UDP-galactose 4-epimerase from *Kluyveromyces fragilis*: existence of subunit independent functional site☆

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Abstract UDP-galactose 4-epimerase from *Kluyveromyces fragilis* is a stable homodimer of 75 kDa/subunit with non-covalently bound NAD acting as cofactor. Partial proteolysis with trypsin in the presence of 5′-UMP, a strong competitive inhibitor, led to a degraded product which was purified. Results from SDS–PAGE, size-exclusion (SE)-HPLC and ultracentrifugation indicated its monomeric status and size between 43 and 45 kDa. 'Two-step assay' with UDP-glucose dehydrogenase as coupling enzyme in the presence of NAD ensured epimerase activity of the monomer. The possibility of transient dimerization of monomeric epimerase during catalysis was excluded by SE-HPLC in the presence of excess substrate and NAD. This truncated enzyme retained catalytic site related properties like K_m for UDP-galactose, 'NADH-like coenzyme fluorescence' and 'reductive inhibition' similar to its dimeric counterpart. Reversible reactivation of the monomer was achieved up to 95% within 3 min from 8 M urea induced unfolded state, indicating that the catalytic site could form independent of its quaternary structure. Equilibrium unfolding between 0 and 8 M urea indicated that the monomer was less stable compared to the dimer. Chemical modification of amino acids and reconstitution with etheno-NAD suggested that the architecture around the catalytic site of the monomer was conserved. Specific modification reagents further confirmed that the cysteine residues required for catalysis and coenzyme fluorophore reside exclusively on a single subunit negating a 'subunit sharing model' of its catalytic site.

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Abbreviations: ANS, 1-anilino 8-naphthalene sulfonic acid; BAL, Na-arsenite; CHD, 1,2-cyclohexanedione; DEPC, diethyl pyrocarbonate; DTT, dithiothretol; diamide, diazene dicarboxylic acid bis (*N,N*-dimethylamide); G–G, glysine–glysine; 2-ME, 2-mercaptoethanol; MMTS, methyl methane thiosulfonate; NEM, *N*-ethyl maleimide; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NBS, *N*-bromo succinimide; PM, pyrene maleimide; PMSF, phenylmethylsulfonyl fluoride; *p*-CMB, *p*-chloromercuribenzoate; STI, soyabean trypsin inhibitor; V_0, void volume; E_{act}, energy of activation; epimerase or epi-D, UDP-galactose 4-epimerase (E.C. 5.1.3.2) where D stands for homodimeric enzyme of 75 kDa/subunit; epi-M, proteolysed functional monomeric epimerase of 43–45 kDa; UDP-glc DH, UDP-glucose dehydrogenase (E.C. 1.1.1.22)

Keywords: UDP-galactose 4-epimerase; Proteolysis; Monomer; Catalysis; *Kluyveromyces fragilis*

1. Introduction

UDP-galactose 4-epimerase (hereafter called epimerase), an obligatory enzyme of galactose metabolism, reversibly converts UDP-galactose (UDP-gal) to UDP-glucose (UDP-glc). Unlike classical dehydrogenases which use NAD as co-substrate (class I oxidoreductases), epimerases act as a model for a rare class of enzymes that requires non-covalently bound NAD as cofactor (class II oxidoreductases) [1,2]. Studies on homodimeric epimerase from *Kluyveromyces fragilis* and *Escherichia coli* are most abundant. Bacterial epimerase was the first whose X-ray crystallographic structure was solved at high resolution. It demonstrated that the dimer contained two NAD binding sites away from the subunit contact region in a symmetry-oriented manner [3,4]. Catalysis by monomers of *E. coli* epimerase provided evidence for binding of NAD to its subunits and its functionality [5]. Mammalian epimerase from calf liver is dimeric [6]. The crystallographic structure of human epimerase shows dimeric structure with NAD binding motif in each subunit [7]. Therefore, dimerization might be a requirement for the stability of these molecules.

Acknowledging that crystallographic structure of *K. fragilis* epimerase is still pending, the architecture of its catalytic site has been mapped using extensive chemical modification reactions [8]. The proposed site constitutes amino acids from both subunits and includes the assumption that stoichiometry of bound NAD is 1/dimer [1,2,8]. Validity of the 'subunit-sharing model' could not be assessed because no experiment could be designed to dissociate dimeric epimerase prior to inactivation. Recent investigation on this enzyme has revealed that the stoichiometry of bound NAD is more inclined to 2/dimer [9]. Further amino acid sequence analysis of several model class I and class II oxidoreductases collectively revealed that each subunit of these enzymes contains a complete NAD binding motif of GXGXXG, popularly known as 'Rossman fold' [10]. Also most of these enzymes can bind one NAD/subunit [9]. Another important feature of this enzyme is its bifunctionality. While its N-terminal domain functions as epimerase, the C-terminal domain is associated with mutarotase explaining its almost double size relative to the *E. coli* enzyme [11]. While

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separating these domains by partial proteolysis, a monomeric fragment was isolated that maintained epimerase activity. Here, we report extensive characterization of the enzyme.

