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Guanidinium chloride- and urea-induced unfolding of FprA, a mycobacterium NADPH-ferredoxin reductase

Stabilization of an apo-protein by GdmCl

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The guanidinium chloride- and urea-induced unfolding of FprA, a mycobacterium NADPH-ferredoxin reductase, was examined in detail using multiple spectroscopic techniques, enzyme activity measurements and size exclusion chromatography. The equilibrium unfolding of FprA by urea is a cooperative process where no stabilization of any partially folded intermediate of protein is observed. In comparison, the unfolding of FprA by guanidinium chloride proceeds through intermediates that are stabilized by interaction of protein with guanidinium chloride. In the presence of low concentrations of guanidinium chloride the protein undergoes compaction of the native conformation; this is due to optimization of charge in the native protein caused by electrostatic shielding by the guanidinium cation of charges on the polar groups located on the protein side chains. At a guanidinium chloride concentration of about 0.8 M, stabilization of apo-protein was observed. The stabilization of apo-FprA by guanidinium chloride is probably the result of direct binding of the Gdm^+ cation to protein. The results presented here suggest that the difference between the urea- and guanidinium chloride-induced unfolding of FprA could be due to electrostatic interactions stabilizating the native conformation of this protein.

The conformational stability of proteins can be measured by equilibrium unfolding studies using guanidinium chloride (GdmCl) and urea, the two agents commonly used as protein denaturants. Analysis of the solvent denaturant curves using these denaturants can provide a measure of the conformational stability of the protein [1,2]. Protein unfolding/folding studies in GdmCl and urea solutions have focussed on the identification of equilibrium and kinetic intermediates [3–5]. Structural characterizations of the partially folded intermediates stabilized during denaturant induced folding/unfolding of proteins have provided significant input on the forces that stabilize these folded intermediates.

Mycobacterium tuberculosis NADPH-ferredoxin reductase (FprA) is a 50-kDa flavoprotein encoded by gene Rv3106 of the H37Rv stain of the pathogen [6]. This is an oxidoreductase enzyme, which is able to take two reducing equivalents from NADPH and transfer them to an as yet unidentified proton acceptor, via the proton-bound FAD cofactor [7]. FprA shows significant sequence homology with adrenodoxin reductase the mammals and with its yeast homologue Arh1p [8], suggesting a possible involvement of this enzyme either in iron metabolism or in cytochrome P450 reductase activity. As these two processes play a major role in survival of the pathogen, studies on the FprA are of significance.

Abbreviations
FprA, NADPH-ferredoxin reductase; GdmCl, guanidinium chloride; λ_{max}, wavelength maximum; SEC, size exclusion chromatography.

Atomic resolution structures of FprA in the oxidized and NADPH-reduced forms have been reported. Structurally, the overall architecture of the FprA protein is similar to that observed for proteins belonging to the family of glutathione reductase [8], of which FprA is a member. The FprA monomer consists of two domains: the FAD-binding domain (residues 2–108 and 324–456) consisting of the N- and C-terminal regions of the enzyme, and the NADPH-binding domain (residues 109–323) consisting of the central part of the polypeptide chain [8]. A small two-stranded β-sheet links the two domains. Our recent studies have demonstrated that the two structural domains of FprA fold/unfold independently of each other [9]. The NADPH-binding domain of FprA was found to be sensitive to cations, which induce significant destabilization of this structural domain. Furthermore, modulation of ionic interactions in FprA (either by cations or by pH) was found to induce coopertivity in the otherwise noncooperative protein molecule [9].

We have carried out a detailed characterization of the structural and functional changes associated with the GdmCl- and urea-induced unfolding of FprA. Various optical spectroscopic techniques such as fluorescence and CD were used to study the changes in the tertiary and secondary structure of the protein during denaturant-induced unfolding. The changes in the molecular dimension of the protein were studied by size exclusion chromatography. Significantly different pathways of FprA unfolding were observed with the two denaturants; with GdmCl showing the stabilization of a compact conformation and a compact apo-intermediate during unfolding of protein, whereas the urea-induced unfolding was found to be a cooperative process without stabilization of any partially folded intermediate.

Results

We have studied the effect of GdmCl- and urea-induced changes in the structural and functional properties of FprA. Time-dependent changes in the structural parameters and enzymatic activity of FprA at increasing GdmCl or urea concentrations (0.5, 1.5 and 4 M) were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within maximum of ≈6 h with no further alterations in the values obtained up to 12 h (data not shown). These observations suggest that a minimum time of ≈6 h is sufficient for achieving equilibrium under any of the denaturing conditions studied.

