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Research Article

A conformationally isoformic thermophilic protein with high kinetic unfolding barriers

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Abstract. The basis for the stability of thermophilic proteins is of fundamental interest for extremophile biology. We investigated the folding and unfolding processes of the homotetrameric *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH). TBADH subunits were 4.8 kcal/mol less stable towards guanidinium chloride (GdmCl) unfolding compared to urea, indicating ionic modulation of TBADH stability. Strongly denaturing conditions promoted mono-exponential unfolding kinetics with linear dependence on denaturant concentration. Here TBADH unfolded >40-fold slower when extrapolated from urea as compared to GdmCl unfolding. A marked unfolding hysteresis was shown when comparing refolding and unfolding in urea. An unusual biphasic unfolding trajectory with an exceptionally slow phase at intermediate concentrations of GdmCl and urea was also observed. We advocate that TBADH forms two distinctly different tetrameric isoforms, and likely an ensemble of native states. This unusual supramolecular folding behavior has been shown responsible for formation of amyloidotic yeast prion strains and can have functional importance for TBADH.

Keywords. Chemical denaturant, *Thermoanaerobacter brockii* alcohol dehydrogenase, kinetic stability, ionic interactions, protein unfolding, conformational isoforms.

Introduction

Thermophilic and hyperthermophilic organisms grow optimally above 60 °C and 80 °C, respectively, where most of the mesophilic proteins are susceptible to denaturation. These organisms use identical amino acid residues in their proteins as their mesophile counterparts, nevertheless they differ in their thermal stability and have adapted their folded state to resist high temperature. Understanding the extraordinary thermal stability of proteins from these organisms is of great interest because it can provide the basis for understanding alternate routes to attain protein stability [1–3]. Apart from that, there are many applications of thermophilic and hyperthermophilic proteins in biotechnological and pharmaceutical industries [4, 5]. Therefore, it is of interest to know whether amino acid sequence, non-covalent interactions, or both play vital roles in maintaining the structural and functional integrity of thermophilic proteins [6]. Comparative studies on mesophilic and thermophilic proteins have not come to a point where

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one single factor could be recognized to be responsible for the higher stability of thermophilic proteins. Our current understanding suggests that a number of factors including ionic networks, increased number of proline residues, packing efficiency through van der Waal's interactions, loop stabilization and oligomerization are involved in higher thermostability [1, 7–9]. So far, most of the effort has been devoted to comparisons of amino acid sequences and available structural analyses. The debate is still open as to whether thermodynamic stability of thermophilic proteins is higher than of mesophilic proteins, or not. It is difficult to obtain the thermodynamic parameters for the stability of thermophilic proteins due to their irreversible nature of unfolding, which restricts the comparison with mesophilic proteins [10]. In addition, we have very limited understanding about the kinetics of unfolding of thermophilic proteins. Although a large number of proline residues and charge clusters indicate a hypothetical high kinetic barrier in the unfolding of thermophilic proteins, very few studies have been reported on this subject. Kinetic studies can provide vital information that is not possible to obtain by sequence comparisons and structural studies, as the differences in the stability should be reflected in the rates of unfolding and refolding [11, 12].

Alcohol dehydrogenases belong to an industrially highly important and interesting class of enzymes because of their use in the production of alcohols and ketones. From an evolutionary point of view this group of proteins provides ideas about the evolution of a catalytic and structural fold [13]. *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH) is an NADP-dependent alcohol dehydrogenase that reversibly catalyzes oxidation-reduction of mainly secondary alcohols into their corresponding ketones. It has been shown that TBADH is specific towards small aliphatic ketones, has a high enantioselectivity [14] and also a high tolerance towards organic solvents [15]. The crystal structure of TBADH reveals a homotetrameric structure (Fig. 1A) in which each subunit is comprised of 352 amino acid residues with a molecular mass of 37.6 kDa [16, 17]. Each subunit has one Zn^{2+} ion present in the catalytic site, while eukaryotic ADH has both one structural and one catalytic Zn^{2+} [18]. TBADH does not contain any disulfide bonds.

The structural information and implicated importance of strategically placed structural determinants obtained from comparative studies of mesophilic *Clostridium beijerinckii* alcohol dehydrogenase (CBADH) [16, 17, 19–21] inspired us to study the unfolding properties of TBADH. We took advantage of the electrostatic nature of GdmCl, and the non-charged denaturant urea. TBADH was found to be surpris-

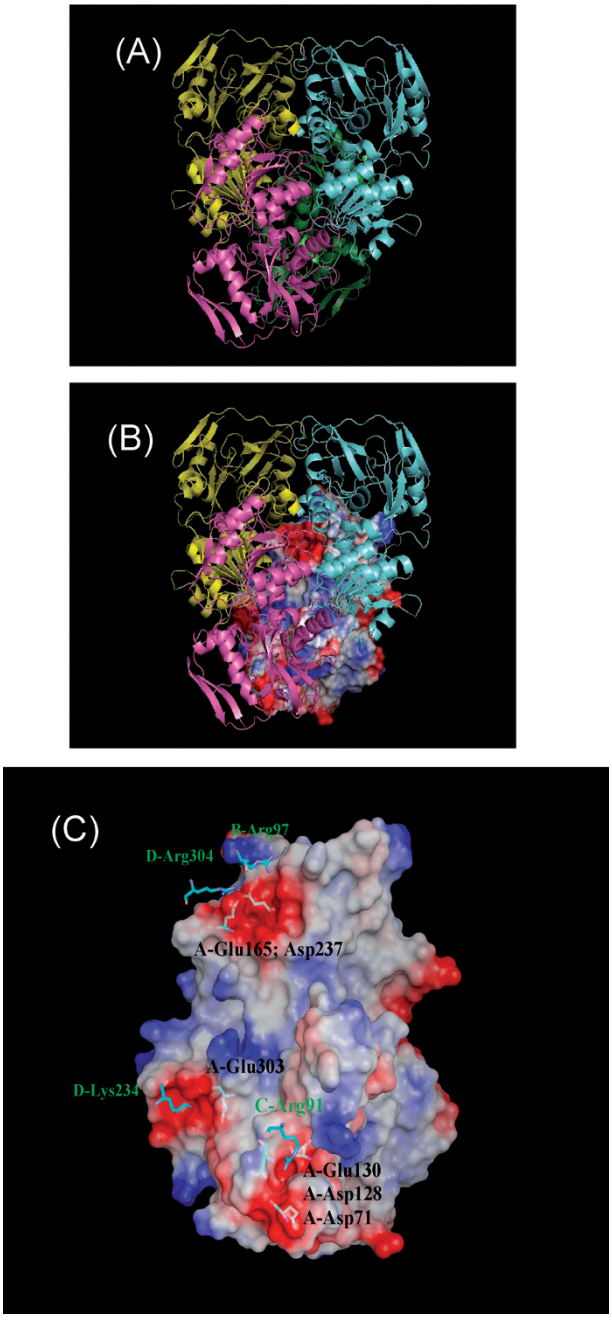

Figure 1. Structure of *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH). (*A*) The overall structure of TBADH with the four domains colored as follows: *A* (green); *B* (cyan); *C* (magenta) and *D* (yellow). (*B*) The overall structure of domains *B*–*C* in relation to the electrostatic potential surface of domain *A*. (*C*) The electrostatic potential surface of domain *A* and the corresponding charged residues of domain *B*–*D* forming the intermolecular salt bridges that were found with WHAT IF (http://swift.cmbi.kun.nl/WIWWWI/). Note that all domains form salt bridges with each separate domain and there are two extensive salt bridge networks in between each domain interface. Figures were generated with PyMOL [39] using the structural data from PDB id 1YKF [16].

ingly sensitive towards GdmCl denaturation, while it was exceptionally resistant against urea denaturation. The stability of TBADH in urea originates from a large kinetic barrier towards unfolding, which likely arises from strategically placed ionic interactions, charge clusters and high abundance of proline residues.

