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Quantitative Modelfree Analysis of Urea Binding to Unfolded Ubiquitin Using a Combination of Small Angle X-ray and Neutron Scattering

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Characterization of the conformational properties of unfolded proteins is essential to our understanding of the molecular basis of protein folding and stability.[1] An accurate description of the unfolded state is also important for the investigation of disease related protein misfolding,[2] as well as to describe the relationship between flexibility and function in intrinsically disordered proteins.[3]

Despite the abundance of biophysical and biochemical data from unfolded proteins in the presence of denaturant, the molecular origin of solvent-induced protein denaturation remains unclear. Over the past 50 years two models have emerged to explain the unfolding effect of urea:[4] The first invokes the disruptive effects on water structure, such that urea acts as a better solvent for hydrophobic groups that are normally buried in water-solvated proteins.[5] An alternative model proposes that urea binds directly to multiple sites on the protein backbone, presumably via hydrogen bonding interaction with the amide groups, thereby destabilizing the native fold relative to the unfolded state.[6] Both models would stabilize the unfolded state in the presence of high concentrations of urea.

Small angle scattering has been used to characterize the change in dimensions of the peptide chain upon protein unfolding[7] and to evaluate the presence of native structure in the unfolded state.[8] The level of chain extension derived from small-angle X-ray data complements NMR analyses of local conformational sampling in the presence of urea, where residual dipolar couplings indicate that backbone dihedral angles sample more extended regions of Ramachandran space,[9,10] in qualitative agreement with fluorescence correlation spectroscopy[11] and pulse field gradient measurements.[12,13]

In this study we apply a powerful combination of small angle neutron (SANS) and X-ray (SAXS) scattering to ubiquitin in 8 M urea solution (H_2O and D_2O at pH 2.5 and 6.5). By comparing the coherent intensities scattered at zero angle from SAXS and SANS and exploiting the different scattering densities of H_2O, D_2O, ubiquitin, and urea for X-rays and neutrons, we are able to quantitatively determine the number of additional urea molecules that are preferentially recruited during the unfolding transition from neutral to acidic pH.

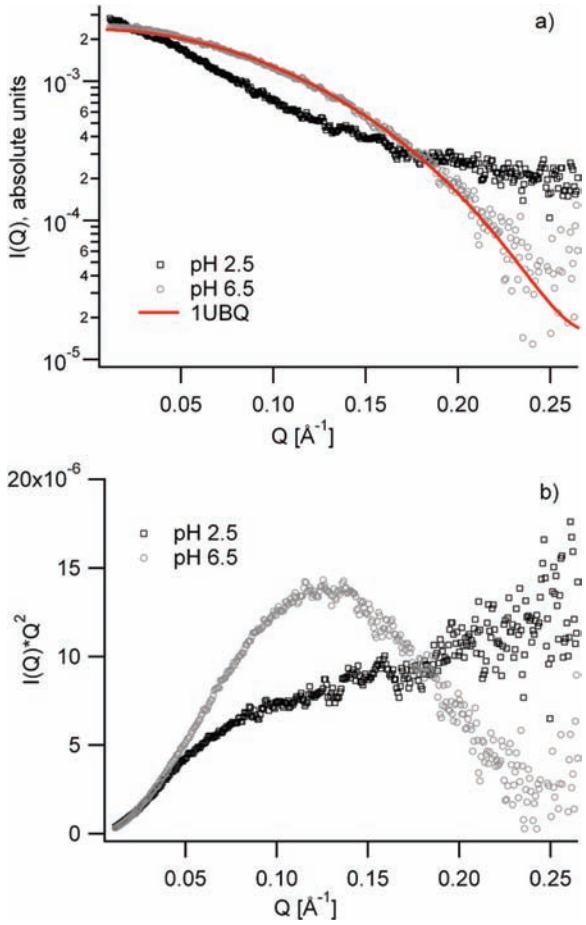

Figure 1. (a) SAXS scattering curves of ubiquitin in 8 M urea/H_2O at pH 6.5 and 2.5 (the curves in 8 M urea/D_2O are very similar within error bars). Protein concentration was 1.7 mM. The red line represents the calculated scattering curve of ubiquitin[14] (PDB 1UBQ) using CRYSOL.[15] (b) Kratky plot[16] of the same data. Data were measured on beamline ID02, ESRF Grenoble, France.