Changes in molecular properties of FprA-associated with GdmCl-induced unfolding

Enzyme activity can be regarded as the most sensitive probe with which to study the changes in enzyme conformation during various treatments as it reflects subtle readjustments at the active site, allowing very small conformational variations of an enzyme structure to be detected. Fig. 1A summarizes the effect of increasing concentrations of GdmCl on the enzymatic activity of FprA. No significant alteration in enzymatic activity of FprA was observed up to ≈0.2 M GdmCl. However, between 0.4 and 0.8 M GdmCl a sharp loss of enzymatic activity (from 93 to ≈2%) of FprA with increasing concentration of GdmCl was observed. At 1 M GdmCl there was a complete loss of enzymatic activity. Furthermore, the enzymatic activity could not be regained on refolding of the 1 M GdmCl-incubated FprA.

The effect of GdmCl on the structural properties of FprA was characterized by carrying out optical spectroscopic studies in the presence of increasing concentrations of GdmCl.

The fluorescent prosthetic groups FAD or FMN present in various flavoproteins exhibit different spectral characteristics in different proteins, reflecting the specific environmental property of isoalloxazine, which is the chromophore present in the molecule [10]. For this reason the FAD group has been used as a natural marker to probe the dynamic microenvironment of the flavin chromophore in flavoproteins [11,12]. FprA contains a tightly bound but noncovalently linked FAD molecule, which in the native conformation of protein is buried in the protein interior, and hence, its fluorescence is quenched [7]. The effect of GdmCl on the FAD microenvironment of FprA is summarized in Fig. 1B where the changes in the FAD fluorescence intensity of FprA on incubation of the enzyme with increasing concentrations of GdmCl are depicted. A large increase, about 20 times, in fluorescence intensity of FAD was observed between 0.25 and 1 M GdmCl. For several FAD-containing proteins it has been shown that enhancement in fluorescence intensity of FAD corresponds to the release of protein-bound FAD on denaturation [12,13]. Hence, the possibility of GdmCl-induced release of FAD from FprA resulting in stabilization of an apo-protein was studied as reported earlier [14]. FprA incubated with 0.8 M GdmCl was concentrated on a 3-kDa cut off Centricon and the presence of FAD in free form (in filtrate) and protein-bound form (in the protein fraction) was monitored by fluorescence spectroscopy. Under these conditions, a major fraction of the FAD was observed in the filtrate (≈ 85% relative fluorescence) with little

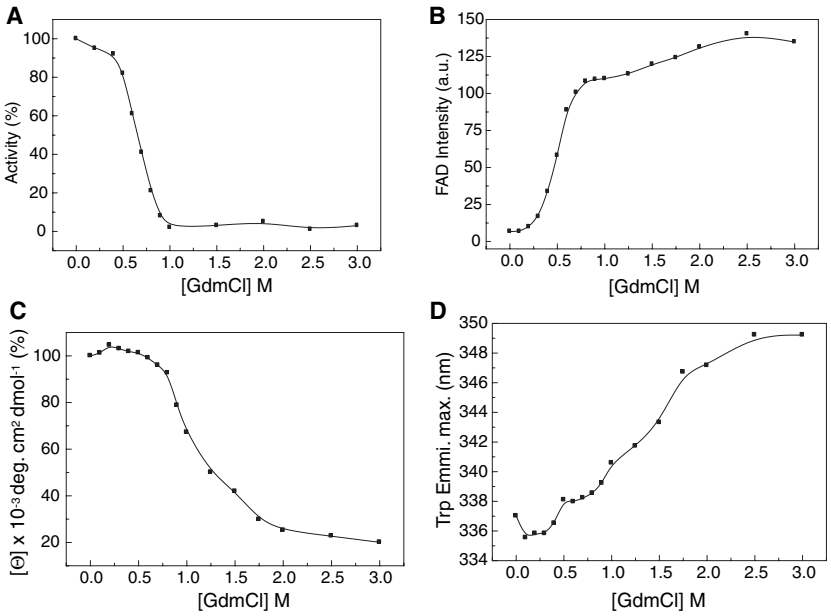

Fig. 1. Changes in functional and structural properties of FprA on incubation with increasing concentration of GdmCl at pH 7.0 and 25 °C. (A) Changes in enzymatic activity of FprA on incubation with increasing concentrations of GdmCl. The data are percentages with enzymatic activity observed for FprA in the absence of GdmCl taken as 100%. (B) Changes in FAD fluorescence intensity of FprA on incubation with increasing concentrations of GdmCl. (C) Changes in CD ellipticity at 222 nm for FprA on incubation with increasing concentrations of GdmCl. Data are percentages with the value observed for FprA in the absence of GdmCl taken as 100%. (D) Changes in tryptophan fluorescence emission wavelength maximum of FprA on incubation with increasing concentrations of GdmCl.