Materials and methods

Chemicals. TBADH (EC 1.1.1.2, Lot 024K4063) and $NADP^+$ (Lot 081K7033) from Sigma-Aldrich (MO, USA). GdmCl and urea from MP Biomedicals (OH, USA), glutaraldehyde (Sigma-Aldrich) and $NaBH_4$ were procured from Aldrich.

Purification of commercial TBADH. Commercial TBADH was purified according to a procedure adapted from Peretz et al. [22]. Samples were loaded onto a Red Sepharose CL-6B XK 26/40 column (Pharmacia), pre-equilibrated with buffer (0.1 M NaCl, 0.1 mM EDTA, 0.1 mM DTT, 25 mM Tris-HCl, pH 7.3), connected to an ÄKTA FPLC system. TBADH was eluted using a linear gradient of 600 ml NaCl (0.1–0.8 M) and fractions containing TBADH were pooled and run through a size exclusion Superose 12 HR 10/30 column (Pharmacia), flow 0.1 ml/min with the buffer described above. TBADH fractions were concentrated by ultrafiltration with a Vivaspin concentrator (Vivascience), 10 000 MW cut-off at 2300 *g*, 4°C, 40 min. TBADH purity was confirmed using NuPAGE 4–12 % Bis-Tris pre-cast gel kit with MES buffer and Coomassie staining and SeeBlue plus2 pre-stained protein standard (Invitrogen Life Technologies). Further purity was confirmed by matrix-assisted laser deionization-time of flight mass spectrometry (MALDI-TOF-MS) (Perspective Biosystems Voyager–DE STR). TBADH concentrations were determined by measuring the absorbance at 280 nm. The extinction coefficient ($\varepsilon_{280} = 30\,940\ M^{-1}\,cm^{-1}$) was calculated as described by Gill and von Hippel [23] and the specific activity of the purified enzyme was 55 units/mg at 40 °C.

Activity assay. TBADH activity was determined using an assay adapted from Kleifeld et al. [24]. All solutions were incubated at 40 °C. Solutions of TBADH (10 mM phosphate, pH 7.5) were added to the assay mixture comprised of 2-butanol (150 mM), Tris-HCl (100 mM, pH 9.0), $NADP^+$ (0.5 mM) to afford a final enzyme concentration of 35 nM and a total sample volume of 0.80 ml. Samples were gently shaken and the absorption increase at 340 nm [ε_{340} (NADPH) = 6.2 $mM^{-1}\,cm^{-1}$] was immediately monitored (40 °C), without preincubation. Activity measurements were also performed with the presence of various concentrations of GdmCl, urea and NaCl in the assay buffer as described above. One unit of TBADH activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol NADPH/min at the initial velocity at the conditions described above.

Denaturation and renaturation. For equilibrium and kinetic denaturation measurements, 30 μg/ml TBADH was incubated at 21 °C in 10 mM phosphate buffer, pH 7.5, containing various concentrations of GdmCl and urea. The concentrations of GdmCl and urea were checked by refractive index measurements. For the chemical cross-linking experiments, the TBADH concentration was 100 μg/ml. Samples were taken at specific time points for the analysis by fluorescence spectroscopy and chemical cross-linking. For equilibrium and kinetic renaturation in GdmCl, TBADH was first denatured in 4 M GdmCl and kept for 12 h before diluting it to various final GdmCl concentrations. Denatured sample were diluted by renaturation buffer (10 mM phosphate buffer, pH 7.5, containing 1 mM DTT) containing various concentration of GdmCl. For renaturation in urea, TBADH was denatured in 8 M urea containing 25 mM HCl (pH 3), for 48 h. Acidic pH was necessary for completely denaturing in 8 M urea. Denatured TBADH was diluted in renaturation buffer to a final concentration of 30 μg/ml TBADH. All the measurements in this study were performed at 21 °C and 30 μg/ml protein concentration unless specified in the figure legends.

The data were fitted to a separate unfolding transition for calculation of the Gibbs free energy of unfolding for the transitions from the native, N, to the unfolded, U, state, respectively (ΔG_{NU}). A linear dependence of the denaturant concentration of the Gibbs free energy of unfolding was assumed [25] according to the formula:

$$\Delta G_{NU} = \Delta G_{NU}{}^{H2O} - m_{NU}\,[\text{denaturant}] \qquad (1)$$

where ΔG^{H2O} denotes the Gibbs free energy of unfolding of the protein in the absence of denaturant and m denotes the slope of the dependence of the stability on the denaturant concentration that reflects the cooperativity of folding and is proportional to the exposure of solvent accessible surface area [26].

Fluorescence spectroscopy. All measurements were done on Spex-Fluoromax-2 at 21 °C. Samples were excited at 295 nm to monitor the tryptophan emission with a slit width of 5 nm for both excitation and emission. Spectra were recorded from 310 nm to

450 nm. The shift in wavelength maximum was used to monitor the conformational change at various denaturant concentrations. All the sample spectra were corrected for background fluorescence.

Chemical cross-linking and SDS-PAGE. Chemical cross-linking with glutaraldehyde was done essentially as described earlier [27]. For equilibrium denaturation experiments, 100 µl TBADH (100 µg/ml) denatured at different urea concentrations in 10 mM phosphate buffer, pH 7.5, were taken at different time points and cross linked by 10 µl glutaraldehyde (25 %) providing a final concentration of 2.5 % glutaraldehyde. After 5 min of reaction, cross-linking was quenched using 10 µl 1 % $NaBH_4$ (prepared in 0.1 M NaOH). Samples were mixed with SDS sample buffer, reduced by 2-mercaptopethanol and boiled for 5 min before loading on to 12 % SDS-PAGE gel. Protein bands were silver stained (Bio-Rad Silver Stain Plus) and quantified by ImageJ (http://rsb.info.nih.gov/ij) analysis. The equilibrium renaturation experiments were conducted as above though using a protein concentration of 44 µg/ml.

Size exclusion chromatography. Native TBADH was injected into a Sephacryl S-300 HR gel filtration column calibrated with high molecular weight standard (GE Healthcare) and was equilibrated with 50 mM phosphate buffer, pH 7.5 containing 100 mM NaCl at 4 °C, using an ÄKTA purification system.

Results

TBADH purity and assembly. Commercially procured TBADH was purified as described earlier [15] and the purity of the enzyme was determined by SDS-PAGE and MALDI-TOF-MS. MALDI-TOF-MS provided a dominant mass-to-charge ratio (*m/z*) at 38 007.4, which corresponds to the monomeric molecular mass of TBADH (theoretical mass 37 646.9 Da) (Fig. 2). The deviation (360.5 Da) in the obtained mass (*m/z*) from the theoretical mass of the monomer is likely due to imprecise determination of these high molecular weights by MALDI-TOF-MS (see more below).

