Structural perturbation of proteins in low denaturant concentrations

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The presence of very low concentrations of the widely used chemical denaturants, guanidinium chloride and urea, induce changes in the tertiary structure of proteins. We have presented results on such changes in four structurally unrelated proteins to show that such structural perturbations are common irrespective of their origin. Data representative of such structural changes are shown for the monomeric globular proteins such as horseradish peroxidase (HRP) from a plant, human serum albumin (HSA) and prothrombin from ovine blood serum, and for the membrane-associated, worm-like elongated protein, spectrin, from ovine erythrocytes. Structural alterations in these proteins were reflected in quenching studies of tryptophan fluorescence using the widely used quencher acrylamide. Stern-Volmer quenching constants measured in presence of the denaturants, even at concentrations below 100 mM, were higher than those measured in absence of the denaturants. Both steady-state and time-resolved fluorescence emission properties of tryptophan and of the extrinsic probe, PRODAN were used for monitoring conformational changes in the proteins in presence of different low concentrations of the denaturants. These results are consistent with earlier studies from our laboratory indicating structural perturbations in proteins at the tertiary level, keeping their native-like secondary structure and their biological activity more or less intact.

In the presence of urea and guanidinium chloride (GdmCl) proteins have been shown to undergo pronounced structural disintegrations at concentrations >1M of these denaturants1,2. However, it is generally accepted that very low concentrations of urea and GdmCl which are often present in the buffer during reconstitution of proteins from the completely unfolded state attained by treatment with 8-10 M urea and 4-6 M GdmCl, do not impart any alterations in their tertiary structure. We have previously shown that low, even millimolar, concentrations of both GdmCl and urea can bring about structural alterations in the tetrameric enzyme, lactate dehydrogenase (LDH) and the hem-containing enzyme, horseradish peroxidase (HRP) at the tertiary level3,5. In this paper, we present an overview of our work on the study of the effect of low concentrations of the commonly used electrolytic denaturant, GdmCl and the uncharged denaturant, urea on the tertiary structure of four proteins, viz. HRP, HSA, prothrombin and the worm-like large elongated protein, spectrin. Since GdmCl is a strong electrolyte but urea is not, such a study with a number of unrelated proteins was considered likely to offer insight into the general mechanism of denaturant-protein interaction.

The denaturation/renaturation of LDH, HRP isoenzyme c (ref. 3-5) and HSA6 by GdmCl and urea have been extensively studied using fluorescence and circular dichroism (CD). Similar studies on prothrombin and spectrin were also done previously. However, the principal emphasis of these studies was to analyze secondary structural changes and different conformational states of the denatured proteins. In the present study, both far-UV and near-UV CD measurements show that in all four proteins very little conformational change takes place upon treatment with up to 1 M GdmCl and urea. The enzymatic activities in LDH and HRP also did not show any change3,5. On the other hand, changes in the steady-state intensity and anisotropy and the excited-state lifetime of tryptophan fluorescence emission, the bimolecular quenching constant (Stern-Volmer constant $K_o$) for acrylamide quenching of tryptophan fluorescence, the steady-state and time-resolved emission parameters of the extrinsic fluorophore, 6-propionyl-2-(dimethylamino) naphthalene (PRODAN),
incorporated into the proteins, all indicate that the presence of low concentrations (10 to 500 mM) of both denaturants brings about changes in their tertiary structures similar to those found earlier in LDH and HRP.

Materials and Methods

HRP (type VIa), HSA, GdmCl, urea, ultrapure acrylamide, N-acetyl-L-tryptophanamide (NATA), and Tris were purchased from Sigma (St. Louis, MO.). PRODAN was procured from Molecular Probes.

Spectrin was isolated and purified from ovine erythrocyte ghosts following published protocol. Prothrombin was isolated from ovine blood serum using barium citrate adsorption technique as described in literature. The purity of the isolated proteins was checked by SDS-PAGE analysis. The concentration of HRP was determined spectrophotometrically assuming a molar absorptivity of 24,000 at 280 nm and 95,000 at 402 nm. Concentrations of spectrin, prothrombin and HSA were determined using an absorbance of 10.7, 14.4 and 5.3 at 280 nm for 1% spectrin, prothrombin and HSA respectively, in a buffer containing 10 mM Tris-HCl, 50 mM NaCl, pH 7.5~8.10. Absorbance measurements were carried out on a Hitachi U-2000 UV-VIS spectrophotometer.