associated with the enzyme (≈ 15% relative fluorescence). For native FprA, a major population of protein-bound FAD (≈ 90%) was observed under the experimental conditions. These observations demonstrate that incubation of FprA with a low concentration of GdmCl (≈ 0.8 M) leads to dissociation of protein-bound FAD.

Far-UV CD studies on GdmCl-induced unfolding of FprA were carried out to study the effect of GdmCl on the secondary structure of the protein. In the far-UV region, the CD spectra of the FprA show the presence of substantial α-helical conformation [15]. Fig. 1C summarizes the effect of increasing GdmCl concentrations on the CD ellipticity at 222 nm for FprA. Up to a GdmCl concentration of ≈ 0.5 M, no significant change in CD ellipticity at 222 nm of FprA was observed. However, between 0.65 and 2.5 M GdmCl, a large sigmoidal decrease in ellipticity at 222 nm from 100 to ≈ 10% was observed. These results suggest that incubation of FprA with higher concentrations of GdmCl results in significant loss of secondary structure of FprA due to unfolding of protein under these conditions.

Changes in the molecular properties of FprA such as enzymatic activity, FAD fluorescence and CD ellipticity at 222 nm at increasing GdmCl concentration showed a sigmoidal dependence; however, the denaturation profiles obtained by monitoring changes in these properties were not super-imposable, suggesting that the GdmCl-induced unfolding of FprA is a multiphasic process with stabilization of intermediates during the unfolding process. Experimental support for this suggestion comes from tryptophan fluorescence studies. The spectral parameters of tryptophan fluorescence such as position, shape, and intensity are dependent on the electronic and dynamic properties of the chromophore environment; hence, steady-state tryptophan fluorescence has been extensively used to obtain information on the structural and dynamic properties of the protein [16]. The FprA molecule contains five tryptophan residues at positions 46, 131, 359, 409 and 423 in the primary sequence of the protein. For FprA at pH 7.0, significant tryptophan fluorescence with an emission λ_{max} at 337 nm was observed. The buried tryptophan residues in the folded protein show an emission λ_{max} at 330–340 nm [17], hence, at pH 7.0 the tryptophan residues in native FprA are buried in the hydrophobic core of the protein. The modification of the tryptophan microenvironment in FprA due to GdmCl treatment was monitored by studying changes in the emission wavelength maximum (λ_{max}) of tryptophan fluorescence as a function of increasing denaturant concentration. Fig. 1D shows the effect of an increasing concentration of GdmCl on the tryptophan fluorescence emission λ_{max} of FprA. An initial decrease in tryptophan emission λ_{max} from 337 to 335 nm was observed on increasing the GdmCl concentration from 0 to 0.25 M. A further increase in GdmCl concentration from 0.3 to 0.8 M reversed this effect, bringing the emission wavelength maxima to 338 nm. A similar change in tryptophan emission maxima of FprA was observed on treatment of protein with increasing concentration of $CaCl_2$ [9]. For FprA incubated with 2.5 M GdmCl a tryptophan emission λ_{max} of 350 nm was observed. Normally, exposed tryptophan residues

in the unfolded protein show emission λ_{max} between 340 and 356 nm [17], indicating that incubation of FprA with a higher concentration of GdmCl results in significant unfolding of the protein molecule.