MALDI mostly produces ions with charge +1, with some of +2 and +3 ions. Tetrameric TBADH is a protein of 150 kDa. Most of the ions produced by MALDI would have a charge of +1, giving an *m/z* of ~150 000 for the tetramer. Peaks corresponding to tetramer (*m/z* 152 931.3), trimer (*m/z* 114 614.4), +2 charged trimer (*m/z* 57 347.4), the dimer (*m/z* 76 108.0) and the +2 monomer (*m/z* 19 073.5) were obtained. The peaks show discrete species but all deviate from the corresponding theoretical mass. The deviation of the observed relative to the theoretical mass increases with the number of subunits, showing that the accuracy of the MALDI-TOF analysis decrease for large TBADH complexes (Fig. 2 inset). However, most importantly the MALDI-TOF spectrum shows TBADH of high purity with discrete peaks representing four different subunit species. The relative abundance from MALDI-TOF-MS should be taken with precaution because low mass species are more prone to vaporization than higher mass species, which leads to underestimation of the amount of, for example, tetramer. The mass spectrum showed that TBADH to a fair degree retained the tetrameric state even in the denaturing conditions used for MALDI-TOF-MS (70 % acetonitrile, 0.1 % TFA). Further the data suggested that TBADH displays symmetric stepwise dissociation of subunits under these conditions.

TBADH activity in denaturants. TBADH catalyzes the oxidation of 2-butanol. TBADH has previously been shown to be very resistant towards organic solvents [15]. We monitored the activity of TBADH in solution (35 nM enzyme in 10 mM phosphate, pH 7.5, 100 mM Tris-HCl, 150 mM 2-butanol; 40 °C) supplemented with urea and GdmCl to assess the resistance towards denaturants. TBADH activity was found to be exceptionally sensitive towards denaturants with C_{50} (concentrations at 50 % activity) values at 0.1 M for GdmCl and 0.3 M urea (Fig. 3A). In GdmCl merely 1 % of activity was retained in 0.75 M GdmCl, and in urea there was a retained activity of 14 % in 2 M urea. The extraordinary sensitivity *versus* the ionic denaturant GdmCl prompted us to investigate how the addition of NaCl affects the enzyme. Surprisingly the C_{50} value for NaCl was 0.2 M with just 6 % activity being retained at 0.75 M NaCl (Fig. 3A).

Equilibrium denaturation-renaturation transition. The structural stability of TBADH towards GdmCl and urea denaturation (in 10 mM phosphate, pH 7.5) was determined by monitoring the change in intrinsic Trp fluorescence. Intrinsic Trp wavelength shift was taken as a measure of the global conformational change at different denaturant concentrations, since each subunit of TBADH has four relatively well distributed Trp's in the structure. In these experiments, we expected TBADH to follow the model where denaturation was dependent on tetramer dissociation, followed by subunit unfolding, and the reverse reaction was initiated by subunit folding followed by tetramerization.

Figure 3B shows the equilibrium GdmCl denaturation-renaturation data. The midpoint concentration of

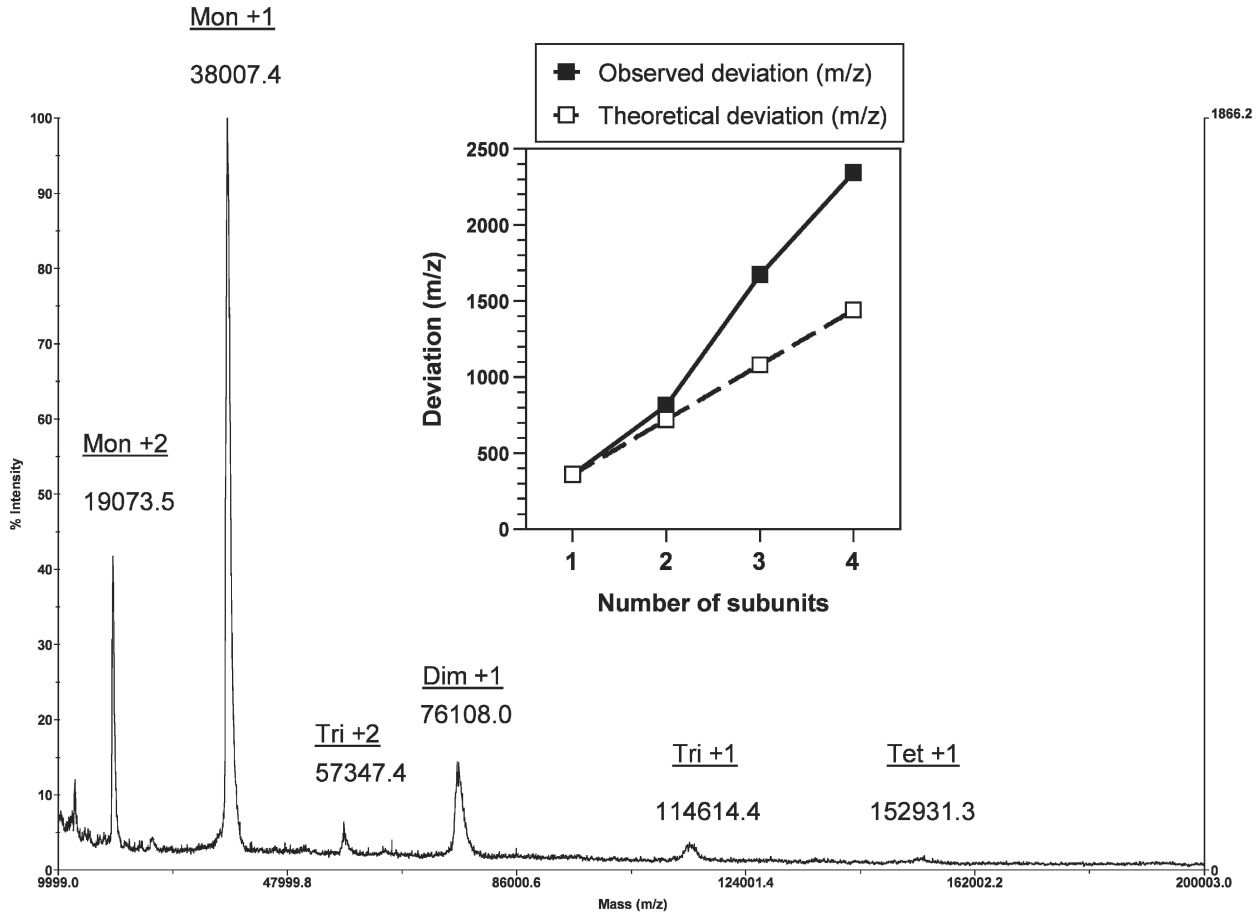

Figure 2. Purity and composition of TBADH by MS. MALDI-TOF-MS spectrum of TBADH (1 mg/ml) mixed 1:1 with in 70% acetonitrile, 0.1% TFA and 10 mg/ml sinapinic acid. The obtained *m/z* values are indicated in the spectrum with the identified species highlighted above each peak. Monomer (Mon), dimer (Dim), trimer (Tri) and tetramer (Tet) of TBADH assemblies of subunits were all identified in the mass spectrum. The inset shows the deviation from the theoretical mass increase (360 Da per subunit obtained from the monomer +1 peak), which is augmented with increasing number of subunits.