Steady-state fluorescence studies were performed using a Hitachi F-4010 spectrofluorometer. Fluorescence emission from tryptophan was measured using excitation at 295 nm to minimise the loss of intensity due to energy transfer to tyrosine. Fluorescence emission from PRODAN was measured using excitation at 360 nm, using 5 nm bandwidth slits for both excitation and emission channels. The concentration of PRODAN in dimethylformamide was determined using a molar absorbance of 18,000 at 360 nm. Typical concentrations of 0.5 µM PRODAN and 7.5 µM HSA, both in presence and absence of the denaturants, were used in all fluorescence measurements. Polarization measurements were performed at an emission wavelength of 340 nm for tryptophan and 450 nm for HSA-bound PRODAN and 520 nm for free PRODAN in aqueous buffer. The time-resolved measurements were performed with 0.5 µM spectrin. The sample temperature was kept at 25°C. The buffer used in all experiments contained 10 mM Tris-HCl, 50 mM NaCl, pH 7.5 (Tris/saline buffer) unless mentioned otherwise.

Quenching of tryptophan fluorescence was measured by serial addition of small aliquots of acrylamide stock solutions, using excitation at 295 nm and recording the emission intensities at 340 nm. Corrections were applied to the observed intensities for dilution of the fluorescent material and for absorption of incident light by acrylamide, the latter being minimal at 295 nm. HSA (5 µM) was incubated with different concentrations of the denaturants at 25°C for 1 hr, in a final volume of 1 ml of Tris/saline buffer, before quenching experiments were performed. Data were fit to the Stern-Volmer relation:

\[
F_0 / F_{corr} = 1 + K_w [Q] \quad \ldots (1)
\]

where \(F_0\) and \(F_{corr}\) are the fluorescence intensities in absence and in presence of the quencher, respectively, [\(Q\)] the quencher concentration, and \(K_w\) the Stern-Volmer (or dynamic) quenching constant. The linear portions of the \(F_0/F_{corr}\)-vs.-[\(Q\)] plots near the origin were used to determine the quenching constants. Error bars for \(K_w\) represent the uncertainties in slopes of the linear fits of quenching data to Equation 1. These errors were smaller than the errors given by the spread in the \(K_w\) values obtained from three independent experiments at a given urea concentration.

Fluorescence lifetimes were determined from total emission intensity decay measurements using a time-domain fluorometer assembled in our laboratory with components from Edinburgh Analytical Instruments (EAI, UK) and EG&G ORTEC (USA) and operated in the time-correlated-single-photon-counting mode. Excitation was provided by a pulsed high-pressure (1.5 atm) N2-lamp operating at 25 kHz repetition rate, the pulse profile having a full width at half maximum (FWHM) of 1.2 ns. The tryptophans were excited at 295 nm and PRODAN at 360 nm. The emission was monitored at 340 nm and 450 nm for the tryptophan and spectrin-bound PRODAN fluorescence respectively. Slits with 8-16 nm bandwidth were used in both excitation and emission channels. The details of the time-resolved measurements were published earlier.
the mean lifetime values in absence and presence of the quencher respectively.

CD spectra of the native and denaturant-incubated proteins were recorded at 25°C on a JASCO J-720 spectropolarimeter using a cylindrical quartz cell of path length 1 mm for far-UV spectra and a rectangular cell of path length 10 mm for the near-UV spectra. The spectra shown are the average of five (three) continuous scans for the far (near)-UV region, corrected by subtracting the appropriate blank runs on protein-free solutions and subjected to a moderate degree of noise-reduction analysis.

Results

The far-UV CD spectra of the four proteins, HSA, HRP, spectrin and prothrombin, shown in Fig. 1, indicate marginal but definite changes in the secondary structure upon treatment with low concentrations of urea or GdmCl. Representative near-UV spectra of HSA and HRP also indicate such changes in the presence of low concentrations of the denaturants (Fig. 2). The wavelength of tryptophan emission maximum remains essentially unchanged for all proteins in presence of 10-500 mM GdmCl or urea. However, the fluorescence intensity decreases appreciably in HSA and spectrin, and marginally in HRP and prothrombin (data not shown).