The $CaCl_2$-induced changes in the tryptophan emission maxima and molecular dimensions of FprA demonstrated an initial compaction of native conformation followed by relaxation of the stabilized compact conformation along with the release of protein-bound FAD [9]. As a similar dependence of tryptophan emission maxima was observed on treatment of FprA with low concentrations of GdmCl (between 0 and 0.8 M). Furthermore, loss of protein-bound FAD was also observed at $\approx$0.8 M GdmCl. Hence, we carried out size exclusion chromatography (SEC) under these conditions to see the changes in the molecular dimension of FprA. Fig. 2 summarizes the results of SEC experiments carried out on FprA on the S-200 Superdex column in the presence and absence of GdmCl at 25 °C. When FprA incubated with 0.25 M GdmCl was loaded onto the SEC column and eluted, a significant increase in the retention volumes to 15.7 mL, as compared to 15.2 mL corresponding to native FprA was observed. This increase in retention volume for the 0.25 M GdmCl-incubated FprA is indicative of significantly reduced hydrodynamic radii for GdmCl-stabilized intermediate of FprA as compared to native protein. This is probably due to GdmCl-induced compaction of the native conformation of the enzyme. For FprA incubated with 0.8 M GdmCl a retention volume to about 15.35 mL was observed which is similar to that observed for native FprA but significantly less than that observed for 0.25 mM GdmCl-stabilized protein. These observations suggest that 0.8 M GdmCl-stabilized FprA has a conformation of which the molecular dimension is similar to that of the native protein but is significantly more open than the protein stabilized by 0.25 M GdmCl. For FprA incubated with 2.5 M GdmCl, a significantly reduced retention volume of $\approx$12.5 mL was observed on SEC, which is indicative of a protein conformation with a significantly larger hydrodynamic radus, i.e., an unfolded protein.

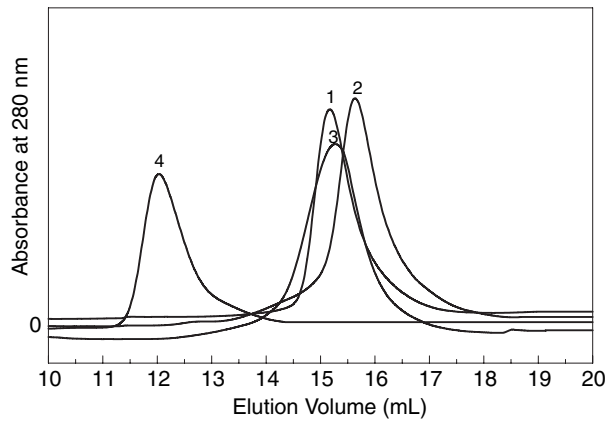

Fig. 2. GdmCl-induced alterations in the molecular dimension of FprA. Size-exclusion chromatographic profiles for FprA and on incubation with increasing concentrations of GdmCl on a Superdex 200 H column at pH 7.0 and 25 °C. Curves 1–4 represent profiles for FprA at pH 7.0 on incubation with 0, 0.25, 0.8 and 2.25 M GdmCl, respectively. The columns were run with the same concentration of GdmCl in which the protein sample was incubated. The samples were incubated for 6 h in GdmCl before column chromatography.

Characteristics of the GdmCl-stabilized compact state of FprA

The structural studies along with SEC experiments reported above demonstrate that low concentrations of GdmCl ($\approx$ 0.25 M) stabilize a compact enzyme conformation. A similar compaction of native conformation of FprA has been reported for the treatment of protein with NaCl and $CaCl_2$ [9]. One of the characteristic properties of the NaCl- or $CaCl_2$-stabilized compact conformation of FprA is that on thermal denaturation it undergoes a complete cooperative unfolding which is in contrast with the partial unfolding observed in case of native FprA [9]. In order to see whether the GdmCl-stabilized compact state is similar to the NaCl- or $CaCl_2$-stabilized compact state, we carried out comparative thermal unfolding studies on the native and GdmCl-stabilized compact state of FprA. Fig. 3 shows the changes in CD ellipticity at 222 nm for native FprA and that treated with 0.25 M GdmCl as a function of

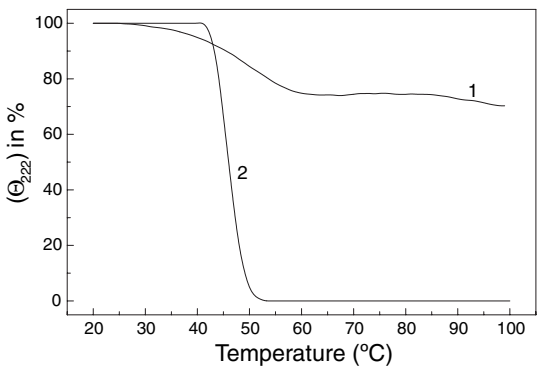

Fig. 3. Changes in thermal denaturation profiles of FprA on incubation with low GdmCl as measured by loss of CD ellipticity at 222 nm. Thermal denaturation profiles of FprA incubated with and without GdmCl. Curves 1 and 2 represent profiles for FprA at pH 7.0, incubated with 0 and 0.25 M GdmCl, respectively. The values for loss of CD signal are percentages with the value observed for protein sample at 20 °C taken as 100%.

temperature. For native FprA, a broad sigmoidal transition between 30 and 65 °C having an apparent T_m (mid point of thermal denaturation) of ≈49 °C and a loss of only ≈27% CD ellipticity at 222 nm was observed, which was same reported earlier [9]. However, for 0.25 M GdmCl-treated FprA, a single sharp sigmoidal transition with a T_m of ≈46 °C and almost complete loss of secondary structure associated with the transition was observed. These observations suggest that low concentrations of NaCl or $CaCl_2$ or GdmCl stabilize a similar compact conformation of FprA.