2. Materials and methods

2.1. Reagents

Fine chemicals were procured as follows: ANS, BAL, CHD, DEPC, DTT, diamide, G-G, MMTS, NEM, NBD-Cl, NAD, NBS, PM, PMSF, *p*-CMB, STI, 5′-UMP, UDP-gal, and UDP-glc from Sigma; DTNB from Pierce; trypsin from SISCO Research Laboratory, Bombay; yeast nitrogen base, peptone, yeast extract, malt yeast extract and agar from HiMedia, Bombay. UDP-glc DH was partially purified from beef liver up to heat denaturation step [12]. To remove trace amount of associated epimerase, the preparation was left under storage condition at pH 5.5 and at −20 °C for 1–3 weeks, whereby the unwanted activity was spontaneously destroyed. Application of 50-fold excess of dehydrogenase to UDP-gal, as compared to the conditions of 'coupled assay', could not detect epimerase activity (also discussed later).

2.2. Purification of epimerase (epi-D)

Yeast strain *K. fragilis* (renamed as *Kluyveromyces marxianus* var. *marxianus*, ATCC No. 10022) was purchased from Microbial Type Collection Center and Gene Bank, IMTECH, Chandigarh, India. Growth of cells, purification of epi-D (UDP-galactose 4-epimerase (E.C. 5.1.3.2) where D stands for homodimeric enzyme of 75 kDa/subunit), demonstration of purity and specific activity achieved have been described recently [11].

2.3. Preparation of monomeric epimerase (epi-M)

Purified epi-D (1 mg/ml) was treated with trypsin (50:1, wt/wt) in 20 mM K-phosphate, pH 8.0, at 4 °C for 4 h in presence of 2.5 mM of 5′-UMP. Residual trypsin was inactivated by the addition of 2-fold molar excess of STI or 5 mM of PMSF. The digest was passed through Waters Protein Pak 125 SE-HPLC column equilibrated with 20 mM Na-phosphate, pH 7.5, at a flow rate of 0.5 ml/min. Elution of proteins was followed at 280 nm. The single major peak eluted corresponding to retention time, $R_t = 9.40 \pm 0.05$ min, was collected [11]. Repeated application of the digest increased the yield. Alternately, 100 μl of the digest was passed through Sephadex G-50 'spin column' to remove excess 5′-UMP, PMSF and generated small peptides [13,14]. Time course of the digestion as followed by SDS–PAGE showed that the rest of the molecule was degraded to undetectable small peptides without accumulation of stable large fragments. The fractions from HPLC or spin column were used without further purification. The preparation was devoid of proteolytic activity as measured with azo-albumin as substrate, detection limit being 0.1% of the amount of trypsin applied originally [15]. Enzyme was stored in 20 mM K-phosphate, pH 8.0 containing 0.5 M NaCl, at −20 °C. Recovery of proteolysed functional monomeric epimerase of 43–45 kDa (epi-M) from 'spin column' was 95 and 60% on the basis of activity and mass, respectively, with respect to epi-D.

2.4. Assay of epimerase

Coupled assay. Unless mentioned otherwise, the activity was measured by coupled assay where continuous conversion of UDP-gal to UDP-glc was monitored in presence of the NAD dependent coupling enzyme UDP-glc DH at 340 nm [16]. In short, 22.5 mM UDP-gal, 12 mM NAD, 5 U of UDP-glc DH, 500 μl of 0.2 M glysine–glysine (G–G) buffer, pH 8.8, and approximately 450 μl of water were added to the cuvette. After incubation for 5 min, 5–50 μl of epimerase was added to initiate the conversion so that in each case final volume of the assay mixture was 1 ml. Such incubation was necessary to remove initial enhancement of catalysis arising from ~0.03% UDP-glc contamination in UDP-gal [17]. One unit of enzyme has been defined as the amount that converted 1 μmol of UDP-gal to UDP-glc per min under standard assay conditions at 25 °C. Calibration curve from 'coupled assay' relating epimerase concentration (2–12 nM) and rate of catalysis was linear. The extrapolated line passed essentially through the origin providing an additional evidence of the absence of epimerase activity in the coupling enzyme.

The following points were ensured for the determination of energy of activation (E_{act}) by 'coupled assay'. None of the enzymes, epi-M, epi-D or UDP-glc DH, was thermally inactivated between the temperature zones studied under assay conditions. Rate of the conversions was considered from the linear propagation of the reaction. The 'coupled assay mixture' contains sufficient excess of coupling enzyme so that it never entered into the rate-limiting step in the expression of epimerase activity; for example, a 4-fold enhancement of epimerase activity was supplemented with 20-fold excess of UDP-glc DH activity. Since coupling enzyme was also activated for rise of temperature, the actual margin was even more.

Two-step assay. It was employed for epimerase to be assayed under any desired condition where the substrate and products are stable. In short, conversion of UDP-gal to UDP-glc by epimerase was continued for a pre-determined period. Then, epimerase was inactivated by vortexing with equal volume of chloroform at 25 °C for 2 min followed by incubation at 85 °C for 15 min. The reaction products were stored in ice. UDP-glc formed was estimated as usual by UDP-glc DH and NAD [13]. Care was taken in all cases that the carried over reagents did not interfere with dehydrogenase assay. Since accumulation of product (UDP-glc) for 15 min was estimated in 'two-step assay', it was separately ensured that the coupling enzyme did not contribute any significant absorption. For a typical experiment, absorbance changes from epi-M and two controls containing coupling enzyme or buffer were 0.450 ± 0.003 and ± 0.005 at 340 nm.