denaturation (C_m) after 1 day of incubation was 1.35 M and the equilibrium shifted towards lower concentrations with increased incubation time (1.2 M at 3 days, 1.1 M at 7 days, 1.05 M at 12 days and 1.0 M at 21 days). To examine the reversibility of the system, renaturation transition curves were obtained from completely denatured protein in 4.0 M GdmCl. Renaturation studies showed a cooperative transition with a C_m of 1.28 M after 1 day of incubation, with essentially complete reversibility of the denaturation process. The 1-day renaturation curve overlaps with the 3-day denaturation curve. Further incubation of the samples for 7 days leads to destabilization of TBADH ($C_m = 0.98$ M) showing that TBADH is very sensitive towards subdenaturing concentrations of GdmCl. This sensitivity towards GdmCl was also seen in the pretransition zone for denaturation at extended incubation times, which was obvious from the drifting base line (Fig. 3B). To evaluate the TBADH subunit stability the Gibbs free energy of unfolding in water ($\Delta G_{NU}{}^{H2O} = 2.5$ kcal/mol) was calculated using Eqn. (1) from the 1-day renaturation transition curve. This is a surprisingly low value for such a large protein and stands in marked contrast to the known thermostability of TBADH.

From the structural analysis, it was evident that ionic interactions play an important role in the stability of TBADH [17, 21]. Therefore, to understand the true effect of chemical denaturation, urea was employed because it is a noncharged chaotrope (Fig. 3C). Denaturation in urea showed an apparent C_m after 1 day of incubation of 7.6 M (Fig. 3C). We obtained a renaturation curve after denaturation of the protein for 2 days in 8 M urea (pH 3). Acidic pH was used during denaturation, because 8 M urea alone was not sufficient to denature the protein completely on a convenient time scale. Complete denaturation was confirmed by fluorescence spectra. Renaturation was achieved by dilution to varying final concentrations of urea with 10 mM phosphate buffer, pH 7.5. Compar-

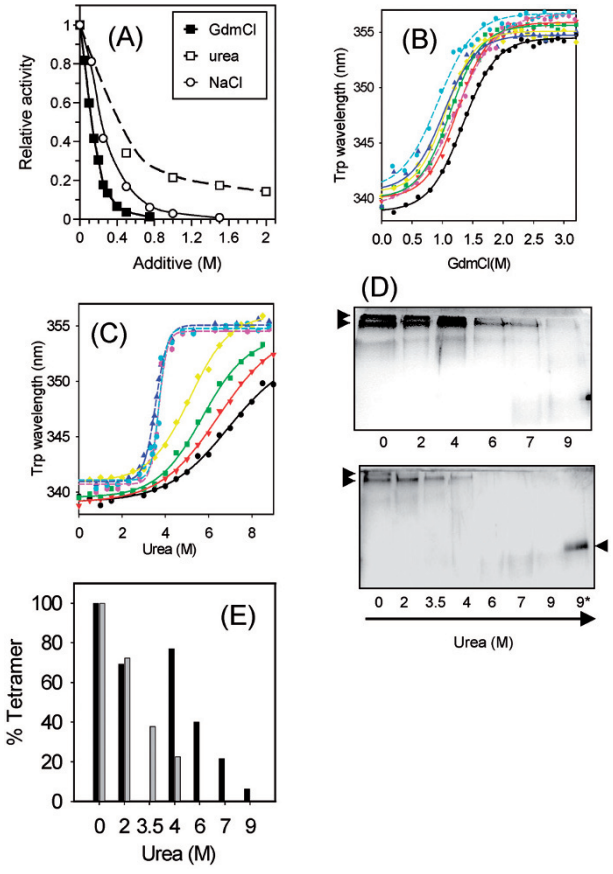

Figure 3. Apparent equilibrium unfolding and refolding transitions of TBADH in guanidinium chloride (GdmCl) and urea. (*A*) Enzyme activity of TBADH in the presence of various concentrations of GdmCl, urea and NaCl without preincubation. (*B*) GdmCl induced denaturation and renaturation transition curves: Denaturing transition curves were made by incubating TBADH in various concentrations of GdmCl for 1 day (black circle, solid line), 3 days (red inverted triangle, solid line), 7 days (green square, solid line), 12 days (yellow rectangle, solid line), 21 days (blue triangle, solid line). The renaturation reaction was carried out by denaturing TBADH in 4 M GdmCl for 24 h and diluting it in various final concentrations of GdmCl after 1 day (pink hexagonal, broken line) and 7 days (cyan circle, broken line). All the measurements were done at 21 °C and 30 μg/ml protein concentration. (*C*) Urea induced denaturation and renaturation transition curves: Denaturing transition curves were obtained by incubating TBADH in various concentrations of urea for 1 day (black circle, solid line), 3 days (red inverted triangle, solid line), 8 days (green square, solid line), 16 days (yellow rectangle, solid line). The renaturation reaction was carried out by denaturing TBADH in 8 M urea (pH 3) for 48 h and diluting it in various final concentrations of urea after 1 day (blue triangle, broken line), after 3 days (pink hexagonal, broken line) and 5 days (cyan circle, broken line). All the measurements were done at 21 °C and 30 μg/ml protein concentration. (*D*) Urea induced denaturation (upper) and reconstitution (lower) of TBADH monitored by chemical cross linking on SDS-PAGE gel (12 % acrylamide). Denaturation was done at 100 μg/ml, and renaturation at 44 μg/ml TBADH concentration. Arrows at the top indicate that the tetramers migrate as a double band and the bottom arrow indicate the presence of the monomer. The last lane of the lower gel (marked 9*) shows the denatured non-cross linked TBADH monomer denatured in 9 M urea. (*E*) Quantification of the tetramer observed on SDS-PAGE by chemically cross-linked TBADH. Amount of tetramer present during unfolding in various concentration of urea is shown by black bar and in reconstitution by gray bar.

ison of the denaturation ($C_m = 7.6$ M) and the renaturation transition ($C_m = 3.7$ M) curves stand in marked contrast, and revealed a significant hysteresis when comparing the 1-day transition curves (Fig. 3C). To evaluate the TBADH subunit stability for comparison with GdmCl renaturation, the Gibbs free energy of unfolding in water ($\Delta G_{NU}^{H2O} = 7.3$ kcal/mol) was calculated from the 1-day renaturation transition curve, and showed that TBADH subunit stability was 4.8 kcal/mol more stable towards urea denaturation than obtained with GdmCl.

The evident hysteresis indicates a large kinetic barrier for the unfolding reaction. Despite the known possible problems with urea forming isocyanate over time that can lead to carbamylation of free amines [28], we decided to continue to observe the denaturation process to establish whether the hysteresis effect had a kinetic origin. Evidently the unfolding of TBADH was very slowly shifted (over 16 days) towards the C_m from renaturation (3.7 M) (Fig. 3C), which supports the hypothesis that this system is kinetically stable. Of note, not even 8.5 M urea (pH 7.5) is sufficient to denature TBADH completely after 8 days.