Characterization of the GdmCl-stabilized apo-FprA

GdmCl-induced denaturation studies on FprA showed that a low concentration of GdmCl induces release of the protein-bound FAD cofactor resulting in stabilization of an apo-protein having molecular dimension, tryptophan microenvironment and secondary structure similar to those of the native protein. Divalent cations such as calcium have been shown to have the same effect [9]. Therefore, to see whether the $CaCl_2$- and GdmCl-stabilized apo-FprA have similar structural characteristics we carried out a comparative GdmCl-induced unfolding study on the FprA and the 0.8 M $CaCl_2$-stabilized apo-protein and analysed it by monitoring the changes in tryptophan fluorescence as summarized in Fig. 4A. For 0.8 M $CaCl_2$-stabilized FprA, a sigmoidal dependence of changes in tryptophan emission maxima with increasing GdmCl concentration was observed between 0 and 4 M GdmCl. Furthermore, the profile for the 0.8 M $CaCl_2$-incubated FprA superimposed significantly with the transition observed between 1 and 4 M GdmCl during GdmCl-induced unfolding of the native protein. A control experiment was also carried out where the GdmCl-induced unfolding of 0.2 M NaCl incubated FprA (which does not show stabilization of an apo-protein) was studied. Under these conditions, a biphasic curve showing two distinct transitions between 0 and 0.8 M and 0.8 and 3 M GdmCl were observed (Fig. 4B). These observations demonstrate that during GdmCl-induced denaturation of FprA the transition observed at low concentrations of GdmCl (0.5–1 M) corresponds to the stabilization of an apo-protein having structural characteristics similar to the $CaCl_2$-stabilized apo-protein.

Changes in molecular properties of FprA associated with urea-induced unfolding

Fig. 5 summarizes the urea-induced changes in functional and structural properties of FprA as studied by

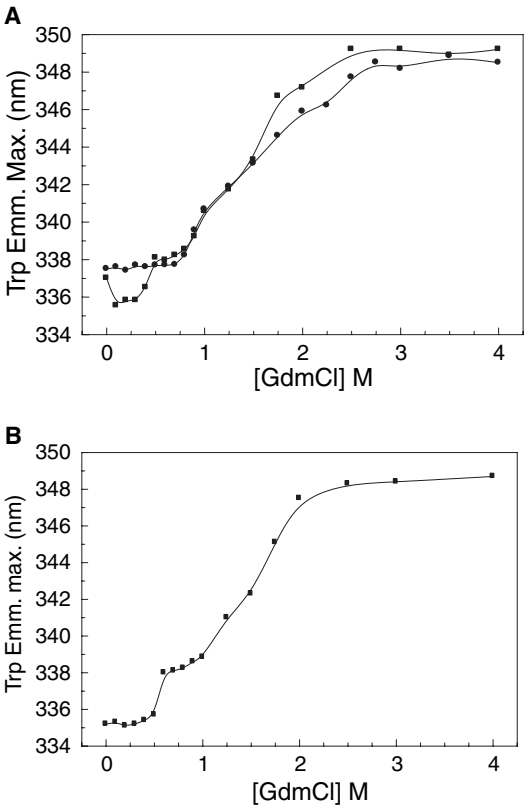

Fig. 4. Effect of $CaCl_2$ or NaCl incubation of FprA on the GdmCl-induced unfolding of protein. Changes in tryptophan fluorescence emission wavelength maximum of FprA and that incubated with 0.8 M $CaCl_2$ (A) and 0.2 M NaCl (B) in the presence of increasing concentrations of GdmCl. In (A) circles and squares represent data for native and 0.2 M $CaCl_2$-stabilized FprA, respectively.

changes in enzymatic activity, FAD and tryptophan fluorescence and CD ellipticity at 222 nm at increasing urea concentration.