2.5. Electrophoresis

PAGE (7.5%) at pH 8.8 and SDS–PAGE (15%) were performed using standard protocols. The following M_w markers (Sigma) were used in SDS–PAGE: carbonic anhydrase (29 kDa), chicken egg albumin (ovalbumin) (45 kDa), BSA (66 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa) and myosin (205 kDa). A linear dependence of log M_w versus band migration was observed. Transverse urea gradient gel electrophoresis between 0 and 8 M urea supplemented with an inverse gradient of 15–11% acrylamide was done at pH 7 to follow unfolding of epi-M at equilibrium [18,19]. Duration of electrophoresis was 8 h at 28 °C.

2.6. SE (size exclusion)-HPLC

A Protein Pak 125 column was applied as described earlier. It was precalibrated with the following markers: alcohol dehydrogenase (150 kDa) (for determination of V_0), BSA (66 kDa), ovalbumin (43 kDa), trypsin (22 kDa), myoglobin (19 kDa) and cytochrome *C* (12.5 kDa). A linear dependence of log M_w versus elution volume (or retention time) was observed. 50 μg of protein was applied per run. Elution was followed at 280 nm.

2.7. Ultracentrifugation

Sucrose (4–20%) density gradient in 20 mM Na-phosphate, pH 7.5, was prepared in 30-ml swing bucket SW-50 tubes of Beckmann Model 50 ultracentrifuge. Samples of 100 μl were applied per tube for a run of 16 h at $100\,000 \times g$ at 4 °C. After centrifugation, 1-ml fractions were collected from the bottom of the tube by a peristaltic pump. The following marker proteins were applied in parallel runs: alcohol dehydrogenase (Baker's yeast, 150 kDa), BSA (66 kDa), hemoglobin (64 kDa), ovalbumin (43 kDa), chicken egg lysozyme (14.5 kDa) and cytochrome C (12 kDa) [5]. Distribution of marker proteins, epi-D or epi-M, was followed by $A_{280\,nm}$ and/or 'two-step assay' as applicable.

2.8. Folding studies

Epi-M (0.5 mg/ml) was incubated with 8 M urea in the presence of 20 mM K-phosphate, pH 7.5, containing 2 mM 2-mercaptoethanol (2-ME) for 10 min at 25 °C. Refolding/reactivation was initiated after 20-fold dilution of the denaturant with the same buffer containing 1 mM of extraneous NAD. These conditions were identical to reversible refolding of epi-D from 8 M urea induced unfolded state [20–22]. Equilibrium unfolded states were generated after incubation of 0.1 mg/ml of epi-M with 0–8 M urea in 20 mM K-phosphate, pH 7.5, containing 2 mM 2-ME for 16 h at 25 °C. The equilibrium-unfolded states were characterized in terms of activity using 'two-step assay', protein fluorescence and coenzyme fluorescence. Transition profiles were drawn using Tanford's relation [23] to generate transition midpoints, $(D_{1/2})_{app}$; cooperativity of transition, $-m_{app}$ and free energy change, ΔG^0_{app}.

2.9. Chemical modifications

Amino acids of epi-M were modified with specific reagents under conditions as were applied to epi-D. These include modifications of arginine by CHD [24], histidine by DEPC [25] and tryptophan by NBS [26]. Cysteine residues were modified by a variety of reagents like BAL, DTNB, MMTS, NBD-Cl, NEM, PM [8], diamide [27] and *p*-CMB [28]. In cases where reagents were initially dissolved in organic solvent, it was ensured that neither the carried over solvent nor the reagent had any inhibitory effect on the coupling enzyme. Coenzyme fluorescence of the modified enzyme was considered taking care of emissions from reagents, if any.

2.10. Spectroscopic methods

Optical measurements were done with a recording spectrophotometer (Analytic Jena Specord 200, Germany) attached to a thermostat water bath (Polyscience, USA). Fluorescence measurements were done with a Hitachi F 4500 recording spectrofluorimeter using a 3-ml quartz cuvette having excitation and emission slit widths of 2.5 nm each. Far UV-CD measurements (200–250 nm) were done on a J 720 (Jasco) spectropolarimeter.

2.11. Other methods

Reconstitution of the enzyme (epi-M) with etheno-NAD was performed after unfolding of the enzyme with 8 M urea followed by 20-fold dilution of the denaturant with buffer containing 1 mM of extraneous etheno-NAD [20,29].

The following buffers were used for testing pH stability of enzyme: 20 mM Na-acetate, pH 4.0–7.0; 20 mM Na-phosphate, pH 7.0–8.0; and 20 mM Tris–HCl, pH 8.0–11.0. Low salt buffer was referred to 1 mM Na-phosphate, pH 7.5. Gel electrophoresis was performed with a mini gel apparatus (Genei, Bangalore, India) and a Hoefer Power supply (model PS 3000), whereby appearance of diffuse bands of epimerase in PAGE was prevented [13]. This was possibly by using thinner gels and glass plates and modified apparatus to facilitate dissipation of heat during electrophoresis. Gels were stained with Coomassie Blue RC-250.