The urea induced unfolding was also characterized by chemical cross-linking in the same buffer (10 mM phosphate, pH 7.5) as denaturation monitored by fluorescence. Chemical cross linking is a powerful tool to study denaturation and renaturation of oligomeric proteins [27]. Glutaraldehyde (final concentration 2.5 %) was used as the cross-linking agent because TBADH has 24 lysine residues in each subunit and glutaraldehyde efficiently cross-links amino groups through inter-subunit lysine side chains. TBADH was denatured for 1 day in various concentrations of urea, and as evident from the gel (Fig. 3D, upper), the intensity of the tetramer band decreases from 100 % at 0 M to 69 % at 2.0 M urea; to 77 % at 4.0 M urea; to 40 % at 6.0 M urea; to 21 % in 7.0 M urea; to 6 % at 9.0 M urea (Fig. 3E). The data are in fair agreement with the denaturation transition curve obtained from the fluorescence spectroscopic measurements, although cross-linking was done at a higher protein concentration (100 μg/ml compared to 30 μg/ml). The intensity of the monomer band could not be monitored because glutaraldehyde-modified TBADH monomers showed diffuse migrating bands. Cross-linking was also performed on the protein in renaturation experiments after 1 day (Fig. 3D, lower). To minimize the aggregation, renaturation experiments were also performed at a lower protein concentration (44 μg/ml) for chemical cross-linking. High urea concentrations (6.0–9.0 M) completely prevented refolding and reassembly of TBADH (Fig. 3E). At 4.0 M urea the amount of tetramer reassembly was 22 %, at 3.5 M urea 38 % tetramer was formed and at

2.0 M urea 72 % TBADH tetramer had reassembled. Again, results were in agreement with the renaturation transition curve as monitored by fluorescence spectroscopy measurements, suggesting that similar structural alterations are monitored by both methods.

Unfolding kinetics. To study the kinetic stability of TBADH in more detail, unfolding kinetics studies were conducted using both GdmCl and urea. The reaction was initiated by manual mixing of native protein with various GdmCl and urea concentrations. At different time points spectra were recorded and the wavelength shifts were monitored. As expected at higher GdmCl concentrations, TBADH unfolded more rapidly, while at lower concentrations it unfolded slower (Fig. 4A). The time course curve of the wavelength shifts can be fitted to a single exponential function from 2.6 M to 4 M GdmCl, while at 2.3 M and 2.0 M GdmCl the unfolding trajectory showed a slow second phase with a linear appearance. By extrapolation of the unfolding kinetics data from the first phase to 0 M GdmCl, a half time of 12 h was determined for TBADH unfolding (Fig. 4C). Unfolding kinetics in urea was carried out from 6.0 to 9.0 M of urea. Compared to GdmCl, TBADH unfolds extremely slowly even at 9.0 M urea (Fig. 4B). At higher urea concentrations (7.5–9.0 M) there is essentially only one kinetic phase, while at lower urea concentrations (6.0 and 6.5 M urea) the unfolding behavior is unusual showing an additional, apparently unrelated, very slow phase (Fig. 4B) with a linear appearance. This behavior is in striking resemblance to that found for the unfolding kinetics at 2.0 and 2.3 M GdmCl. Extrapolation of the unfolding rate constants of the first phase (7.5 – 9.0 M) to 0 M urea yields a half life of 20 days of unfolding in buffer for TBADH, which is 40-fold slower than that obtained from GdmCl.

Refolding kinetics. From the equilibrium data, it was evident that TBADH refolds much faster than it unfolds. To understand the hysteresis effect further, refolding kinetics was carried out by manual mixing into various concentrations of GdmCl and urea. As shown in Figure 5A and B, a substantial folding phase has already occurred during the dead time (45 s) of mixing and measurement. Chemical cross-linking experiments after 2 min of refolding showed that no tetramer assembly has occurred at this early time point (data not shown). This indicates that > 50 % of the fluorescence blue shift (from ~ 354 to ~ 346 nm) is due to monomer folding and the measured phase (~ 346 to ~ 340 nm) is likely an assembly phase. As expected, increasing denaturant concentrations of GdmCl (0.1 – 0.4 M, Fig. 5A) and urea (0.5 – 2.5 M, Fig. 5B) in the refolding buffer, leads to slower rates of

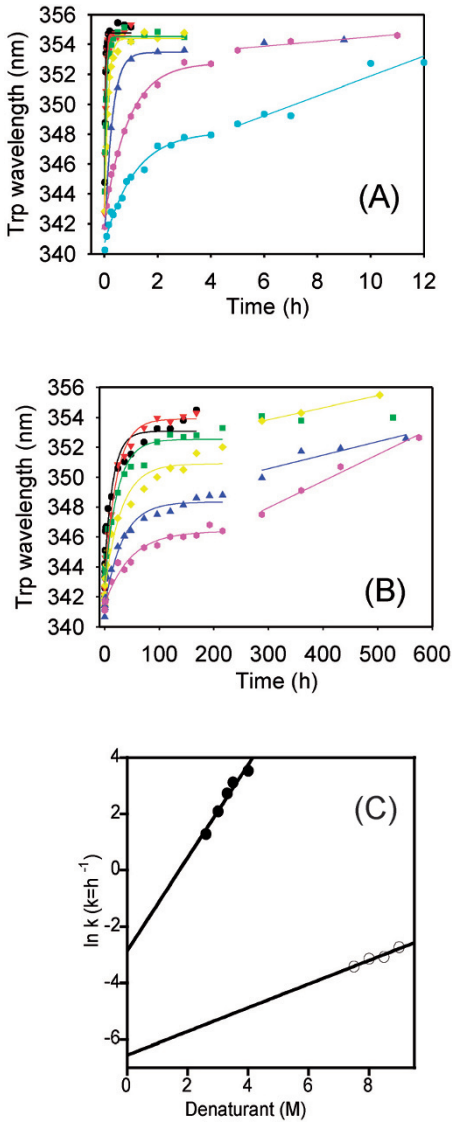

Figure 4. Unfolding kinetics of TBADH in GdmCl and urea. (*A*) The unfolding kinetics of TBADH in 4.0 M (black circle), 3.5 M (red inverted triangle), 3.3 M (green square), 3.0 M (yellow rectangle), 2.6 M (blue triangle), 2.3 M (pink, hexagonal) and in 2.0 M (cyan circle) GdmCl. The fitted solid line shows single exponential fit. The slow phase could not be fitted into an exponential function, but the data points reveal an additional distinct linear phase at lower concentrations of GdmCl. All the measurements were done at 21 °C and 30 µg/ml protein concentration. (*B*) Urea denatured unfolding kinetics was carried out in 9.0 M (black circle), 8.5 M (red inverted triangle), 8.0 M (green square), 7.5 M (yellow rectangle), 6.5 M (blue triangle) and in 6.0 M (pink, hexagonal) urea. The fitted solid line shows single exponential fit. The slow phase could not be fitted into an exponential function but the data points reveal an additional distinct linear phase at lower concentrations of urea. A line at 7.5 M urea is also shown where a minor fraction of the unfolding trace can be attributed to a slow phase. Note the different time scales in (*A*) and (*B*). (*C*) An assumed linear dependence of the natural logarithm of the unfolding rate constant (k in h^{-1}) of TBADH in various concentrations of GdmCl (closed circle) and urea (open circle). The rate constants were obtained from data fitted to single exponential function and extrapolated to 0 M denaturant concentration.

refolding. Despite a large difference in the unfolding rates induced by GdmCl and urea (compare Fig. 4A and B**),** refolding is fast in both denaturants. Extrapolating the refolding rate constants to 0 M leads to a half time of ~5 min for both denaturants (Fig. 5C). This fast rate of refolding corroborates the evidence of the presence of large kinetic barriers towards unfolding of TBADH.

