UDP-galactose 4-epimerase from *Escherichia coli*: Equilibrium unfolding studies

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UDP-galactose 4-epimerase from *Escherichia coli* is a homodimer of 39 kDa subunit with non-covalently bound NAD acting as cofactor. The enzyme can be reversibly reactivated after denaturation and dissociation using 8 M urea at pH 7.0. There is a strong affinity between the cofactor and the refolded molecule as no extraneous NAD is required for its reactivation. Results from equilibrium denaturation using parameters like catalytic activity, circular-dichroism, fluorescence emission (both intrinsic and with extraneous fluorophore 1-amino 8-naphthalene sulfonic acid), 'reductive inhibition' (associated with orientation of NAD on the native enzyme surface), elution profile from size-exclusion HPLC and light scattering have been compiled here. These show that inactivation, integrity of secondary, tertiary and quaternary structures have different transition mid-points suggestive of non-cooperative transition. The unfolding process may be broadly resolved into three parts: an active dimeric holoenzyme with 50% of its original secondary structure at 2.5 M urea; an active monomeric holoenzyme at 3 M urea with only 40% of secondary structure and finally further denaturation by 6 M urea leads to an inactive unfolded state with only 20% of residual secondary structure. Thermodynamical parameters associated with some transitions have been quantitated. The results have been discussed with the X-ray crystallographic structure of the enzyme.

UDP-galactose 4-epimerase (hereafter called epimerase) is an obligatory enzyme of galactose metabolism and is ubiquitously present in all organisms. The enzyme catalyses a freely reversible epimerisation at the C-4 position of the carbohydrate residue between UDP-galactose (UDP-gal) and UDP-glucose (UDP-glu). The enzyme from *Escherichia coli* is a homodimer of 39 kDa subunit and contains tightly, but non-covalently bound NAD as the cofactor. During catalysis, NAD is transiently reduced to NADH. Its 'oxidoreductase' type of mechanism of action is well documented.

Among epimerases, the X-ray crystallographic structure of the bacterial enzyme alone is known at present at high resolution, which is used as reference. It shows that the enzyme has two nucleotide binding sites in symmetry oriented positions away from the subunit interface. Whether these two sites are catalytically functional or not, is not known. Subunits are composed of two domains of roughly equal size; the N-terminal (1-181) and C-terminal (182-336) domains. The N-terminal domain contains the nucleotide binding site and the substrate binding site is situated at the cleft formed between the two domains.

The unusual quaternary structure of the enzyme offers an unique system to be studied, including its folding behaviour.

Reversible folding and reactivation of this molecule after denaturation and dissociation by 8 M urea at pH 7.0 has been reported earlier. It showed that in spite of incomplete recovery of secondary structure during refolding, reactivation was complete. Moreover the refolded molecule was able to associate with its own stoichiometric amount of NAD, indicating strong affinity of the ligand with the protein. Subsequently, kinetics of the reactivation process was analyzed and it was concluded that an internal rearrangement of the unfolding intermediate shielded from solvent was the rate limiting step in reactivation (also manuscript communicated elsewhere). Here we report properties of the unfolding intermediates of this enzyme equilibrated between 0-8 M urea at neutral pH. It may be mentioned that the kinetic and equilibrium folding studies of epimerase from yeast (*Kluyveromyces fragilis*) have also been carried out in this laboratory over the years.

Materials and Methods

Reagents

Urea (E. Merck, India, AR) was recrystallized from hot ethanol to remove possible contamination of cyanate ions. It was dried to constant weight and stock
solutions were made gravimetrically. All fine chemicals e.g., UDP-gal, NAD, ANS (1-anilino 8-naphthalene sulfonic acid), EDTA, 2-mercaptoethanol (2-MCE), Sephadex G-50 and molecular weight markers were from Sigma (USA). Acrylamide (molecular biology grade, Sigma) was used as quencher. Other laboratory reagents (AR grade) were purchased locally. UDP-glucose dehydrogenase was partially purified from beef liver up to the heat denaturation step. The preparation was left in storage buffer at pH 5.5 for two weeks when the background epimerase activity was lost.

Epimerase purification and assay

The enzyme was isolated from a high yielding strain with respect to epimerase, kindly donated by Prof D B Wilson of Cornell University. It was purified to homogeneity after Wilson and Hogness with minor modifications. The preparation was stored in 20 mM KH₂PO₄ buffer, pH 8.5, containing 5 mM 2-MCE and 1 mM EDTA. It showed a single band in SDSPAGE corresponding to molecular mass of 39 kDa. Specific activity of the enzyme was 7600 U/mg of protein.