The most noticeable effect of pre-incubation of the proteins in the denaturants was observed in experiments on acrylamide quenching of tryptophan fluorescence, as shown by the Stern-Volmer plots in Fig. 3 for HSA and spectrin. The linear regions of the plots showed a definite increase of slope, implying increased accessibility of the tryptophan residues to the quencher molecules, brought about by the presence of low concentrations of the denaturant. Quenching studies were also performed by time-resolved fluorescence measurements in spectrin and Ksv determined from the slope of the plot of τ/τ against the acrylamide concentration (Fig. 3b). The quenching efficiency showed a definite increase, from (4.07±0.03) M⁻¹ for native spectrin to (5.10±0.02) M⁻¹ and (4.78±0.04) M⁻¹ for spectrin in presence of 20 mM GdmCl and 50 mM urea respectively, pointing to structural perturbations in spectrin. As a control experiment, similar acrylamide quenching studies were also carried out on the model tryptophan
compound, N-acetyl-L-tryptophanamide (NATA). A value of \( (16.5 \pm 0.3) \, M^{-1} \) for \( K_{sv} \) was obtained independent of the concentration (upto \( 1 \, M \)) of the denaturants in the solution in which NATA was incubated, indicating that the changes in \( K_{sv} \) reported here are specific for the proteins. Table 1 summarizes the results of quenching experiments for \( K_{sv} \) in all four proteins at different non-denaturing concentrations of the denaturants.

We have also studied the effect of urea and GdmCl on the conformation of the proteins by monitoring the change in emission properties of the extrinsic fluorophore PRODAN incorporated in the proteins. PRODAN is a hydrophobic probe which is known to readily associate with the hydrophobic regions of proteins and showed changes in its fluorescence characteristics\(^{14}\). Fig. 4 shows a set of representative spectra of PRODAN when bound to HSA in presence of varying concentrations of GdmCl. The emission maxima of PRODAN blue-shifted from 520 nm in the aqueous buffer to 450 nm when bound to HSA, and to 430 nm when bound to spectrin\(^{14}\). The emission intensity of HSA-bound PRODAN also changes in the presence of low concentrations of both denaturants (Fig. 4), suggesting structural modifications consistent with the changes in emission maximum of PRODAN bound to serum albumin.

Time-resolved measurements of both tryptophan and PRODAN fluorescence decay yielded a mean lifetime of 3.8 ns for the tryptophans in spectrin, and 1.1 ns for PRODAN in aqueous buffer and 4.6 ns PRODAN bound to native spectrin. This increase in lifetime is consistent with the localization of PRODAN in the interior of the protein, whereby a number of non-radiative pathways for its decay are inhibited. More significantly, the lifetime changes even in the presence of millimolar concentrations of urea and GdmCl, as shown in Table 1. Representative decay

Table 1—Mean lifetimes of tryptophan and PRODAN emission, and Stern-Volmer constants for acrylamide quenching of tryptophan fluorescence, in presence and absence of low concentrations of denaturants for four different proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>( \tau ) in ns for Trp (PRODAN)</th>
<th>( K_{sv} ) (M(^{-1}))</th>
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<tbody>
<tr>
<td></td>
<td>- Denaturant</td>
<td>+ Denaturant</td>
</tr>
<tr>
<td>Spectrin</td>
<td>3.81 (4.64)</td>
<td>3.87 (4.84)</td>
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<tr>
<td></td>
<td>(10 mM GdmCl)</td>
<td></td>
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<tr>
<td>Horseradish peroxidase</td>
<td>2.34 (2.13)</td>
<td>2.69 (2.13)</td>
</tr>
<tr>
<td></td>
<td>(100 mM GdmCl)</td>
<td></td>
</tr>
<tr>
<td>Prothrombin</td>
<td>4.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>4.50 (3.04)</td>
<td>4.43 (2.82)</td>
</tr>
<tr>
<td></td>
<td>(50 mM Urea)</td>
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</table>
Fig. 4—Fluorescence spectra of Prodan (1 μM) bound to HSA (15 μM) in Tris-saline buffer, pH 7.5, preincubated with 2 M, 0 M, 50 mM and 100 mM GdmCl for one hr (in order of increasing intensity at 450 nm).

profiles of the fluorescence intensity of both tryptophan and spectrin-bound PRODAN are shown in Fig. 5.