Native TBADH is a mixture of isoformic tetramers. The observed double band of the cross-linked tetrameric TBADH in the 12% SDS-PAGE gel in the absence of denaturant indicated the possibility of the presence of more than one species of native protein (Fig. 3D). In addition, unusual biphasic unfolding kinetic profiles of TBADH at intermediate concentrations of GdmCl (2.0–2.3 M) and urea (6.0–6.5 M), were observed, which also indicates that TBADH comprises more than one native species. Here the second slow phase in the unfolding kinetics at 2.0–2.3 M GdmCl and 6.0–6.5 M urea reached a final Trp wavelength maximum of ~352–355 nm, representing essentially total protein denaturation with Trp completely exposed to the surrounding aqueous media after the very long incubation time. Hence, the second slow phase is not caused by, for example, aggregation or partial denaturation to a molten globule state, but instead represents the total denaturation reaction of TBADH, not kinetically accessible during the first phase. Taken together these indications initially led to an investigation of the quaternary composition of native TBADH. The protein was analyzed using another size exclusion chromatography (SEC) setup. Interestingly, on the Sephacryl S-300 column, there were two peaks in the chromatogram, with one species eluted at 85 ml and the second at 98 ml (Fig. 6A). This would correspond to a molecular mass of just 10–15 kDa for the two peaks. The molecular weight marker aldolase (158 kDa) is shown for reference (Fig. 6A). The uncharacteristic trace for TBADH was unexpected because previous SEC analysis of TBADH has been performed in 50 mM Tris-HCl, 100 mM NaCl in which TBADH migrated as a 160-kDa protein [29]. Hence, it was not possible to determine the molecular mass by our SEC setup because of severe retention on the column. This was most likely due to the large number of charge clusters on the surface of TBADH that are differently shielded in Tris as opposed to phosphate that we used in our SEC analysis. That TBADH is affected by Tris-buffer was evident from apparent stabilizing effects during GdmCl denaturation (Fig. 6C). Accordingly, separation of these species was performed at low ionic concentration (50 mM phosphate, pH 7.5, 0.1 M NaCl) to use conditions that were as native as possible

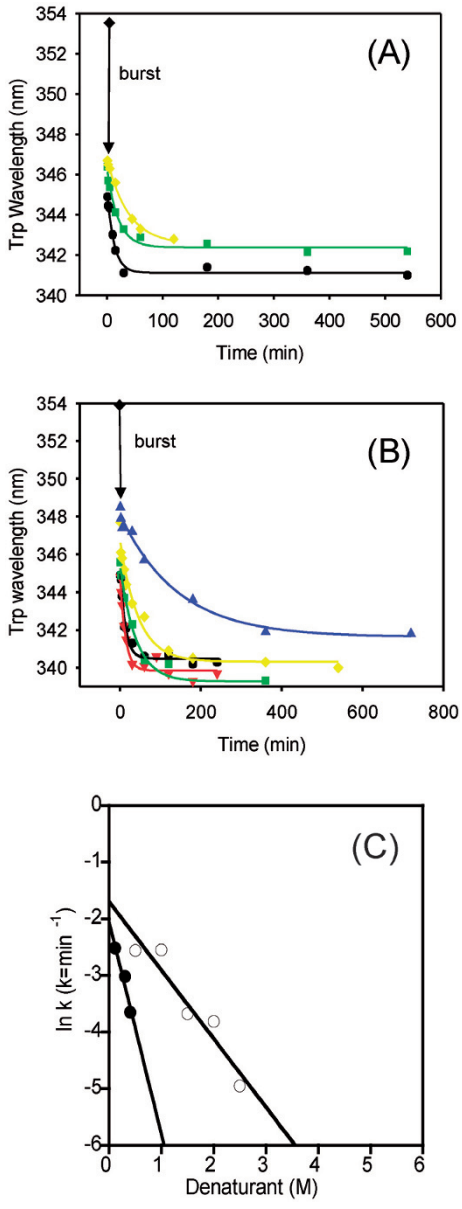

Figure 5. Refolding kinetics of TBADH from GdmCl and urea. Denatured TBADH was diluted by manual mixing with a dead time of 45 s. The arrows (noted burst phase) indicate the monomer folding phase within the dead time starting from 354 nm. (*A*) Refolding kinetics in the presence of 0.1 M (black), 0.3 M (green) and 0.4 M (yellow) GdmCl concentrations. (*B*) Refolding kinetics in the presence of 0.5 M (black), 1.0 M (red), 1.5 M (green), 2.0 M (yellow) and 2.5 M (blue) urea concentrations. (*C*) An assumed linear dependence of the natural logarithm of the folding rate constant (k in min^{-1}) concentrations of GdmCl (closed circle) and urea (open circle). The rate constants were obtained from data fitted to single exponential function and extrapolated to 0 M denaturant concentration.

considering the inactivating effect of NaCl on TBADH (Fig. 3A). Chemical cross-linking with glutaraldehyde was performed on the contents of the two peaks to investigate if these might migrate differently on an SDS-PAGE gel. The two species could be

resolved on a lower concentration of gel (7.5% acrylamide) (Fig. 6B), where both cross-linked TBADH tetramers move as >250-kDa proteins. A control experiment without cross-linking showed identical monomeric bands (37 kDa) on the gel, which indicated that each constituent subunit was identical in mass in both species (Fig. 6B), highly consistent with previous SDS-PAGE analysis of non-cross linked TBADH [29]. That both cross-linked TBADH isoforms migrate in SDS-PAGE as proteins larger than 250 kDa can originate from an elongated shape of the protein or charge clusters in the cross-linked protein. The shape of cross-linked TBADH denatured in SDS is not known, but the crystal structures of the tetramer reveal a crossed double dumbbell shape, with a "chest" width of 93 Å and a "waist" width of 51 Å (Fig. 6F). The two different isoforms might be cross-linked differently causing them to form two separate bands in SDS-PAGE. Further support for a non-covalent difference originates from the purified TBADH protein by MALDI-TOF-MS, which only showed one monomer mass peak (Fig. 2). In MALDI-TOF-MS spectra, tetrameric, trimeric and dimeric species were observed (Fig. 2). No higher molecular masses than those corresponding to the tetramer were detected (i.e., a pentamer is not present), although a hexameric structure cannot be entirely excluded, as the trimer carries a +2 charged hexamer. The data presented above are indicative of TBADH forming two tetrameric isoforms with the same subunit composition, but with different packing and exposure of charged residues, giving the isoforms different, and abnormal, migration times on the Sephacryl S-300 column and in SDS-PAGE after chemical modification of charged side chains. There are several reasons why we conclude that this corresponds to two distinct tetrameric isoforms: (1) TBADH was crystallized as a tetrameric protein in all reported structures [16, 17, 30]; (2) TBADH elutes as one peak from size exclusion Superose 12 HR10/30 column; (3) MS only revealed a tetrameric mass peak and symmetric smaller dissociation products, pealed off one subunit at a time; (4) both tetramers are enzymatically active, showing that tetramer 1 (eluting early) is not a misfolded species; (5) in retrospect, during refolding, significant concentrations of tetramer 1 (upper band) appears only at 0 M urea and not at 2.0 M urea (Fig. 3C, lower gel) – an indication of a less-stable state. Further, during unfolding tetramer 1, migrating as a larger species, is less stable. Larger oligomers are often more stable than smaller oligomers; and (6) despite the unusual migration behavior, both species migrate very closely on the SDS-PAGE gel, making a tetrameric and an octameric species unlikely, although a hexamer cannot be excluded. Differing charges (modified by glutaraldehyde) likely accounts for the different diffusion lengths. The same holds true for the gel filtration separation (Sephacryl S-300 HR) where tetramer 2 was severely retarded on the column.