Figure 1a shows SAXS curves of ubiquitin in 8 M urea/H_2O (pH 6.5 and 2.5) as well as a calculated scattering curve of native ubiquitin[14] (PDB 1UBQ) using the program CRYSOL.[15] Figure 1b shows a Kratky plot[16] of the data. At pH 6.5 the predicted scattering curve is very similar to the experimental curve over the entire Q-range, indicating that, in agreement with early

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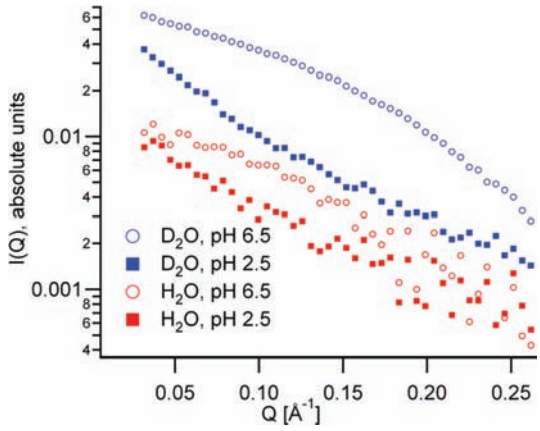

Figure 2. SANS curves from ubiquitin in 8 M urea/H_2O (red) and D_2O (blue) at pH 6.5 (open) and 2.5 (closed). Data were recorded on small angle neutron diffractometer D22 at the Institut Laue Langevin, Grenoble, France.

observations,[17] the protein is in its native conformation even when dissolved in high concentrations of denaturant. Lowering the pH to 2.5 results in a change in overall shape of the scattering curve along with a significant increase in radius of gyration (Table 1), suggesting that under these conditions the protein unfolds into a disordered conformation. These findings are corroborated by the Kratky plot that shows the characteristic features for a transition from a native folded into an unfolded state.

SANS data were recorded under the same conditions, the pH being lowered by adding a small amount of 7.7 M HCl to the sample in between two neutron exposure times (details in Supporting Information). The corresponding curves are shown in Figure 2. They display, in both 8 M urea/H_2O and D_2O, and in analogy to the SAXS data, a transition from a folded (pH 6.5) to an unfolded state (pH 2.5).

A remarkable feature, observed by both SAXS and SANS, is the change in the intensities scattered at zero angle, $I(0)$, by lowering the pH: an increase is observed by SAXS upon denaturation, while SANS in D_2O shows a significant decrease while the situation in SANS in H_2O is ambiguous due to large errors in our data (Figures 1a, 2, and Supporting Information Figure 1).

Both the amplitude and the sign of the changes in $I(0)$ can be explained quantitatively by assuming that a certain number ΔN_{urea} of urea molecules are being recruited by ubiquitin upon unfolding and by considering the different scattering densities of ubiquitin, water, and urea molecules for X-rays and neutrons (Figure 3). In the context of the invariant particle hypothesis[18] for a scattering particle composed of a single molecule of ubiquitin and N_{urea} urea molecules, $I(0)$ can be related to the scattering densities of the protein, the urea molecules, and the bulk solvent (ρ_{prot}, ρ_{urea}, and ρ_S, respectively) as follows:

$$\sqrt{I(0)} \propto (\rho_{prot} - \rho_S)V_{prot} + N_{urea}(\rho_{urea} - \rho_S)V_{urea} \qquad (1)$$

V_{prot} and V_{urea} are the solvent-excluded volumes of ubiquitin and urea. The bulk solvent density ρ_S refers to the 8 M urea/(H_2O or D_2O) mixtures. For X-rays, the densities can be expressed as electrons per $Å^3$ while their unit is cm^{-2} for neutrons. In the latter case, the number of exchangeable hydrogens must be taken into account when calculating the scattering densities in a D_2O solvent.

This differential behavior with respect to neutron and X-ray scattering allows for a quantitative and model-free identification of the nature and extent of solvent binding to the unfolded protein. We use Eq 1 to determine ΔN_{urea} (e.g., the number of urea molecules $N_{urea}^{pH2.5} - N_{urea}^{pH6.5}$ recruited during unfolding) from the relative changes of $I(0)$ measured by SAXS and by SANS in D_2O and H_2O upon denaturation. To this end we made several assumptions: (1) The specific volumes of the protein and of urea in pure water remain unchanged in 8 M urea solutions. (2) They do not change during the binding process. (3) All exchangeable hydrogens (in the protein and urea) are exchanged to a fraction corresponding to the molar ratio of deuterium in the 8 M urea/D_2O solution. (4) The changes of bulk solvent scattering density as a function of pH can be neglected (further details in Supporting Information).

Table 1. Radii of Gyration, R_g, and Relative Intensities Scattered in the Forward Direction, $I(0)$, Determined by the Guinier Approximation[30] as well as Number of Urea Molecules, ΔN_{urea}, Recruited by Ubiquitin during Unfolding Assuming $N_{urea}^{pH6.5} = 0$ (Eq 1 and Supporting Information Eq 2)[a]

solvent	method	R_g [Å]	$[I(0)/I_{pH6.5}(0)]^{1/2}$	ΔN_{urea}
pH 6.5 (H_2O)	SANS	14.0 ± 2.0	1	-
	SAXS	14.4 ± 0.5	1	-
pH 6.5 (D_2O)	SANS	13.4 ± 0.6	1	-
	SAXS	14.3 ± 0.6	1	-
pH 2.5 (H_2O)	SANS	22.0...37.5	0.94...1.23	−5...19
	SAXS	28.0 ± 3.5	1.10 ± 0.03	16 ± 5
pH 2.5 (D_2O)	SANS	32.5 ± 2.0	0.90 ± 0.02	25 ± 7
	SAXS	28.4 ± 4.0	1.11 ± 0.02	17 ± 5

[a] The radii of gyration are compatible with values from literature for ubiquitin (native:[14] 14.0 Å, unfolded:[8] 25.2 Å).