Discussion

The mechanism of action of urea and GdmCl on protein denaturation is still obscure. According to one school of thought, urea stabilizes both the native and denatured protein conformations. The larger number of binding sites exposed in the unfolded state favours denaturation\(^{15-17}\). Crystallographic studies of \(\alpha\)-chymotrypsin in the presence of GdmCl and urea have shown that both denaturants bind to the surface of the folded protein, with the urea molecules (but not the charged GdmCl) also permeating the interior, occupying small cavities and somewhat perturbing the close-packed interior\(^{18}\). A recent molecular dynamics simulation study also suggests that the preferential adsorption of urea molecules onto the charged hydrophilic residues on protein surfaces initiates the denaturation of proteins\(^{19}\). Mokhatadze and Privalov have studied the interaction of urea and guanidinium chloride with proteins using calorimetry\(^{20}\). They were able to describe the observed heat effects in terms of a simple binding model and could estimate the number of apparent binding sites of the denaturants for the three proteins ribonuclease A, lysozyme and cytochrome C. The binding of urea to the small globular proteins, bovine pancreatic trypsin inhibitor (BPTI) and PEC-60 was also investigated by NMR spectroscopy and the average binding constants were found to lie within 5-10 M\(^{-1}\) at room temperature\(^{21}\). Assuming equilibrium bimolecular binding between protein and the denaturants, the occupancy of the binding site should be higher than 50% for denaturant concentrations larger than the inverse binding constant, suggesting that even submolar (10\(^{-2}\) to 10\(^{-1}\) M) concentrations of the denaturants can bind to micromolar concentration of proteins and may thus, lead to structural alterations in the compact folded state of the protein.

Both steady-state and time-resolved fluorescence and CD are very useful techniques for studying the structure and dynamics of proteins\(^{22,23}\). The intrinsic tryptophan fluorophores of proteins, and extrinsic probes such as ANS, PRODAN etc., are reporters that can sense the microenvironment\(^{18}\). Acrylamide is also used as a quencher of protein fluorescence to detect conformational changes in proteins. We have followed the change in the quenching constant (\(K_w\)) for all proteins, in presence and absence of low...
concentrations of the denaturant, to probe any local conformational change in the neighbourhood of the sole tryptophan residue in HRP and HSA. The present work extends our earlier studies done with LDH and HRP to generalize the fact that the change in the tertiary structure of proteins takes place in all the four completely unrelated proteins, the monomeric enzyme HRP, the monomeric serum proteins, HSA and prothrombin, and the worm-like elongated cytoskeletal protein, spectrin in presence of non-denaturing concentrations of urea and GdmCl. In all proteins, such a structural relaxation was indicated by an increase in the Stern-Volmer constant for acrylamide quenching in presence of 10-500 mM urea or GdmCl concentrations (Table 1).

The basic features of the far-UV spectra (Fig. 1), characteristic of the folded conformation of the peptide backbone, as well as the magnitude of helicity do not show major changes for denaturant concentrations upto 500 mM. The enzymatic activities of LDH and HRP also remained practically unchanged when the enzymes were incubated in the denaturant concentrations upto 1M (ref. 3-5). Some minor changes in the microenvironment of the aromatic side chains of HSA and HRP were reflected in the near-UV CD spectra (Fig. 2). The conformational changes of HRP and HSA in submolar GdmCl or urea, detected by the fluorescence quenching measurements, are associated with a probable relaxation of the single tryptophan residue in them (Fig. 3). The fluorescence changes associated with HSA-bound PRODAN (Fig. 4) also indicate conformational changes in the protein, in the neighbourhood of its PRODAN binding site upon incubation in low denaturant concentrations. It is very probable that PRODAN binds to a distinct hydrophobic patch and senses denaturant-induced conformational change in its near vicinity.

An extension of our previous study was undertaken to investigate whether such a low concentration of any protein denaturant could induce structural perturbations in the protein through direct binding and whether there are differences between the altered structures brought about by urea and GdmCl. Results shown in this paper indicate that chemical denaturants could induce measurable changes in the tertiary sturcture of proteins. This study, confirms the importance of protein-denaturant interaction at low concentrations of the denaturant and suggests that such denaturant-induced perturbations may be common to a large class of proteins.

References