No significant effect of urea on the enzymatic activity, FAD fluorescence, tryptophan fluorescence and CD ellipticity at 222 nm of FprA was observed up to a urea concentration of 2.0 M. However, between 2.0 and 5 M urea there was a sharp sigmoidal decrease in enzymatic activity from 100% to almost complete loss of activity, ≈10 times enhancement in FAD fluorescence intensity, an increase in tryptophan emission λ_{max} from 335 to 350 nm, and ≈80% loss of CD signal at 222 nm (Fig. 5A–D). These observations suggest that urea induces a cooperative unfolding of the FprA molecule. Fig. 5F summarizes the results of SEC experiments carried out on FprA on the S-200 Superdex column in the presence and absence of urea at 25 °C. For FprA incubated with 6 M urea, a significant decrease in the retention volume to 12.1 mL, as compared to

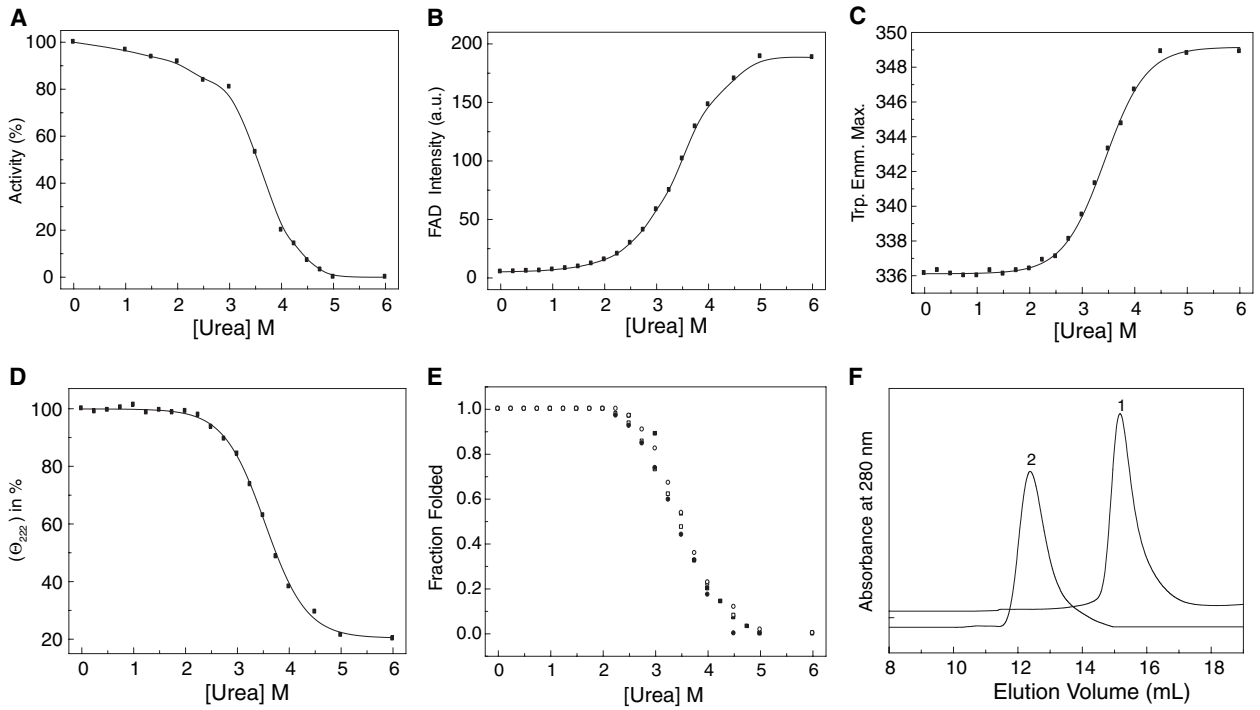

Fig. 5. Changes in functional and structural properties and molecular dimension of FprA on incubation with increasing concentrations of urea at pH 7.0 and 25 °C. (A) Changes in enzymatic activity of FprA on incubation with increasing concentrations of urea. Data are percentages with enzymatic activity observed for FprA in the absence of urea taken as 100%. (B) Changes in FAD fluorescence polarization of FprA on incubation with increasing concentration of urea. (C) Changes in CD ellipticity at 222 nm for FprA on incubation with increasing concentration of urea. Data are percentages with the value observed for FprA in the absence of urea taken as 100%. (D) Changes in tryptophan fluorescence emission wavelength maximum of FprA on incubation with increasing concentrations of GdmCl. (E) Urea-induced unfolding transition of FprA as obtained from enzymatic activity (A, ■), FAD fluorescence intensity (B; □), tryptophan emission maxima (C; ●), and ellipticity at 222 nm (D; ○). A linear extrapolation of the baseline in the pre- and post-transitional regions was used to determine the fraction of folded protein within the transition region by assuming two-state mechanism of unfolding. (F) Size-exclusion chromatographic profiles for FprA and on incubation with and without urea on Superdex 200 H column at pH 7.0 and 25 °C. Curves 1 and 2 represent profiles for FprA at pH 7.0 and that on incubation with 6 M urea, respectively. The columns were run using same urea concentration at which the protein sample was incubated. The samples were incubated for 6 h in urea before column chromatography.