3. Results and discussion

3.1. Homogeneity and molecular weight of epi-M

Taking advantage of resistance against trypsinization upon binding of 5′-UMP, a strong competitive inhibitor of epimerase [30], a truncated form of the enzyme (epi-M) was isolated. epi-M (10 μg) so obtained demonstrated a single band in 7.5% PAGE at pH 8.8. Diffused nature of the band with higher amount of enzyme (30 μg) was a general property of *K. fragilis* epimerase [16] (Fig. 1A). A 15% SDS–PAGE also demonstrated a single band having identical migration with ovalbumin and, thus, M_w of 45 kDa was attributed to epi-M (Fig. 1B). Overloading of any gel and staining with silver nitrate instead of Coomassie Blue did not reveal further bands. A 0–8 M transverse urea gradient gel electrophoresis showed a single band throughout the electrogram. It demonstrated that even under strongly denaturing condition, no adhering protein or proteolyzed fragment could be detected (Fig. 1C). In Protein Pak 125 SE-HPLC column (fractionation range 5–80 kDa), epi-M was eluted as a single symmetrical peak of $R_t = 9.40 \pm 0.05$ min. This corresponded to M_w of 45 kDa relative to the calibration curve (Fig. 1D, and inset). No undigested or fragmented products were detected in the chromatogram. The dimeric protein under similar conditions was eluted at $R_t = 5.88 \pm 0.05$ min that corresponded to the void volume (V_0). Rechromatography of epi-M recovered from HPLC after storage for one month at 4 °C showed an identical retention time.

The distribution profiles of epi-M and epi-D in ultracentrifuge under defined conditions were monitored by protein estimation and epimerase assay. With respect to a calibration

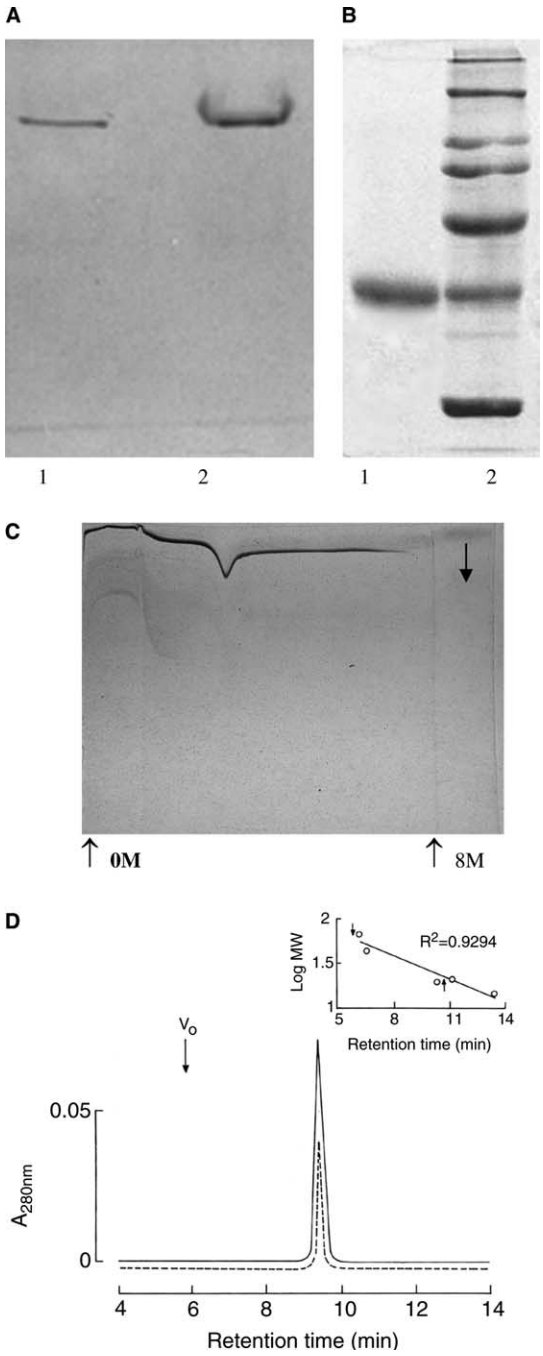

Fig. 1. (A) 7.5% PAGE, pH 8.8, of 10 and 30 μg of epi-M (lanes 1 and 2, respectively). (B) 15% SDS–PAGE of 20 μg of epi-M (lane 1) and 20 μg of marker proteins (lane 2). (C) 0–8 M transverse urea gradient gel profile at pH 8.8 of epi-M. (↓) and (↑) indicated direction of protein migration and urea concentrations, respectively. 250 μg of epi-M was applied. (D) Protein-Pak 125 SE-HPLC profile of epi-M. Chromatographic conditions have been described in the text. The solid and dotted lines represent the chromatogram of epi-M (50 and 25 μg) in 20mM Na-phosphate, pH 7.0, in the absence and presence of 50 μM of UDP-gal and NAD, respectively. (Inset) Calibration curve showing dependence of log M_w versus retention time. Positions of void volume (V_0), molecular weight markers and epi-M have been indicated by (○), (↓) and (↑), respectively. R^2 is regression coefficient.