Enzyme activity was measured according to 'coupled assay' where formation of UDP-glu from UDP-gal was monitored continuously using UDP-glucose dehydrogenase as the coupling enzyme in presence of NAD at 340 nm. To avoid possible assistance of reactivation by NAD during coupled assay, an alternate protocol, viz. 'two-step assay' was also employed. In short, epimerization of UDP-gal was done in presence of 20 mM KH₂PO₄ buffer, pH 8.5, containing 0-8 M urea for a stipulated duration. Epimerase was then inactivated by vortexing with chloroform and the converted UDP-glu was estimated as described above.

Denaturation and renaturation

The procedures were essentially as described earlier. In short 5 μl (10 mg/ml) of the enzyme was denatured in presence of 20 mM KH₂PO₄ buffer, pH 7.0 containing 0-8 M urea for 16 hr at 25°C. Corresponding buffers in absence of the enzyme served as controls in spectroscopic measurements. Renaturation was initiated after dilution of the fully denatured enzyme with buffer containing variable concentration of urea and was left for 16 hr. The fully denatured state may be reached by 8 M urea in mere 5 min.

Spectroscopic methods

Enzyme assay and ultra-violet absorption spectra (240-300 nm) were recorded by a Hitachi 3200U recording spectrophotometer. CD measurements were made with a Jasco V-720C spectropolarimeter. Fluorescence experiments were performed with a Hitachi F-4020 spectrofluorimeter. Scattering of proteins were measured by the same spectrofluorimeter having excitation and emission wavelengths set at 320 nm. In fluorescence quenching experiments equilibrium intermediates between 0-8 M urea at an interval of 0.5 M were treated with quenchers of the following concentrations: acrylamide (0-0.8 M); KI (0-0.5 M) and TCE (0-0.4 M). The following relations were applied:

'Stern-Volmer' plot: F/F₀ = 1 + Kₛᵥ [Q] where F₀ and F are fluorescence intensities in absence and presence of quenchers, Kₛᵥ is the Stern-Volmer constant and [Q] is the quencher concentration in molarity. In case a linear dependency is observed, the slope of the equation gives Kₛᵥ. This parameter indicates quenching efficiency of a ligand.

'Lehrer' plot: F₀/F = (1/₁fₛᵥ)[₁/₁[V]) + ₁fₛᵥ where ΔF = F₀ - F and ₁fₛᵥ is the fraction of the fluorophore population accessible to the quencher at a concentration [Q]. ₁fₛᵥ is obtained by extrapolating ₁/₁[V] to 0.

Fluorescence intensity was corrected for inner filter effect when the absorbance of the solution at the wavelength of excitation and emission exceeded 0.02 using the relation F_corr = F₀ antilog (O.D.ₐₓ + O.D.ₑₚₐₜ)/2. This was found to be negligible with the three quenchers used.

The change of hydrophobic environment of tryptophan residues of the equilibrium intermediates were probed by using second derivative emission spectra after excitation at 295 nm. Corrected spectra were obtained using respective solvents. The ratio R of the derivative values at 338.5 nm and 358.0 nm was used as an index of the average hydrophobicity of tryptophan residues. Detailed description of generation and interpretation of such data have been mentioned elsewhere (also manuscript communicated).

Interaction of ANS with epimerase was followed fluorimetrically (ex: 375 nm; em: 485 nm). An aqueous solution of the fluorophore was made fresh before experiments. Its concentration was 120 μM in the reaction mixture. Baseline corrections were made in all fluorescence experiments.

Formation of soluble aggregated intermediate was followed by measuring turbidity at 320 nm by a spectrophotometer or from light scattering with a spectrofluorimeter having excitation and emission wavelengths set at 320 nm (slit width 2.5 nm). The protein had no absorption at 320 nm (O.D. = 0.001).
Reductive inhibition

NAD bound to epimerase could be quantitatively converted to NADH by incubating with 2 mM L-arabinose and 0.5 mM 5'-UMP (a strong competitive inhibitor) at 25°C for 16 hr. This specific mode of reduction of NAD is known as 'reductive inhibition' and has been applied to different conformers of epimerase to enhance the sensitivity of quantitation of the cofactor. Enzyme bound NADH was subsequently estimated fluorimetrically (ex: 353 nm; em: 441 nm) with reference to a control epimerase sample taken as 100%.