15.1 mL corresponding to native FprA was observed. This suggests a significant enhancement in the molecular dimension of FprA on treatment with a high concentration of urea, which is possible only when the protein undergoes extensive unfolding under these conditions.

The changes in the tertiary and secondary structure of FprA, as monitored by changes in the enzyme activity, tryptophan fluorescence and CD ellipticity at 222 nm associated with urea-induced unfolding of protein all occurred between 2 and 5 M urea; $\approx$1.5 M urea was required to half denature the protein (Fig. 5E). This observation suggests that during urea-induced unfolding of FprA there is a concomitant unfolding of the tertiary and the secondary structure of protein with no partially folded intermediate being stabilized during this process.

Discussion

The equilibrium unfolding of FprA in urea and GdmCl suggests dramatically different pathways and mechanism for the two denaturants as summarized in Fig. 6. The urea-induced unfolding of FprA was found to be a cooperative process in which the protein molecule undergoes unfolding without stabilization of any partially unfolded intermediate. However, GdmCl-induced unfolding of FprA was a noncooperative process. At low GdmCl concentration ($\approx$ 0.25 M), compaction of the native conformation of the enzyme is observed. An increase in GdmCl concentration to $\approx$0.8 M results in removal of protein-bound FAD from the enzyme and hence, an apo-protein is stabilized under these conditions. The apo-protein could not be converted back to holo-protein even when refolding

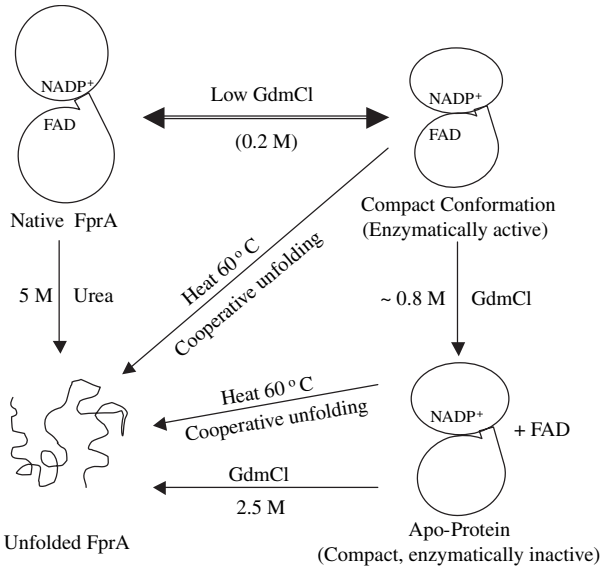

Fig. 6. Schematic representation of the urea- and GdmCl-induced structural and functional changes in FprA.

was carried out in the presence of excess FAD. Higher concentrations of GdmCl induce irreversible unfolding of FprA.

The exact molecular mechanism/s of the denaturing action of urea and GdmCl has not yet been clearly defined [18,19]. It has been presumed that both urea and GdmCl molecules unfold proteins by solubilizing the nonpolar parts of the protein molecule along with the peptide backbone CONH groups and the polar groups in the side chains of proteins [20,21]. According to this mechanism the unfolding of FprA should follow the same path with both denaturants. However, significant differences in the unfolding pathway of FprA were observed for urea and GdmCl. This prompted us to look for other possible differences between the two denaturants, which would explain their different effects on the unfolding process.

GdmCl is an electrolyte and therefore is expected to ionise into Gdm^+ and Cl^- in aqueous solution. From a structural point of view, urea and Gdm^+ are very similar; however, urea is a neutral (uncharged) molecule whereas the guanidinium ion has a positive charge delocalized over the planar structure. At high concentrations, GdmCl is a denaturant because the binding of Gdm^+ ions to the protein predominates and pushes the equilibrium towards the unfolded state; this results in denaturation of protein. However, at low concentrations Gdm^+ ion can preferentially adsorb onto the protein surface due to interactions with the negatively charged amino acid side chains present in protein molecule. This would lead to perturbations and/or weakening of the optimized electrostatic interactions present in the native conformation of protein, and as a result stabilization of intermediates can be observed under these conditions.