From the experimentally determined changes of $I(0)$ (Table 1), we found that ~20 molecules of urea are recruited by ubiquitin during the unfolding process. This number is not found to be very sensitive to $N_{urea}^{pH6.5}$ (Supporting Information Figure 2). Absolute calibration by SANS (8 M urea/H_2O) against water[19] suggests that the number of urea molecules bound to native ubiquitin is unlikely to exceed 10, compatible with values found in literature for small proteins (see Supporting Information for details).[20−22] Under the conditions of this study, using methods developed by Schellman,[23] we can estimate the binding constant of urea to each of the sites. Assuming that urea preferentially binds to the peptide bonds of the protein, 72 sites will be available in ubiquitin leading to a binding constant of urea to each of the sites S_i of 0.048 M^{-1}. This estimate compares quite closely with reported values derived from solubility,[24] NMR,[25,26] end-to-end diffusion,[27] and calorimetry measurements.[28]

These results appear to rule out preferential hydration by water molecules at pH 2.5: indeed by comparing the contrast $\Delta\rho$ of the individual components in SANS (8 M urea/D_2O) (Figure 3, left), one sees that $\Delta\rho$ of D_2O molecules and ubiquitin are of the same sign. Binding of D_2O would therefore *increase* the scattering density.

We can equally rule out a major influence of a solvent shell of different density than the bulk solvent[29] on our results (see Supporting Information). On the contrary, our data rather suggest that the overall volume of the hydration water of denatured ubiquitin can be only slightly larger than the one of the native protein and/or that it is less dense. While hydration water might play a minor role, the interpretation of our results in terms of a recruitment of urea molecules upon unfolding is capable of

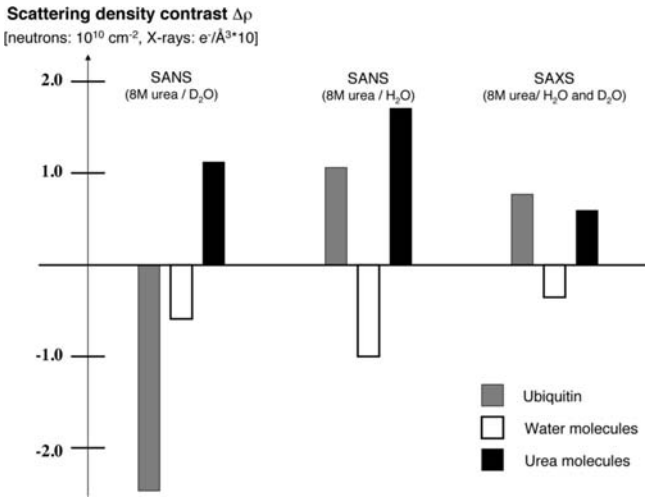

Figure 3. Scattering contrast $\Delta\rho = \rho - \rho_S$, calculated for ubiquitin, water, and urea molecules for neutrons in 8 M urea/(D_2O and H_2O) and for X-rays (identical values for 8 M urea/H_2O and 8 M urea/D_2O). For details, see Supporting Information. Opposite signs in scattering density of two components associated in solution induce an apparent diminution of the molecular weight while identical signs lead to an increase.

explaining all data sets (SAXS and SANS) simultaneously in a consistent manner.

In conclusion, in this study we demonstrate that comparison of the complementary scattering properties of H_2O, D_2O, ubiquitin, and urea for small angle neutron and X-rays allows a quantitative and model-free analysis of the interaction of urea with the protein ubiquitin under denaturing conditions. Our results suggest that a pH change from 6.5 to 2.5 triggers a recruitment of ~20 urea molecules from the bulk solution per ubiquitin molecule during the unfolding process. This information will contribute to the development of a more detailed molecular understanding of the mechanisms of protein denaturation and qualitatively supports the model of direct binding of urea to the protein backbone. More generally this analysis reiterates the strong complementarity of small-angle X-ray and neutron scattering for the study of the conformational behavior of chemically denatured and intrinsically disordered proteins in solution. Combination of these data with explicit ensemble descriptions of the unfolded state[31] will hopefully provide previously unavailable insight into protein−solvent interactions in these highly flexible systems.

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Supporting Information Available: Details of sample preparation, experimental procedures, calculation of scattering densities, and data analysis. Complete ref 8. This material is available free of charge via the Internet at http://pubs.acs.org.

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