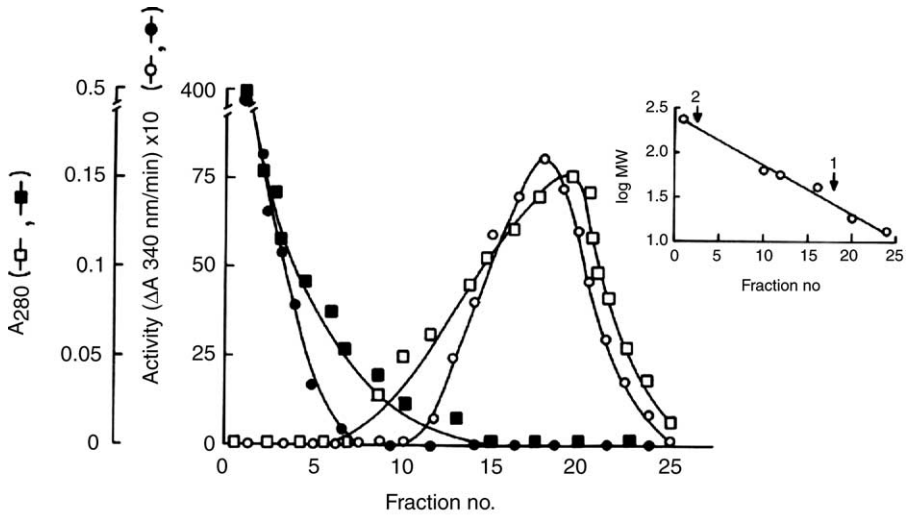

Fig. 2. Ultracentrifugal patterns of epi-M (0.8 mg) and epi-D (1 mg). Experimental conditions have been described in the text. The solid and open symbols represent epi-D and epi-M, respectively. The asymmetric nature of distribution of epi-D is due to accumulation of the molecules at the bottom of the tube. (Inset) Calibration curve of the ultracentrifugal run showing dependence of log M_w versus fraction number. The open circles, 1 and 2 represent position of marker proteins, epi-M and epi-D, respectively.

curve, the M_w of these two species were found to be 43 and 135 kDa (Fig. 2, and inset). In case of epi-M, no component of high molecular weight showing epimerase activity was detected. These results collectively ensure homogeneity, size between 43 and 45 kDa and monomeric state of epi-M.

3.2. Catalytic properties

Time dependent formation of UDP-glc from 0.22 mM of UDP-gal by 66 and 165 nM of epi-M has been confirmed by 'two-step assay'. Under these conditions, the reactions were essentially irreversible because at most 10% of the substrate was converted. The reactions followed first order kinetics having $k = 0.090 \pm 0.011$ and 0.144 ± 0.025 min^{-1}, respectively. The variation between the ratio of reaction rates and enzyme concentrations (1:1.6 versus 1:2.5) was possibly due to incomplete inactivation of epimerase by chloroform treatment during termination of the reactions. To ensure that epi-M did not form a transient dimer during catalysis, SE-HPLC was done as stated earlier where the column was equilibrated with 20 mM Na-phosphate, pH 7.5, containing 50 mM each of UDP-glc and NAD (substrate and cofactor for epimerase) as were present in the assay mixture. The chromatogram indicated the absence of multimerization of the enzyme (Fig. 1D, dotted line).

For convenience and accuracy, the rest of the activity measurements were done by coupled assay. Epi-M was prepared in the presence of 5′-UMP and the excess ligand was subsequently removed. In a control experiment, it was confirmed that the carried over enzyme bound stoichiometric 5′-UMP was ineffective in altering catalytic parameters of the enzyme. Epi-M demonstrated standard Michaelis–Menten relation with respect to UDP-gal. Corresponding Lineweaver–Burk plot yielded a K_m of 0.10 mM. This was comparable to 0.13 mM for epi-D [1,16,20]. Specific activity of epi-M was found to be 115 as compared to 60–70 for epi-D. These corresponded to turnover numbers of 10 360 and 9100 for the two enzymes.

3.3. Physical properties

Spectroscopic parameters. Comparative fluorescence emission (ex: 280 nm, em: 300–500 nm) of 66 nM of epi-D and epi-M showed em_{max} at 335.5 and 335.4 nm, respectively, with 42% reduction of emission intensity for the latter. Assuming uniform distribution of aromatic amino acids along the polypeptide chain, this was apparently due to 40% reduction of mass upon proteolysis. Circular dichroism spectra of epi-M in the far UV-zone showed maximum molar ellipticity at 208 nm typical of a globular protein containing significant amount of α-helix (Fig. 3A). Analysis of Chen and Yang [19] yielded 27.1% of α-helix for epi-M, which was 23.4% for epi-D. Thus, a substantial portion of the secondary structure was retained after proteolysis.

Coenzyme fluorescence. Epi-D offers a characteristic NADH like emission (ex: 353 nm; em: 450 nm) possibly from Cys-SH...NAD weak interaction [1,2,8]. This 'coenzyme fluorescence' is considered as a sensitive index for retention of finer integrity of the catalytic site and had been widely used as a probe to monitor perturbation of that site [8,20,21,24–27]. A functional enzyme devoid of coenzyme fluorescence, called 'dark enzyme', is also known. Epi-M showed similar coenzyme fluorescence (em_{max}: 450.5 nm). Emission intensity was 95% compared to an equimolar concentration of epi-D, indicating retention of integrity of the catalytic site (Fig. 3B).

Reductive inhibition. Another characteristic property of this enzyme is reduction of surface bound NAD to NADH by a reducing sugar like L-arabinose (100 mM) in the presence of 5′-UMP (1.25 mM) at pH 7.5, with concomitant inactivation and gain of NADH fluorescence [30]. This is also a probe to monitor its catalytic site [8,24–27]. When subjected to such reduction at an enzyme concentration of 66 nM, epi-M was completely inactivated similar to epi-D; the rate constants being 2.13×10^{-2} and 1.41×10^{-2} min^{-1}, respectively, at 25 °C.