Other Methods

Proteins (100 µl) were separated from ligands or denaturants by loading onto a pre-spun (2.9×0.7 cm) Sephadex G-50 column - usually known as 'spin column'. The column was pre-equilibrated with standard renaturing buffer and was eluted by low-speed centrifugation. Recovery was 90-95%. Elution profiles of epimerase pre-equilibrated under different denaturing conditions were followed from a LKB Ultropack TSK G3000SW SE-HPLC column (0.75×30 cm) in presence of 50 mM NaH2PO4 buffer, pH 7.0 containing 0-8 M urea. Elution was monitored at 280 nm at a flow rate of 0.5 ml/min.

Calibration of the HPLC column was done with the following marker proteins under native conditions: aldolase (158 kDa); hemoglobin (64 kDa); ovalbumin (43 kDa) and carbonic anhydrase (27 kDa). A linear dependency of log Mw vs elution time (Vi) was observed. Native and fully denatured epimerase were eluted at Vi = 15.5 and 10.8 min respectively which corresponded to native molecular mass of around 80 kDa. Molecular mass of the unfolded state was not characterized as the column was not calibrated with unfolded proteins.

Calculation of transition parameters

In the unfolding of proteins at equilibrium, the transition profiles could be used to generate transition midpoints, (Dye)app, cooperativity of transition, -mapp and free energy change, ΔG°app from the relations derived by Tanford. Standard equations have been described in detail earlier.

Results

Disorganization at the catalytic site-inactivation

Epimerase from E. coli is a stable enzyme that retains full activity at pH 8.5 and also at pH 7.0 in presence of 3 M urea for 16 hr at 25°C. Earlier, in a different context it has been shown that for equilibrium unfolding intermediates inactivation occurred between 3-3.5 M urea. In the course of the experiments of the present report, it has been observed that unfolding transition profile of fluorescence emission (referred below) was variable to some extent for enzymes purified from different batches. In order to see whether such variation exists during inactivation, transition profiles were constructed from four different batches of enzyme. Sharp transition of inactivation was observed between 3-4 M urea with insignificant batch wise variation of the enzyme. About 40% increase of activity was consistently observed with the intermediate at 2 M urea. Its origin has not been investigated.

Reductive inhibition

Relative abundance of NAD with unfolding intermediates to native state was estimated fluorimetrically by reductive inhibition. It showed that by 3 M urea there was 85% loss of fluorescence intensity of the product that ultimately decreased close to zero by 5 M urea. The transition was not so sharp having midpoint at around 2.0 M urea.

Interaction with ANS

ANS is an extensively used nonspecific fluorophore that interacts with the hydrophobic patches of proteins with enhancement of fluorescence intensity and red shift of emission maxima. In case of epimerase both from yeast and E. coli, the interaction with ANS is specific at the substrate binding site because most of the fluorescence could be replaced by 5'-UMP (an active site directed competitive inhibitor) with concomitant quenching of emission. Interaction of partially and fully denatured epimerase with ANS showed a monotonous decrease of fluorescence intensity between 0-4 M urea. However, compared to 75% quenching of intensity with 0.5 mM 5'-UMP in the native state, effect of the inhibitor was only significant up to 2 M urea.

Secondary structure

Native epimerase shows maximum negative molar ellipticity at 222 nm typical of an α-helix containing
Fig. 1—Relative secondary structure of unfolding intermediates of epimerase measured by ellipticity at 222 nm from CD spectroscopy. [Secondary structure of the native conformer in absence of the denaturant was considered as 100%. The arrow pointing left indicates relative molar ellipticity of native conformer after 16 hr of incubation with buffer. The upward arrows indicate midpoints of the two transitions].

Therefore, relative loss of secondary structure of the intermediates were monitored at that wavelength which showed a continuous pattern of unfolding (Fig. 1). The profile represents an initial relatively sharp transition followed by a not so sharp one leading to a state that finally corresponds to 20% of residual structure as reported earlier. Thus it is a three state transition (N→I→D). Transition profiles constructed with arbitrary values of the secondary structure of the intermediate (I) shows that 60% yielded the best fit. The fitness was supported by the linear dependency of the thermodynamical characters of each step and the transition mid-points derived were 1.8 and 3.2 M urea (described later).

Fig. 2—(a): Fluorescence intensity of unfolding intermediates of epimerase at emission maxima equilibrated between 0-8 M urea after excitation at 280 nm. [Protein concentration was 25 μg/ml]. (b): Transition profile of epimerase in terms of 'R' (an index obtained from second derivative emission spectra) against concentration of urea.