In FprA, modulation of ionic interactions present in the native conformation of the protein by monovalent cations has been shown to result in stabilization of a compact conformation [9]. Low GdmCl concentration ($\approx$ 0.25 M) was also found to stabilize a compact conformation of the native protein which showed a cooperative complete unfolding on thermal denaturation similar to that observed for the cation stabilized compact state of FprA. These observations suggest that the stabilization of a compact conformation of native FprA at low GdmCl concentration is due to interaction of the Gdm^+ cation with the negatively charged side chain moieties; this leads to optimization of the electrostatic interactions present in the native conformation of the protein thus resulting in compaction.

The most interesting observation during GdmCl-induced denaturation of FprA is the stabilization of an apo-protein in presence of $\approx$0.8 M GdmCl. This GdmCl-stabilized apo-FprA showed a molecular dimension comparable to that of the native protein, thus demonstrating that it has a compact conformation. The release of protein bound-FAD from FprA by GdmCl could result from either specific interaction between GdmCl and the GdmCl-stabilized compact intermediate (at 0.25 M GdmCl) through binding, or from the effect of Gdm^+ ion on the electrostatic shielding of protein through an ionic strength effect. The GdmCl-induced release of FAD from FprA is not likely to be a result of electrostatic shielding. There are two strong reasons for this belief: firstly, interaction of monovalent cations with FprA does not bring about any significant change in the FAD microenvironment of protein [9]; secondly, inclusion of NaCl during the GdmCl study, to maintain the ionic strength, showed no significant effect on the GdmCl stabilization of the compact apo-intermediate of the protein (Fig. 4B). This implies that the stabilization of a compact apo-intermediate of FprA by GdmCl is probably due to specific interaction of Gdm^+ cation with the protein.

The differences in the GdmCl and urea denaturation of FprA are probably due the fact that electrostatic interactions within the protein molecule play an important role in its stability. The GdmCl molecule, due to the presence of the Gdm^+ ion can modulate the ionic interactions stabilizing the native conformation of FprA leading to stabilization of intermediates. However, the neutral urea molecule does not have the capacity to modulate the electrostatic interactions

present in the protein and hence no stabilization of any intermediate is observed during urea-induced unfolding of FprA.

Experimental procedures

All chemicals were from Sigma and were of highest purity available.

Methods

Overexpression and purification of FprA

Cloning, overexpression and purification of the FprA was carried out as described earlier [7]. The ESI-MS and SDS/PAGE of the purified FprA showed that the preparation was > 95% pure.

GdmCl and urea denaturation of FprA

FprA (7 μm) was dissolved in sodium phosphate buffer (50 mM, pH 7) in the presence/absence of increasing concentration of GdmCl/urea and incubated for 6 h at 4 °C before the measurements were made.

Enzymatic activity

Diaphoreses activity of the enzyme was measured at 25 °C with potassium ferricyanide as electron acceptor and NADPH as reductant as described earlier [7]. For studies using increasing concentration of urea or GdmCl, the assay buffer contained concentrations of denaturant similar to those in which the enzyme was incubated.

Fluorescence spectroscopy

Fluorescence spectra were recorded with Perkin-Elmer LS 50B spectrofluorometer in a 5-mm path length quartz cell. The excitation wavelength for tryptophan and FAD fluorescence measurements were 290 and 370 nm, respectively, and the emission was recorded from 300 to 400 nm and from 400 to 600 nm, respectively.

CD measurements

CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium(+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity [θ], which is defined as $[\theta] = 100 \times \theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in mol residue·l^{-1}, and l is the length of the light path in centimetres. CD spectra were measured at an enzyme concentration of 7 μM with a 1-mm cell at 25 °C. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of denaturant under similar conditions.

Size exclusion chromatography

Gel filtration experiments were carried out on a Superdex 200 H 10/30 column (manufacturer's exclusion limit 600 kDa for proteins) on AKTA FPLC (Amersham Pharmacia Biotech, Sweden). The column was equilibrated and run with 50 mM phosphate buffer pH 7.0 containing the desired GdmCl or urea concentration at 25 °C with a flow rate of 0.3 mL·min^{-1}.

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