The two separated isoforms were further characterized by measuring the unfolding kinetics in 6 M urea where the fast phase (0.28/h) of tetramer 1 was fourfold faster than that of tetramer 2 (0.069/h), showing that the most populated species (tetramer 2) is the most kinetically stable protein (Fig. 6D). To our surprise, however, both isoforms retained their unusual biphasic kinetic profiles, although with different amplitudes of the two phases. The retained biphasic kinetic profiles indicate that TBADH shows more biophysical diversity in its unfolding behavior that cannot be fully explained by the two isolated isoforms. In addition, a longevity study was also performed. The separated isoforms of TBADH were stored for 1 year in phosphate buffer (pH 7.5) at 4 °C. TBADH tetramer 2 retained 42% activity and tetramer 1 retained 20% activity even after this prolonged storage in buffer, compared to freshly purified enzyme. The samples were subjected to glutaraldehyde cross-linking and SDS-PAGE analysis, which demonstrated that the two species retained their respective isoformic state even after 1 year of storage at 4 °C in buffer. A small amount of tetramer 1 had formed in the sample of tetramer 2, and a small amount of tetramer 2 had formed in the tetramer 1 sample (compare gels in Fig. 6B and E).

Discussion

Thermophilic proteins are unusual in their resistance towards high temperatures and chemical denaturants. Recently, it was shown by computational analysis and molecular simulations that two sources account for the high thermal stability, one based on structure and the other on sequence [6]. In the structure-based mechanism, the compactness of the molecule appears accountable, while in the sequence-based mechanism, a limited set of interactions is primarily responsible for elevated stability, which arise from particular residues [6]. TBADH has 75% sequence identity with the mesophilic CBADH [29]. From the structural studies of TBADH, a number of factors emerged that could be responsible for the high thermal stability. The most important are salt bridges, hydrogen bonds, van der Waal's contacts and a greater number of proline residues. Amino acid sequence analysis revealed that 21 proline residues are present in each TBADH subunit compared to 13 residues in the mesophilic counterpart CBADH [17]. Mutational analysis of

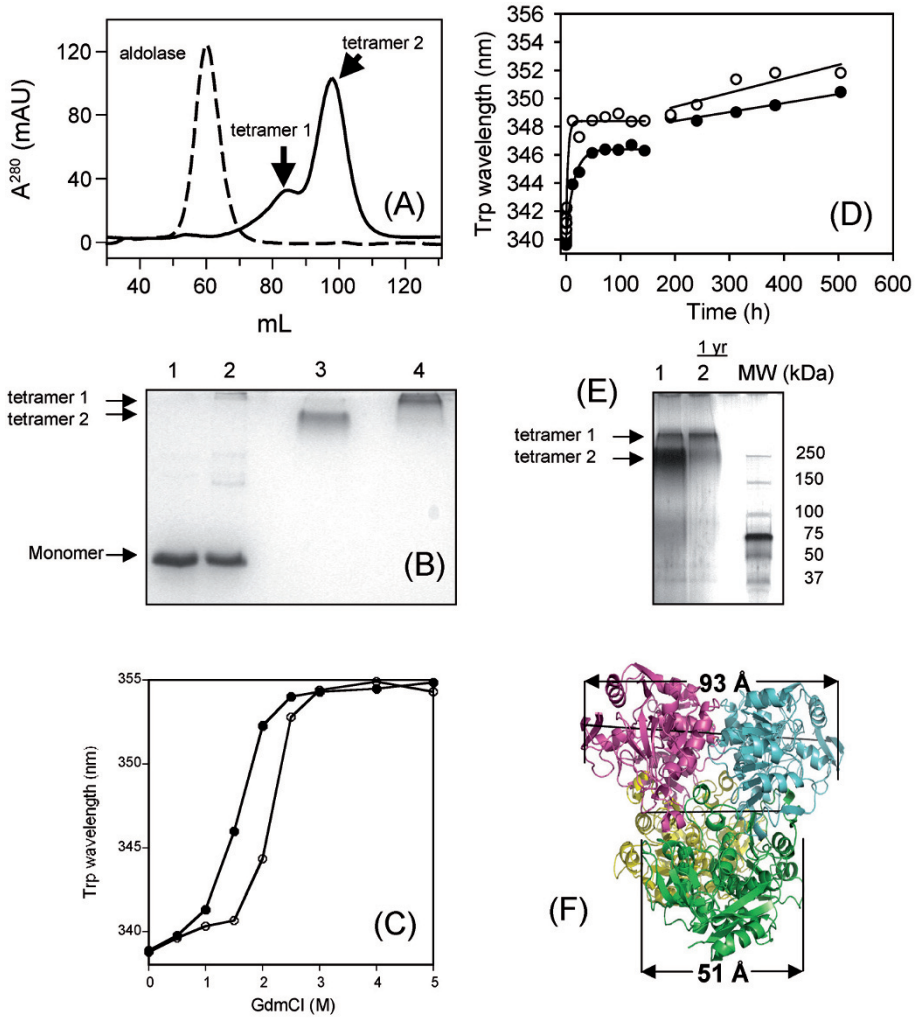

Figure 6. Conformational isoforms of native TBADH. (*A*) Separation of native TBADH by size exclusion chromatography where tetramer 1 eluted at 85 ml and tetramer 2 eluted at 98 ml (solid line). The chromatographic trace for aldolase (158 kDa) is shown for reference (dashed line). (*B*) Glutaraldehyde cross-linking of tetramer 1 and tetramer 2 analyzed by SDS-PAGE (7.5% acrylamide). Lane 1: tetramer 2 no cross-link; lane 2: tetramer 1 no cross-link; lane 3: tetramer 2 cross-linked; lane 4: tetramer 1 cross-linked. (*C*) GdmCl denaturation of TBADH after 1-day incubation in (filled symbols) 10 mM phosphate buffer, pH 7.5 and (open symbols) 100 mM Tris-sulfate buffer, pH 7.5. (*D*) Unfolding kinetics of tetramer 1 (open symbols) and the tetramer 2 (filled symbols) in 6.0 M urea, monitored by shift in tryptophan wavelength emission. (*E*) Chemical cross-linking of tetramer 1 and tetramer 2 analyzed by SDS-PAGE after 1 year storage at 4 °C. (7.5% acrylamide). Lane 1: tetramer 2 cross-linked; lane 2: tetramer 1 cross-linked; MW, molecular weight standard from Bio-Rad® (250–10 kDa) resolved between 250–37 kDa on the precast 7.5% acrylamide gel. (*F*) Distances in the crossed double dumbbell-shaped tetrameric TBADH crystal structure showing the "chest" distance (93 Å) and the "waist" distance (51 Å). The atypical migration pattern of the cross-linked TBADH isoforms in SDS-PAGE (>250 kDa) can originate from an elongated structure or from charge clusters. Differences in cross-linking of surface located lysine residues could explain the separation of the two isoforms on SDS-PAGE. Figure was generated with PyMOL [39] using the structural data from PDB id 1YKF [16].