Interaction with ANS. 1-ANS is an extrinsic fluorophore that anchors on the hydrophobic patches of biomolecules resulting

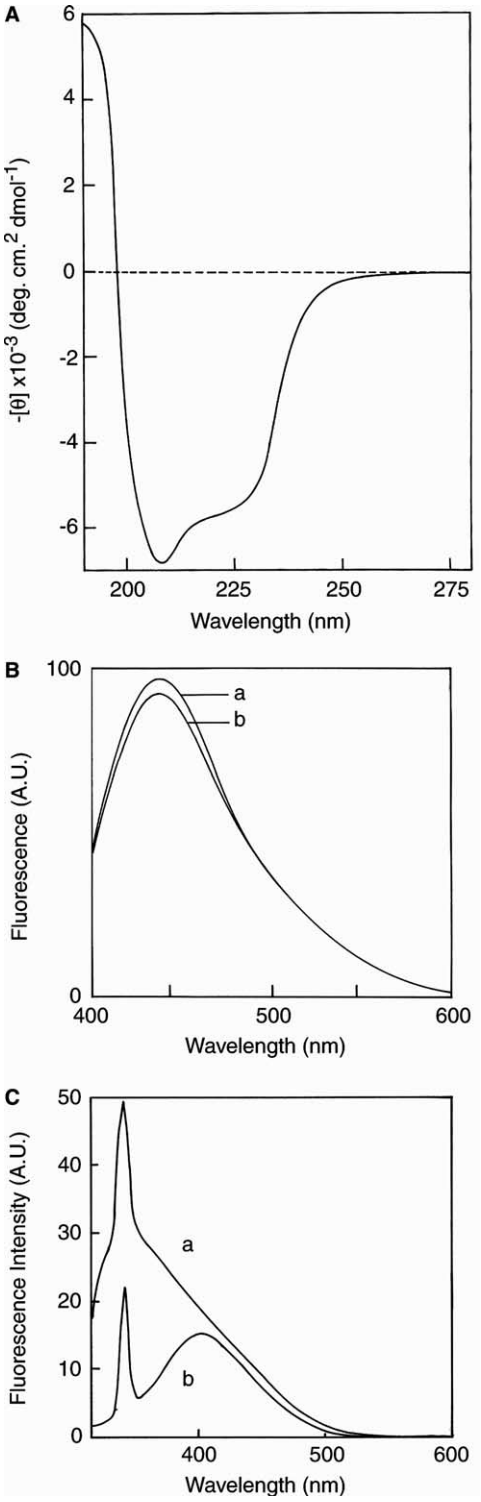

Fig. 3. (A) Far-UV CD of 33 nM of epi-M between 190 and 280 nm in the presence of 20 mM K-phosphate, pH 7.5. Data were presented after solvent correction ($n = 5$). (B) NADH-like coenzyme fluorescence of 132 nM of epi-D (a) and epi-M (b) after excitation at 353 nm. (C) Emission spectra of epi-M reconstituted with etheno-NAD (a). Emission from an equimolar concentration of free etheno-NAD has been presented in (b).

in maximum 100-fold enhancement of fluorescence intensity together with blue shift of emission (ex: 375 nm; em: 520 → 480 nm). ANS (65 μM) offers such emission properties after its interaction with epi-D (em_{max} : 471.0 nm) [31]. Under defined conditions as stated earlier, epi-M interacts with ANS showing enhancement of emission intensity (em_{max} : 471.6 nm) that was 70% compared to epi-D (result not shown). This indicated surface hydrophobic character of epi-M.

Reconstitution with etheno-NAD. Etheno-NAD, a non-functional structural analog of NAD, has been used as a fluorescence probe to monitor the hydrophobic milieu of the nucleotide binding site of dehydrogenases, including epi-D [29]. epi-M was reconstituted with etheno-NAD after reversible folding by 8 M urea [20]. It showed more than 2-fold enhancement of fluorescence emission and also changes of emission pattern over a control of equimolar concentration of the free fluorophore (ex: 305 nm; em: 315–500 nm) (Fig. 3C). This interaction was similar to epi-D, indicating that the microenvironment around NAD binding site of epi-M was largely retained as par epi-D.

E_{act}. 'Arrhenius plots' from the rates of conversion of UDP-gal to UDP-glc by epi-D and epi-M were constructed by altering coupled assay temperature between 25–45 and 18–40 °C, respectively. Derived E_{act} were 22.8 ± 1.8 and 15.5 ± 1.5 kcal/mol, respectively. The lower E_{act} for epi-M is an indication towards stabilization of the transition state arising from truncation of the parent molecule.

3.4. Reversible folding

While epi-D could be reversibly refolded and reactivated to between 80% and 90% after denaturation by 8 M urea [20–22], it was of interest to verify whether epi-M could reactivate efficiently under identical conditions. It was ensured that under the stated denaturing conditions, $[\theta]_{222\,nm}$ of the monomer was reduced to 15% indicating almost complete loss of its secondary structure. Refolding by dilution with buffer in the presence of 1 mM extraneous NAD led to 95% reactivation by 3 min. The rate of active enzyme formation was 0.362 μmole min^{-1} at 25 °C (Fig. 4A). The refolded state was not stable; activity was retained for 5–10 min followed by inactivation within 30 min. Under similar conditions, refolded dimeric state was stable for about 2 h [20].

