UDP-galactose 4-epimerase from *Escherichia coli*: existence of a catalytic monomer

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Abstract UDP-galactose 4-epimerase from *Escherichia coli* is a homodimer of molecular mass 39 kDa/subunit and requires NAD as a co-factor. X-ray crystallographic studies indicate two pyridine nucleotide co-factor-binding sites of the dimeric molecule situated in a symmetry-oriented manner. Size-exclusion HPLC of an equilibrium intermediate at 3 M urea suggests a monomeric holoenzyme structure that is catalytically active. Ultracentrifugal studies of the native enzyme in a 5–20% sucrose gradient at low protein concentration also indicate existence of a catalytic monomer. The monomer resembles the dimeric protein in stability and most of its physico–chemical properties.

Key words: UDP-galactose 4-epimerase; Subunit dissociation; Catalytic monomer; Size-exclusion HPLC; Ultracentrifugation

1. Introduction

UDP-galactose 4-epimerase (hereafter called epimerase) reversibly converts UDP-galactose (UDP-gal) to UDP glucose (UDP-glu). This enzyme from *Escherichia coli* is a non-covalently linked homodimer of molecular mass 39 kDa/subunit and requires bound NAD as a co-factor [1,2]. Though it was believed that only one molecule of NAD combines with the dimer, it became clear from the X-ray crystallographic studies (in the presence of the substrate analog UDP benzene) that the dimer contains two pyridine nucleotide-binding sites opposite each other in a symmetry-oriented fashion. The nucleotide-binding sites were found to be adjacent to the substrate-binding sites [3]. Thus, each subunit holoenzyme is expected to be functional provided the subunits maintain their native structure and that there is no allosteric regulation of catalysis. This report indicates that *E. coli* epimerase subunits are stabilized under certain conditions where they can function catalytically. The origin of stability of the dimeric structure also has been addressed.

2. Materials and methods

2.1. Purification and assay epimerase

Epimerase was purified from a high-yielding *E. coli* strain with respect to UDP-galactose 4-epimerase kindly donated by Professor D.B. Wilson, Cornell University, Ithaca, NY [4]. It was homogeneous both in SDS-PAGE and in native PAGE. The preparation was stored in 20 mM potassium phosphate buffer pH 8.5 containing 2 mM 2-mercaptoethanol and 1 mM EDTA where it maintains its dimeric structure [1].

Epimerase was assayed according to the coupled assay protocol where formation of UDP-glu from UDP-gal was monitored continuously by coupling with UDP-glucose dehydrogenase and NAD at 340 nm [4]. This assay requires a high salt buffer and the absence of the denaturant for the functioning of dehydrogenase. Alternatively, a two-step assay procedure was also employed in which epimerase is assayed under any desired state of equilibrium where both the substrate and the product are stable. In short, UDP-gal was epimerised to UDP-glu for a pre-determined time and the reaction was terminated by destroying epimerase with chloroform. UDP-glu in aqueous layer was estimated as usual by NAD and UDP-glu dehydrogenase [4]. Care was taken to check that the carried over denaturant did not inhibit dehydrogenase assay.

2.2. SE-HPLC

A Waters Protein Pak 300 SW column (7.8 mm × 30 cm) was equilibrated with 20 mM potassium phosphate buffer pH 7.0 containing 0–6 M urea. Protein samples were equilibrated in the presence of the denaturant for 20 h. 50 μg of protein was loaded per run and its elution was followed by A_{280nm} at a flow rate of 0.5 ml/min.

2.3. Ultracentrifugation

Sucrose gradient (5–20%) in either 20 or 1 mM potassium phosphate buffer pH 8.5 was prepared in 5-ml ultracentrifugation tubes and 100-μl epimerase samples pre-equilibrated with the same buffer for 20 h at 25°C were layered over it. Protein concentration in the equilibrate was 10–350 μg/ml. Samples were run at 42000 rpm in a Beckman L8-80 M ultracentrifuge at 4°C for 10 h. Fractions (250 μl) were collected from the bottom of the tube by a peristaltic pump and were assayed for epimerase activity by coupled assay method. Marker proteins (1–5 mg/ml) were also run in parallel and the eluted fractions were followed by A_{280nm}. The marker proteins used were cytochrome *c* (12 kDa), ovalbumin (45 kDa), BSA (66 kDa), hemoglobin (64 kDa) and alcohol dehydrogenase (150 kDa).