Tertiary structure
Change of tertiary structure was monitored from fluorescence emission spectrum after excitation at 280 nm. A continuous red shift of em_{max} from 336.8→356.0 nm and a discontinuous change of emission intensity at λ_{max, em} was observed with progress of unfolding (Fig. 2a). The profile shows a linear dependency of emission with increase of denaturant concentration both in the pre- and post-transition zones possibly from interference with buffer. For enzymes purified from different batches, the onset and termination of the transition varied between 2-3 and 3-4 M urea on repetition but the transition was sharp and was essentially complete within 1 M of urea. Figure 3a is therefore a representative data.

For further information about the hydrophobic character of the environment of tryptophan residues, the index 'R' derived from second derivative emission spectra was considered. Unlike neighbouring amino acid to affect fluorescence emission, 'R' is known to be only dependent on the hydrophobic environment. Derived 'R' as described in the methods was found to be 0.066 for the native states. Such low values are indicative of strong hydrophobic milieu around tryptophans in folded conformation. 'R' changed to 0.46 for the unfolded state indicating exposure of the indole nucleus to aqueous solvents. A plot of 'R' against denaturant concentration between 0-8 M urea shows a transition between 2-4 M urea (Fig. 2b). This is similar to that observed in protein fluorescence (Fig. 2a).
Quenching of intrinsic protein fluorescence by ligands like acrylamide, KI or TCE was used as a more sensitive probe to monitor surface accessibility and polarity of the environment of tryptophan residues both in the native and unfolded states. In all cases quenching was observed and the normalized plots of emission in presence and absence of these quenchers showed a distinct blue shift of emission as follows:

(a) Native state (emission intensity, 100; em\text{max}, 337.0 nm); 0.8 M acrylamide (residual intensity 30%; 329.8 nm); 0.4 M KI (75%; 335.8 nm); and 0.4 M TCE (6%; 327.2 nm); (b) Unfolded state (100%; 350.8 nm); 0.8 M acrylamide (5%; 346.2 nm); 0.4 M KI (35%; 349.4 nm); and 0.4 M TCE (9%; 346.0 nm).

Change of $f_0$ from native to unfolded state was as follows: acrylamide 0.8-1.0; KI 0.5-1.0 and TCE 1.0-1.0. Thus, the unfolding process increases only the accessibility of KI to any significant extent. The Stern-Volmer plots of the unfolding intermediates between 0-8 M urea using 0-0.8 M acrylamide were upward in nature. Deviation from linearity is an indication of heterogeneous environment of tryptophan residues. The modified Stern-Volmer (Lehrer) plot shows a linear dependency. These data were used to generate $K_\alpha$ and $f_\alpha$ for the intermediates. Similar plots were generated for KI and TCE. The summary of the result was that for KI, change of $K_\alpha$ with unfolding was not prominent. However distinct increases were observed with acrylamide and TCE from 3.0-10.0 and 3.25-9.5 respectively between 2-8 M urea in a continuous fashion. As stated earlier change of $f_\alpha$ was significant only for KI where it was changed from 0.5 to 1.0 between 0-4 M urea consistent with the protein fluorescence data. Thus the influence of the neighbouring amino acids around tryptophans appears to change continuously during unfolding, leading to higher affinity of the quenchers.

**Quaternary structure**

It has been demonstrated earlier that epimerase could maintain dimeric structure in presence of 2.5 M urea. Stability of quaternary structure depends on protein concentration and unfolding/refolding intermediates are prone to aggregation with variation of external conditions. Therefore, dissociation profiles of epimerase were constructed using Protein Pak 300 SE-HPLC with 10-100 μg/ml of unfolding intermediates. It was observed that elution volumes were reduced with gradual unfolding but a discontinuity was observed between 2.5-3.0 M urea. Such discontinuity could only be explained from dissociation of subunits. No concentration dependent change of transition profile was observed and no aggregated state could be identified in the HPLC profile.

Since in SE-HPLC proteins are diluted several fold, concentration dependent dissociation during chromatography could not be ruled out. Also aggregated states formed may be unstable to column matrix. Ability of the intermediates to scatter light as an aggregate was verified spectrophotometrically and spectrofluorimetrically (Fig. 4). It shows that the intermediate at a very low concentration of urea, $c_f$ 0.1 M can indeed form an aggregate. The actual aggregated state has been confirmed from SE-HPLC where 23% of the protein was eluted at the void volume (Fig. 4 inset).

**Thermodynamical parameters associated with denaturation**

The dependence of $\Delta G_{\text{app}}$ with concentration of denaturant was derived from transition curves of unfolding using three parameters, viz. inactivation, sec-
state were identical. The corresponding R values were 0.066 and 0.072.