Equilibrium unfolding of epi-D and -M between 0 and 8 M urea at pH 7.0 showed different profiles in transverse gradient gel electrophoresis. Migration of epi-D was linear without appearance of any inflection point [32]. The process involved both denaturation and dissociation of subunits and as a result the migration remained unaltered [33]. epi-M, however, showed reproducible inflection point at 3.3 M urea though the migration of the native and unfolded states was similar (Fig. 1C). No multiple unfolded states, presumably arising from proline *cis–trans* isomerization, were observed. The inflection point corresponded to the transition mid-point of protein fluorescence (stated below).

The equilibrium unfolded states of epi-M during unfolding by 8 M urea were characterized in terms of inactivation, loss or quenching of coenzyme and protein fluorescence. Transition profiles were constructed and ΔG^0 of these processes were derived from the extrapolated linear relation of ΔG versus molar urea concentration (Fig. 4B). Other parameters available from this relation were $(D_{1/2})_{app}$ (transition mid-point) and $-m_{app}$ (cooperativity index); corresponding values for epi-M and epi-D have been provided in Table 1. Transition midpoints of inactivation and loss of coenzyme fluorescence of

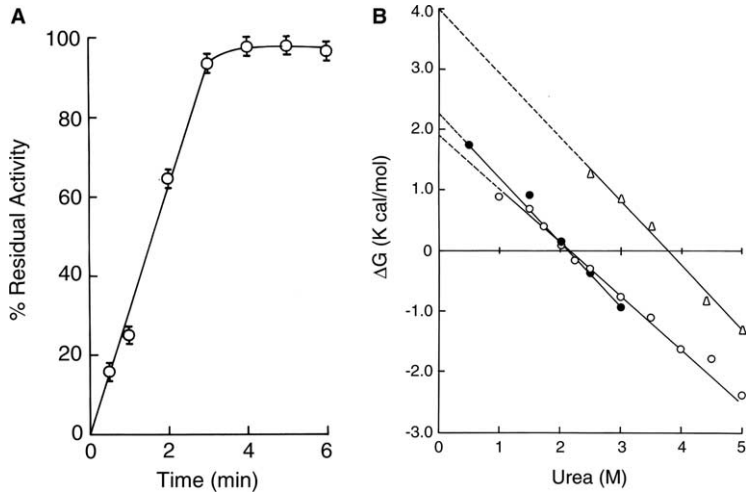

Fig. 4. (A) Reactivation kinetics of epi-M (final concentration 66 nM) after denaturation by 8 M urea at pH 7.5 for 10 min at 25 °C. The bars represent variation of four experiments. (B) Equilibrium unfolding profiles of epi-M between 0 and 8 M urea at pH 7.5 constructed with the following parameters, (●) residual catalytic activity; (○) 'coenzyme fluorescence' and (△) protein fluorescence.

Table 1
Physical properties of dimeric epimerase (epi-D) and monomeric epimerase (epi-M)

Properties	epi-D	epi-M
Activity	100%	90%
Sp. activity	60	115
K_m (for UDP-gal, in mM)	0.13	0.10
Protein fluorescence (ex: 280 nm)		
λ_{max}em	336.0 nm	335.4 nm
Fluorescence intensity	100%	58%
Coenzyme fluorescence (ex: 353 nm)		
λ_{max}em	436.1 nm	436.5 nm
Fluorescence intensity	100%	95%
Interaction with ANS (65 μM) (ex: 375 nm)		
λ_{max}em	471.2 nm	471.6 nm
Fluorescence intensity	100%	72%
Thermal stability (residual activity at 45 °C after 40 min incubation)	70%	0%
Equilibrium unfolding parameters ($D_{1/2}, -m, \Delta G^0$)		
Inactivation	1.3, 5.67, −8.8	2.1, 0.94, −1.98
Coenzyme fluorescence	2.5, 1.9, −4.6	2.2, 1.02, −2.25
Protein fluorescence	n.r.	3.8, 1.32, −5.0
Urea gradient gel electrophoresis		
Inflection point (M urea)	Linear profile	3.3
α-Helix content	23.4%	27. 1%
E_{act} (kcal/mol)	22.8 ± 1.8	15.5 ± 1.5

n.r., not reported.

epi-M and epi-D were comparable between the ranges of 1.3 and 2.5 M urea. However, cooperativity index of the monomer was between 0.94 and 1.02 as compared to 1.9 and 5.67 for the dimer. This suggested lesser stability of the catalytic site of the former. This instability was also reflected in the respective ΔG^0_{app} (−1.9 and −2.2 for epi-M versus −8.8 and −4.6 for epi-D). The transition midpoint of tertiary structure disruption for epi-M at 3.8 M urea was comparable to the midpoint of transition of urea gradient gel electrophoresis at 3.3 M. A respective G^0_{app} of −5.0 for this transition was within the range as observed from unfolding of small single domain proteins [23]. Physical properties of epi-M and -D have been compared in Table 1.