2.4. Modification by phenylglyoxal

Epimerase (0.6 μM) was incubated in 50 mM potassium phosphate buffer containing 7.2 mM phenylglyoxal (PG, a group-specific reagent for modification of arginine residues) for 3 h. The residual activity was then reduced to 15%. PG (100 mM) was dissolved in dimethyl sulfoxide.

2.5. Spectroscopic methods

Enzyme assay was carried out using a Beckman DU6 spectrophotometer. Far UV CD measurements (200–250 nm) were done on a Jasco V-720C spectropolarimeter. Fluorescence experiments were done on a Hitachi F 4020 spectrofluorimeter. All fine chemicals were purchased from Sigma (St. Louis, MO).

3. Results and discussion

The elution profile of a non-covalently linked multimeric protein from a SE-HPLC at equilibrium with varying concentrations of the denaturant affected by two opposing factors viz., denaturation increases the Stokes radius and thus accelerates the migration and dissociation decreases the Stokes radius and thus retards migration. The retention time (V_t) of epimerase in Protein Pak 300 SW SE-HPLC column equilibrated in 0–6 M urea is shown in Fig. 1A. It shows a dis-

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Abbreviations: Epimerase, UDP-galactose 4-epimerase (EC 5.1.3.2.); UDP-gal, UDP galactose; UDP-glu, UDP glucose; SE-HPLC, size-exclusion HPLC; ANS, 1-anilino 8-naphthalene sulphonic acid; PG, phenyl glyoxal; CD, circular dichroism

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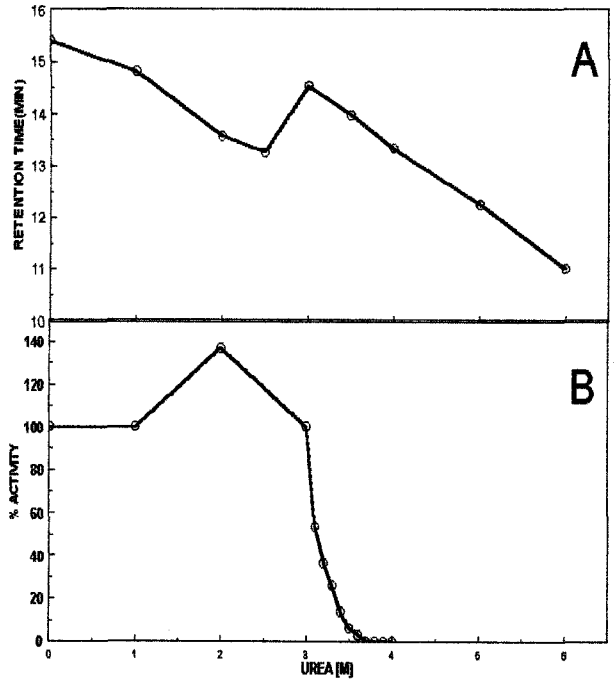

Fig. 1. (A) Dependence of retention time of epimerase in a Waters Protein Pak 300SW SE-HPLC column equilibrated in 0–6 M urea. Protein samples and the column were equilibrated at the respective denaturant concentration. (B) Residual catalytical activity of epimerase equilibrated in the same denaturant concentrations, as estimated by two-step assay.

continuous pattern. The V_t of the enzyme shows a gradual drop with increase of urea concentration between 0 and 2.5 M as well as between 3 and 6 M. However, between 2.5 and 3 M urea, there is an increase of V_t in spite of further unfolding of the molecule as evidenced in the CD spectroscopy. CD data of epimerase in 2.5 and 3 M urea indicate retention of 51 and 42% molar ellipticity at 222 nm (results not shown). Therefore, the discontinuity can be only explained by the dissociation of subunits. Identical discontinuous pattern of V_t was obtained using a LKB Ultropak TSK G3000SW SE-HPLC column. A similar phenomenon of disproportional expansion of the molecule due to dissociation of subunits has been observed in the case of complete unfolding of the enzyme [5].