Discussion

X-ray crystallography structure of the bacterial epimerase shows that the N-terminal domain contains a classic NAD⁺ binding motif with seven strands of parallel β-pleated sheet flanked on either side of α-helices. The smaller C-terminal domain contains three strands of β-pleated sheets, two major α-helices and one helical turn which are involved in substrate binding. The present report indicates that around 40% of the secondary structure that is lost in the first transition of CD, is not required for catalysis (Fig. 1). Earlier it was observed that 35% of the secondary structure could not be recovered during reversible folding in spite of complete reactivation. Thus a substantial portion of the structure of the enzyme, possibly from the C-terminal domain, is not involved in maintaining the stability or functionality of the catalytic site. Existence of a strong hydrophobic core of epimerase having 20% secondary structure that denied unfolding in presence of 8 M urea has been indicated earlier. Similar result has been obtained with unfolding intermediates (Fig. 1).

It is interesting to note that epimerase could be heat denatured to an amorphous precipitate. This could be refolded after dissolving in 8 M urea followed by dilution with buffer with 95% recovery of activity (S Nayar, unpublished observation). Such phenomenon is also an indication of existence of strong hydrophobic core denying thermal denaturation. Aggregation is considered to be a common feature of proteins in solution that can be observed under moderate to strong denaturing conditions. Evaluation of aggregation...
gation by light scattering and HPLC shows that for epimerase it is significant only at very low concentration of urea (0.1 M), ahead of all transitions followed so far. This is a major advantage of its folding studies.

A compilation of results for transition from different parameters associated with denaturation indicated an overall stability of the dimeric structure. X-ray crystallographic structure shows at least 7 ionic interactions, numerous hydrogen bonding and hydrophobic interactions at the subunit contact region. These prevent dissociation of the monomers up to 3 M urea. Major structural destabilization occurs between 3.0-3.2 M urea affecting inactivation, tertiary structure and the second transition with CD. Monitoring integrity of the catalytic site by interaction with ANS or ‘reductive inhibition’ was not successful because the signals disappeared in spite of functionality of the partially unfolded enzyme.

Tryptophan residues of proteins are often used as sensitive monitors for conformation changes because of their selective fluorescence emission. Epimerase contains 5 tryptophans (170, 300, 312, 326 and 328) which lie in the C-terminal domain. INSIGHT-11 program was used to view the three dimensional picture and all the tryptophans were found to lie in a 4 A cube. Therefore, exposure of these residues to 3 M urea could be primarily attributed to collapse of the C-terminal domain (Fig. 2a). Second derivative spectral analysis to follow unfolding also indicates the same (Fig. 2b). Further, the refolded/reactivated state shows an overlapping tryptophan derivative spectra with the native state strongly indicating regeneration of the original tryptophan environment. Fluorescence quenchers were employed to address surface accessibility of tryptophans. It was observed that acrylamide and trichloroethanol could reach 80-100% of these residues with 70-95% quenching of intensity indicating their environment to be uncharged and hydrophobic. This is consistent with the fluorescence emission data. KI, on the other hand, could reach 50% of these residues probably due to partial positive character somewhere around their interacting sites. Such interpretations are common for multi tryptophan proteins. Fluorescence data with CsCl also indicated a similar situation when all 5 tryptophan residues were found to be quenchable thus residing on the surface26.

Small single domain monomeric proteins unfold in a cooperative manner where \( \Delta G^{\circ}_{\text{app}} \) varies between -5 to -10 kcal/ mole20. Epimerase being constituted of two domains, the biphasic character of the CD profile may be attributed to domain melting. Evidences are now more inclined to assume that partial melting of the N-terminal domain is associated with the first CD transition having low \( \Delta G^{\circ}_{\text{app}} \) of -2.62 kcal/mole. The other domain being more rigid destabilizes around 3.0-3.2 M urea having higher \( \Delta G^{\circ}_{\text{app}} \) of -6.12 kcal/mole. The second transition is more cooperative in nature.

The unfolding process of epimerase from E. coli may be summarized in the schematic presentation:

\[ N^* \leftrightarrow N^*_1 \leftrightarrow N^*_2 \leftrightarrow 2 \text{I} \leftrightarrow 2 \text{U} \]

where \((N^*-N^*)\) is the native dimer, (●) is NAD, \((N^*_1\) - \(N^*_2\) ) is the native like dimer with 30% loss of secondary structure, \(N^*_1\) is the functional dissociated monomer at 3 M urea, [I] is the intermediate at 6 M urea which had not been well characterized and U is well characterized unfolded state devoid of the cofactor. The striking difference of the equilibrium unfolding pattern of yeast epimerase with its bacterial counterpart is that dissociation of the cofactor occurs prior to subunits dissociation12.

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