CBADH reveals that replacements of proline are more effective at certain strategic positions for stabilizing TBADH [19, 31]. Proline residues are known to be important for enhancement of the thermal stability, because they decrease the entropy of the unfolded state and thereby increase the free energy of this state, which in turn will stabilize the native state [32]. The subunit interface of TBADH has an increased number of salt bridges, hydrogen bonds and hydrophobic interactions in comparison to the mesophilic CBADH [16, 17]. An earlier study indicated that there are 31 salt bridges in TBADH compared to 24 in CBADH, and that some of them are involved in extensive networks [17]. We performed an additional search for salt bridges, using a structure with a slightly higher resolution than in the earlier work (PDB id 1YKF) with the Web version of the WHAT IF software, which revealed 14 intermolecular salt bridges. Indeed, a role of ionic networks for thermal stability has been demonstrated [21]. A closer examination of the structural determinants showed that they are strategically placed at the

subunit interface (e.g., Fig. 1A–C), which emphasizes the importance of oligomerization in the thermal stability of proteins [20].

To resolve the stability mystery of thermophiles, it might be necessary to look beyond the structure and sequence analysis of the proteins. Equilibrium and kinetic refolding and unfolding experiments can enhance our understanding about the nature of thermophilic proteins. Surprisingly, few studies have focused on the kinetics of unfolding of hyperthermophilic proteins [11, 12, 33]. To address these issues, we used a charged (GdmCl) and a neutral (urea) denaturant for unfolding and refolding studies of TBADH. To our surprise, TBADH was a very unstable protein in GdmCl with a modest C_m of 1–1.3 M and having a native state subunit stability of merely 2.5 kcal/mol. Furthermore, the TBADH enzyme activity showed an exceptionally low tolerance against both GdmCl and NaCl, which indicates a conformational change in the active site or direct inhibition of substrate binding. We hence turned to urea and detected a more stable protein with a C_m of 3.7 M and a significantly more stable native state subunit (7.3 kcal/mol). In addition, TBADH retained significant enzyme activity in urea solutions up to 2.0 M urea. Nevertheless, these numbers were not very impressive for a thermophilic protein. Refolding kinetics in the presence of non-denaturing concentrations of both denaturants, urea and GdmCl showed very fast refolding and assembly that was independent of type of denaturant (Fig. 4C). When we turned to unfolding experiments, the exceptional kinetic stability of urea revealed another story. An apparently large hysteresis shown at equilibrium arises from slow unfolding of the protein and showed that high energetic barriers for unfolding of TBADH that provide a kinetic origin for the unusual stability of this protein. A drastically different phenomenon, where urea is a more potent denaturant than GdmCl, has been observed for kinetically stable transthyretin (TTR). The unusual behavior of TTR was explained by ionic shielding by chloride ions stabilizing the tetramer [27]. The unfolding kinetics of TBADH was carried out to gain information about the kinetic stability of the protein. Unfolding kinetics represent the difference between the stability of the native state and the transition state of unfolding. Under the conditions used, i.e., high concentrations of denaturant, unfolding is essentially the only reaction needed to be taken into account since the refolding reaction is largely suppressed under these conditions. The transition state for unfolding will be very native-like under strong denaturing conditions as dictated by the Hammond postulate. The unfolding kinetics of TBADH has a distinctly different behavior at lower and higher denaturant concentrations. Extrapolation of the unfolding rate constant to 0 M denaturant leads to the conclusion that TBADH has a 40-fold longer half-life in urea than in GdmCl. However, the value in urea is highly underestimated, because only the fast phase of unfolding (originating from data collected at 7.5–9.0 M urea) has been taken into account. The physical origin of the high kinetic stability may be traced in the sequence and structure of TBADH. Structural analysis of the TBADH has shown that ion pairs and ion-pair networks are important factors for the stability of this protein. These interactions are more prominent at the inter-subunit interface and some of them are involved in an extensive network as illustrated in Figure 1A–C. Notably, several Arg residues are located at the inter-subunit interaction networks. It has been shown in the thermophilic glutamate dehydrogenase that ion pair networks provide more stability to the protein molecules than isolated ion pairs, and this phenomenon is particularly effective at higher temperatures [34]. The role of salt bridges in the thermal stability of TBADH was demonstrated earlier by Bogin et al. [21]. In addition, disruption of an ionic network around Arg20 accelerates the unfolding rate in D-glyceraldehyde-3-phosphate dehydrogenase [35]. GdmCl, which is an Arg-guanidinium mimic, is capable of disrupting ionic interactions by rupturing the ion pair network with the Glu and Asp residues, which leads to the efficient unfolding of the protein. Urea does not disrupt these ionic networks, likely resulting in slow unfolding kinetics compared to unfolding in GdmCl. In both cases, higher concentrations of denaturant led to faster unfolding of TBADH with only one kinetic phase (Fig. 4A, B), while at lower concentrations the protein showed unusual biphasic kinetics. Since this phenomenon was observed for both GdmCl and urea, it cannot be attributed to differences in the nature of the denaturant nor to isocyanate formation in aging urea, rather it must reflect an intrinsic behavior of the protein. We therefore investigated if TBADH forms more than one native species. TBADH was found to exist in two isomeric forms, with the same subunit composition by MALDI-TOF-MS, chromatography, chemical cross-linking and SDS-PAGE. It is possible that these species are of two different oligomeric sizes. Such an observation was made for hyperthermophilic lactate dehydrogenase from *Thermotoga maritima*, where it was shown to exist in homo-octameric and homo-tetrameric forms [36]. The several reasons motivating the conclusion that this corresponds to two distinct tetrameric isoforms (tetramer 1 and 2), exhibiting quite different behavior and stability, are presented in the results section.

The diversity of the TBADH isoforms appears not to end there. Although tetramer 1 unfolds fourfold faster

compared to tetramer 2 (Fig. 6D), we still observed the extremely slow unfolding phase in 6.0 M urea for both isoforms, although with different amplitudes. Hence, it is possible that TBADH forms even more than two stable isoformic tetrameric species, possibly an ensemble of isoformic species. TBADH can appear to disobey a central dogma of protein folding, postulated by Anfinsen, that a protein will fold into its most thermodynamically stable state under given environmental conditions [37]. However, for TBADH, it is more likely that significant kinetic barriers separate the conformational isoforms rather than ground state thermodynamic stability, which was supported by our longevity study. What can be the basis for the exceptional behavior of TBADH? Hypothetically, stable alternate conformations of TBADH could form from proline isomers and each subunit of TBADH has 21 Pro residues (8 more than CBADH). These alternate conformations may give rise to extremely slow unfolding phases and the ensemble of conformers. The biological relevance of these reported conformational isoforms of TBADH can only be hypothetical at this point. It has not escaped us that the suggested isoformic variation of TBADH is reminiscent of the hypothetical conformational variations reported for the pathogenic mammalian prion protein, or as that demonstrated for yeast prion strains [38]. In yeast cells, different prion isoforms are used for modulation of catabolism or translation termination. For a bacterium like *T. brockii*, which thrives under tough conditions, protein conformational variability may possibly be important for functional plasticity such as regulation, stability and substrate promiscuity to name a few.

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