collapsed subset of the ensemble of denatured states. However, the observation of a small fraction of dimer under strongly denaturing conditions is also consistent with the remarkably high thermodynamic stability of dimeric Ure2, even when in the form of a partially folded intermediate [13].

Association, dissociation, and unfolding of Ure2

The transition observed by SAXS (Fig. 3) coincides with the transition observed by intrinsic fluorescence, near- and far-UV circular dichroism under the same buffer conditions [2,13]. The SAXS data show that dissociation does not occur prior to the onset of unfolding. This is consistent with thermodynamic analysis of Ure2 unfolding, which indicates that Ure2 derives a significant proportion of its stability from formation of a dimer, so that even at submicromolar protein concentrations, native-like or partially folded monomeric intermediates of Ure2 remain less stable than their dimeric counterparts throughout the GdmCl denaturation curve [13].

Even at the high protein concentrations necessary for SAXS experiments, the native dimer showed little tendency to aggregate into higher-order oligomers. However, under partially denaturing conditions, an aggregated state corresponding to a hexamer was formed. This is an interesting observation in light of two recent findings: (1) investigation of the effects of solution

conditions on the kinetics of amyloid formation suggests that the mechanism of amyloid nucleation involves partial unfolding of the native state [13] and (2) the nucleus for amyloid nucleation has been suggested to represent a hexamer [37].

Despite the hint of residual structure, the position of the final baseline in SAXS experiments implies that the denatured state is predominantly monomeric and this is confirmed by the appearance of monomer under strongly cross-linking conditions (Fig. 5). The cross-linking results in Tris–HCl buffer, pH 8.4, where unfolding monitored by optical probes appears two-state and dissociation is difficult to detect, compared to phosphate buffer, where dissociation of the dimeric intermediate is readily observed, are extremely similar. This is consistent with the proposal of a unified mechanism for Ure2 denaturation: $N_2 \Longleftrightarrow I_2 \Longleftrightarrow U$, over this range of conditions [13].

Acknowledgments

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