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TBADH activity in water-miscible organic solvents: correlations between enzyme performance, enantioselectivity and protein structure through spectroscopic studies

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The enantioselective reduction of 2-pentanone to (*R*)- and (*S*)-2-pentanol by *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* alcohol dehydrogenase (TBADH) in mixtures of water and water-miscible organic solvents was investigated. Significant enzymatic activity was retained in up to 87% methanol, ethanol and acetonitrile. The changes in enzyme activity as a function of organic solvent were correlated to structural alterations of TBADH with a series of spectroscopic studies (fluorescence, fluorescence quenching and circular dichroism (CD)). Interestingly, this study shows that the tetrameric form of TBADH is not critical for catalytic performance.

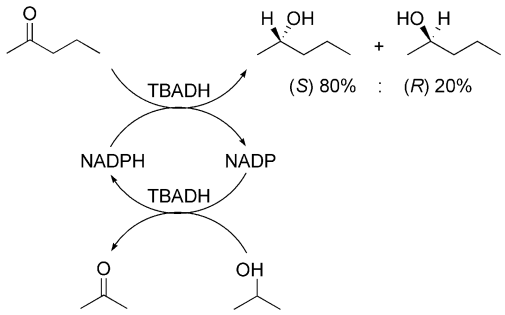
Introduction

The utility of enzymes for the catalysis of asymmetric reactions in aqueous media is well established.[1] Nonetheless, it can be advantageous, or even necessary, to employ organic media, *e.g.* for increased substrate/product solubility and stability, substrate specificity, regiospecificity and enantiopurity.[2,3]

The *Thermoanaerobacter* (formerly *Thermoanaerobium*) *brockii* (*T. brockii*) alcohol dehydrogenase (TBADH) is an oxidoreductase derived from *T. brockii*, a thermophilic bacterium isolated from the hot springs of Yellowstone National Park (USA). TBADH has been shown to possess stability towards elevated temperatures and some organic solvents[5,6] and demonstrates a specificity for small aliphatic ketone substrates and exhibits high product enantioselectivity.[4,7,8] In its native form, the enzyme is a homotetramer with a subunit molecular weight of 37 652 Da,[9] with each monomer consisting of a cofactor domain and a catalytic domain.[10,11] Furthermore, each monomer contains four tryptophan residues, rendering TBADH amenable for fluorescence-based spectroscopic studies.

Although the potential of TBADH for asymmetric synthesis in organic media is implicit from many previous studies, no thorough investigation has examined the potential and consequences of the use of TBADH in syntheses involving high concentrations of water-miscible organic solvents.

In this study, the influence of a range of water-miscible protic and aprotic organic solvents [methanol, ethanol, 1-propanol, acetonitrile (ACN) and tetrahydrofuran (THF)], on the enantioselectivity and enzymatic performance of TBADH in the reduction of 2-pentanone to (*R*)- and (*S*)-2-pentanol is presented, Scheme 1. Furthermore, the influence of the organic solvents on the observed enzyme activities has been correlated to secondary, tertiary and quaternary structural features of the protein, using a series of fluorescence and circular dichroism (CD) studies. The relative resilience of TBADH to these solvents is demonstrated in part by the product enantioselectivity and yield, together with the collective insights gained from the fluorescence and CD studies.



Scheme 1 Reduction of 2-pentanone to (*R*)- and (*S*)-2-pentanol by TBADH in a coupled substrate reaction in which the cosubstrate 2-propanol is oxidized to acetone to achieve regeneration of the cofactor NADPH.[4]

Results and discussion

Enzymatic reduction of 2-pentanone

In order to investigate the utility of this enzyme for use in monophasic asymmetric organic synthesis, the influence of a series of protic and aprotic water-miscible organic solvents on the enzymatic reduction of 2-pentanone by TBADH at 20 °C was examined, Fig. 1. 2-Pentanone was chosen as substrate since it has previously been used in an extensive investigation of factors influencing the TBADH-mediated reduction of ketones.[4] Assay procedures previously developed by Keinan *et al.*,[4] using 2-propanol (22%, v/v) as a cosubstrate for cofactor recycling were used in conjunction with commercial TBADH (used as received). Reactions performed under these conditions, *i.e.* 78% (v/v) buffer, afforded the products (*R*)- and (*S*)-2-pentanol in 60% total yield as determined by GC-MS analysis using a chiral capillary column.