Enzyme activity of epimerase equilibrated in 0–6 M urea is shown in Fig. 1B. It shows that, though partial unfolding at 3 M urea occurs, the enzyme is fully active in the two-step assay. To rule out the remote possibility that an apoenzyme structure is formed at 3 M urea, and that the reassociation of the co-factor is facilitated by the substrate during assay, epimerase equilibrated at 3 M urea is passed through the SE-HPLC column to remove the co-factor, if any. The eluted protein shows full activity, indicating an original holoenzyme structure. Expression of full activity with 72% recovery of secondary structure has also been observed in the reversible folding of this molecule [5]. However, initial rate of conversion of UDP-gal to UDP-glu could not be measured accurately as the reaction was complete by 1 min in each case. It was neither a case of substrate depletion nor was the enzyme destabilized at the very high dilution. Addition of BSA as an inert carrier did not alter the reaction rate.

Though these experiments suggest the existence of catalytic subunits, the state described above is partially unfolded. Therefore, dissociation under native conditions was sought for. Multimeric proteins tend to dissociate spontaneously under low salt buffers, e.g. as low as 1 mM [1], and also in a very dilute solution [6]. But, under these two conditions, there are a very limited number of methods by which the quaternary structure of a protein can be determined. Analytical ultracentrifugation and detection of the protein profile by biological activity gives a suitable solution. Epimerase at concentrations of 10–60 μg/ml, when incubated in 1 mM potassium phosphate buffer pH 8.5 for 20 h at 25°C and subjected to ultracentrifugation, migrated slower than the native dimer. A comparison of the molecular mass with marker proteins reveals that the dimeric protein is ≈80 kDa and t..hat of the slower-migrating protein is 40 kDa (Fig. 2, and inset). This species can also perform normal catalysis in the two-step assay. The combination of HPLC and ultracentrifugation studies strongly indicates functional monomers.

Influence of ionic strength of the media and protein concentration required for dissociation were also investigated. 1–35 μg of epimerase in 100 μl potassium phosphate buffer pH 8.5 was incubated for 20 h at 25°C and was subjected to ultracentrifugation where it was eventually distributed in ≈2 ml of the gradient. The relationship of the percentage of monomers to the protein concentration was as follows:

Table 1
Physical properties of epimerase in dimeric and monomeric states

Characteristics	Dimeric state	Monomeric state
Activity	100%	90%[a]
$CD[\theta]_{222nm}$	100%	95%[a]
Protein fluorescence (ex 280 nm)		
λ_{max} em	338 nm	337 nm
Fluorescence intensity[b]	100%	150%
Interaction with ANS (65 μM) (ex 375 nm)		
λ_{max} em	471.2 nm	494.6 nm
Fluorescence intensity[b]	100%	62.3%
Quenching by 5′-UMP (10 mM)	67%	45%
PG-modified protein		
Interaction with ANS (ex 375 nm)		
λ_{max} em	514 nm	514 nm
Fluorescence intensity[b]	15% of native	15% of native
Quenching by 5′-UMP	7.5% of native	7.5% of native
pH stability	Stable at pH 5.5–10	Same as dimer
Thermal stability	Starts inactivating at 40°C	Same as dimer

[a]5–10% inactivation and structural perturbations occurred when native epimerase was incubated at 25°C for 20 h.
[b]Fluorescence intensity at λ_{max} em.

350 μg/ml – 0%; 210 μg/ml – 20%; 150 μg/ml – 70%; 60–10 μg/ml – 100%. To check the salt dependency, sucrose gradients were made in 50, 20, 5 and 1 mM potassium phosphate buffers pH 8.5, a fixed concentration of 60 μg/ml of epimerase was layered on each and subjected to ultracentrifugation. There was no appreciable change in the dissociation pattern. Low protein concentration was found to be essential for dissociation, even then we performed most of the experiments in 1 mM salt to demonstrate the stability of the protein.

Various physico–chemical properties of the functional monomer which could be measured (in its stabilizing conditions) are summarised in Table 1. There is hardly any change of secondary structure, as evidenced from the far UV CD (200–250 nm) (Fig. 3), but certain changes of tertiary structure are observed from the protein aromatic amino-acid fluorescence. Moreover, both the dimeric and monomeric proteins are found to be stable at pH 5.5–10.0. Rate of inactivation of both species at pH 4.0 and at temperatures of 45–65°C is identical.