3.5. Chemical modifications

Arginine, histidine and tryptophan residues were identified previously at the catalytic site of epi-D by group specific reagents. Reactions were done with these reagents for epi-M under conditions at par epi-D (23–25). Inactivation to the extent of 85–90% occurred in cases of CHD and DEPC in a time dependent manner similar to epi-D (Table 2). Inactivation by DEPC was reversed after treatment with hydroxylamine, HCl indicating that the inactivation was caused by histidine and not tyrosine residue/s [34]. NBS, however, failed to inactivate epi-M in contrast to epi-D. Thus, the role of tryptophan at the catalytic site of epimerase remains speculative at this moment. Of particular interest is the role of cysteine residues.

Table 2
Chemical modification of amino acids other than cysteine residues

Reagents	Amino acid modified	% Residual acitivity	
		epi-D	epi-M
Diethyl pyrocarbonate (DEPC)	Histidine	5	0
Cyclohexanedione (CHD)	Arginine	2	20
N-Bromosuccinimide (NBS)	Tryptophan	5	95

Two such residues were identified for epi-D, one responsible for catalysis and the other for maintenance of coenzyme fluorescence. These were believed to be contributing from different subunits; – an outcome of 'subunit sharing model'. The distinction was based on sensitivity towards cysteine modification reagents like DTNB, *o*-iodosobenzoic acid, MMTS, NBD-Cl, NEM, PM, BAL, *p*-CMB and diamide and reversibility after reduction with DTT [8]. Results of these modification reactions, including reversibility by DTT on epi-M and -D, have been summarized in Table 3. It shows that the overall effects were similar, indicating retention of the architecture of the catalytic site. The results of fluorescence emission after DTNB, NEM and diamide modifications have been shown in Fig. 5. While the effects of DTNB and NEM were comparable, difference was observed for diamide where coenzyme fluorescence was reversibly gained after treatment with DTT only for epi-M.

Table 3
Chemical modification of cysteine residues

Reagents	% Residual coenzyme fluorescence		% Residual activity	
	epi-D	epi-M	epi-D	epi-M
DTNB	5	17	15	0
MMTS	n.r.	100	10	15
NEM	5	6	5	0
PM	n.r.	100	20	55
BAL	n.r.	100	18	20
p-CMB	5	100	0	0
Diamide	0	4	0	0

n.r. – not reported.

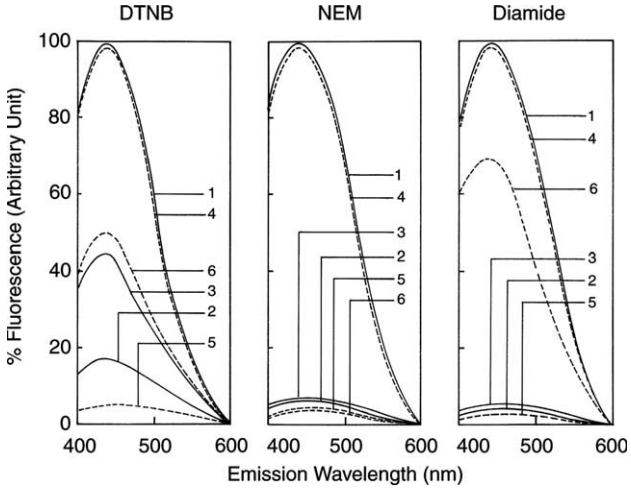

Fig. 5. Effect of cysteine modification on coenzyme fluorescence of epimerase. (Left panel) DTNB, (middle panel) NEM and (right panel) diamide. In all sets, 1, 2 and 3 represent emissions from epi-D, after modification with respective reagents and after reduction with DTT, respectively. Emissions from epi-M under identical conditions have been presented by 4, 5 and 6, respectively. Protein concentration in all sets was 66 nM.

3.6. Stability

Under normal storage conditions, epi-M became inactive within a week, while epi-D retained 80% of its activity after a month. Incubation at 45 °C for 3 h at 0.5 mg/ml in 20 mM Na-phosphate, pH 7.5, showed that the residual activities for epi-M and -D were 0 and 60%, respectively. Even at 25 °C, under similar conditions epi-M lost 40% of its activity. Incubation of epi-M between pH 4.0 and 9.0 at 25 °C for 4 h followed by 'coupled assay' showed that the residual activities at the extreme pH's were 8% and 24%, respectively, with respect to a control serving as 100% at pH 7.0. epi-D was inactivated after incubation in low salt buffer presumably after dissociation [35]. Epi-M also became inactivated in that buffer at a faster rate. General instability of epi-M was indicated from its unfolding studies.

4. Conclusion

Over the years, considerable efforts were made to generate functional monomer of *K. fragiils* epimerase to resolve essentiality of dimeric structure for catalysis. These included partial unfolding with urea [21], modification with *p*-CMB [27] and incubation with low salt buffer [35]. Unfortunately, in all cases inactivation preceded dissociation. Partial proteolysis with trypsin as described, however, led to epi-M, – a functional monomer with retention of all major structural and catalytic parameters. Thus, the age-old notion of 'subunit-sharing model' does not hold anymore. In retrospect, we tried to assess on what basis the sharing model was proposed. A crucial experiment was the cross linking between two cysteine residues, assumed to be one from each subunit, by diamide with concomitant inactivation and loss of coenzyme fluorescence [8,26]. While explaining the phenomenon, the possibility of cross-linking between non-functional cysteine residues followed by alteration of the enzymatic and physical properties was not considered. Such a possibility cannot be ruled out after estimation of cysteine residues. Moreover, prediction of modification of 2–4 cysteine residues from a total number of 32 residues/dimer by the method of subtraction also remained questionable.

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