1-ANS, an extrinsic fluorophore, reacts with the dimeric epimerase, resulting in an enhancement of fluorescence intensity and a blue shift of the fluorescence emission. This fluorescence is quenched by 5′-UMP, a competitive inhibitor [7], hence the interaction is considered as substrate-binding site directed. In the monomeric molecule, 5′-UMP (10 mM) could quench the ANS (65 μM) intensity only upto 45% as compared to 67% of the dimeric protein under identical conditions. Thus, one may expect the generation of a new interacting site of ANS arising out of dissociation, presumably at the subunit contact region which is insensitive to 5′-UMP replacement. To verify this, epimerase was treated with phenylglyoxal (PG) which modifies an arginine residue at the active site, leading to inactivation and failure to interact with ANS [8]. The PG-modified protein showed ≈15% residual activity and an equivalent proportion of bound ANS intensity.

This intensity could be further proportionally quenched by 5′-UMP. The PG-modified monomeric protein showed identical behaviour with ANS and 5′-UMP, thus suggesting generation of no new ANS-binding site. Dissociation possibly induces a conformational change of insignificant amplitude at the substrate-binding site because of which the inhibitor could not replace ANS as efficiently as the native dimer.

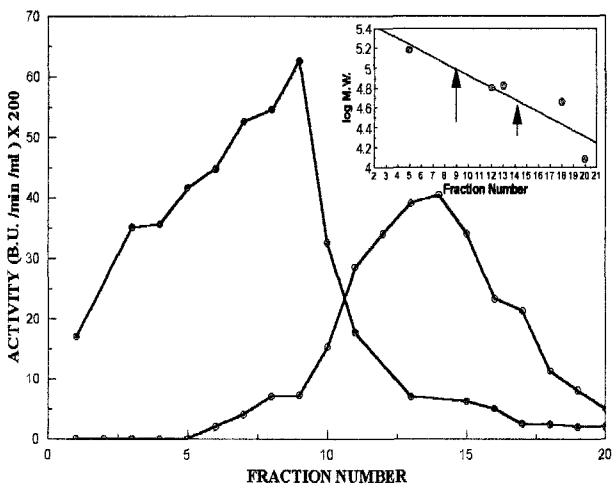

Fig. 2. 5–20% sucrose gradient ultracentrifugation pattern of 0.35 mg/ml of native epimerase in 20 mM potassium phosphate buffer pH 8.5 (●) and 0.06 mg/ml of incubated epimerase in 1 mM potassium phosphate buffer pH 8.5 (○). Estimation of protein was by coupled assay method. Activity shown here represents a 5-fold diluted sample of the native epimerase. Migration of the sample was from fraction numbers 20 to 1. (inset). Dependency of log (M.W.) vs. migration of marker proteins. Position of peak top of activity of the two epimerase samples are marked by arrows.

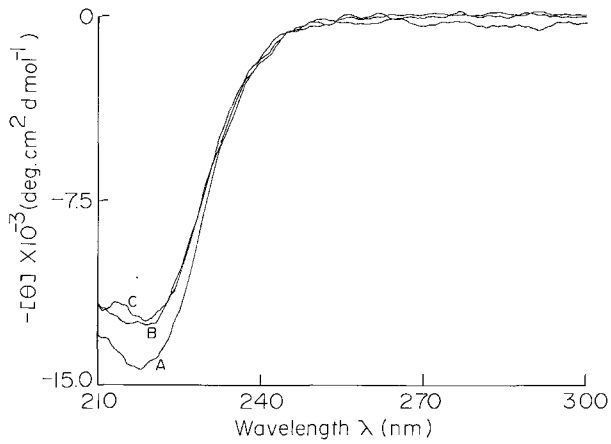

Fig. 3. Far UV circular dichroism spectra of epimerase under different conditions. (A) Native protein in 20 mM potassium phosphate buffer pH 7.0, (B) native protein after incubation at 20°C for 20 h and (C) same sample after incubation in 1 mM potassium phosphate buffer pH 7.0 under identical conditions. Protein concentration was 60 μg/ml.

It is not clear whether the hydrophobic interactions at the subunit contact region contribute mostly to the stabilization of the dimeric structure. X-ray crystallographic analysis shows several ionic interactions (at least 7), numerous hydrogen bonding and also hydrophobic interactions at the contact region. Whatever may be the origin of stability of the dimer, the monomer also has stable structure and can function catalytically. This is in sharp contrast to the epimerase from yeast *Kluyveromyces fragilis* that is also a NAD-bound dimeric holoenzyme. In this case, dissociation always leads to inactivation and extended chemical modifications suggest that the active site is at the interface of the subunits [1,9,10].

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