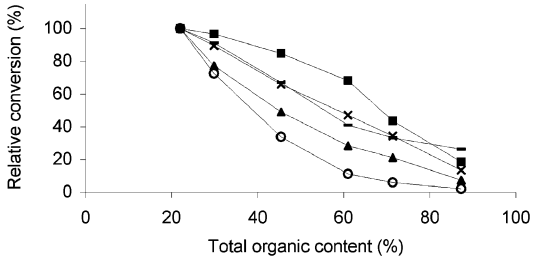


Fig. 1 Conversion of 2-pentanone to (*R*)- and (*S*)-2-pentanol by TBADH at 20 °C in methanol (■), ethanol (×), 1-propanol (▲), ACN (–) and THF (○), all samples containing 22% 2-propanol as cosubstrate for cofactor recycling. At no data point did the standard deviation exceed 4.5% and the average standard deviation was 1.2%.

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Table 1 Characteristics of organic solvents

Solvent	C_{50}[a]	DC[b]	log P[c]	ε[d]	I[e]
Methanol	68	30.5	−0.74	32.7	5.1
Ethanol	58	54.4	−0.32	24.6	4.3
ACN	55	64.3	−0.34	35.9	5.8
1-Propanol	45	69.2	0.34	20.4	3.9
THF	38	100	0.46	7.6	4.0

[a] The solvent organic content in percentage at which enzyme activity is 50% of the activity in buffer and cosubstrate mixture (22% total organic content), see Fig. 1. [b] Denaturation capacity.[13] [c] Partitioning coefficient of the solvent in the water–1-octanol biphasic system.[13] [d] Solvent dielectric constant at 25 °C.[14] [e] Polarity index.[15]

Increasing concentrations of methanol, up to 60% total organic content, produced a decline in conversion efficiency, to 70%, Fig. 1. Higher concentrations of methanol resulted in a significant decline in activity, to 20% conversion at 87% total organic solvent content. The use of ethanol and 1-propanol presented similar trends, though the decrease in enzyme efficiency was more pronounced, and appeared to be related to the relative hydrophobicities of the three alcohols, Table 1. Interestingly, despite the reduction in enzyme efficiency in media of higher hydrophobicity, the enantiomeric excess (ee) of the product (*S*)-alcohol remained relatively constant, 60 ± 4%, in all organic solvent compositions and concentrations studied (not determined for 1-propanol, 87% total organic solvent concentration, due to low yield). Improvement of ee could be achieved by interruption of reaction at 50% conversion in order to decrease time dependent racemization,[4] and by optimizing temperature and cosubstrate concentration.[12]

In the cases of the aprotic solvents, ACN and THF, the former resulted in a reactivity profile similar to that obtained with the ethanol series, though enzyme reactivity was somewhat less influenced by higher concentrations of this solvent. In THF, the influence of this solvent on enzyme efficiency is already quite apparent at lower concentrations, though again, and as also for ACN, the product ee was stable at 60 ± 4% (ee determination for THF at 71% and 87% not possible due to low yield).

From these data it is evident that TBADH is capable of efficient function and product enantioselectivity at relatively high concentrations of water-miscible organic solvents, though elevated hydrophobicities result in successively lower conversions. Several physical parameters have previously been used to characterize enzyme catalysis in organic media, Table 1. While the trends in Fig. 1 indicate a good correlation between TBADH activity (C_{50}), denaturation capacity (DC) and log P, at 87% total organic content correlation with the dielectric constant (ε) and polarity index (I) of the solvent appear best.

However, based upon the solvent systems studied, all parameters support the general relationship between solvent hydrophobicity and enzyme activity described above.

The robustness of this protein suggested studies on the influence of these organic solvents on the structure of the TBADH tetramer.

Purification of commercial TBADH for spectroscopic studies

Commercial TBADH was purified for use in the subsequent spectroscopic studies. As seen in the SDS-PAGE gel, Fig. 2, purified enzyme (lane 3) migrated as a single band with an apparent molecular weight of ~38 kDa per subunit as confirmed by the SeeBlue protein standard band of alcohol dehydrogenase at 38 kDa (lane 4).

Intrinsic tryptophan fluorescence of TBADH

Fluorescence studies were performed to investigate the possibility of correlating the organic solvent induced decrease in TBADH activity to changes in TBADH tryptophan (Trp)

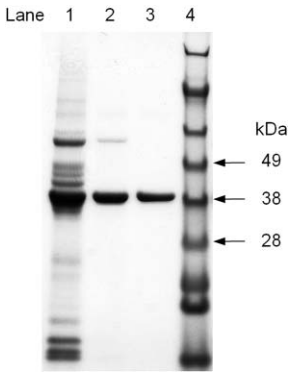

Fig. 2 SDS-PAGE analysis of TBADH purification steps showing commercial TBADH (lane 1), fraction from Red Sepharose column (lane 2), fraction from Superose column (lane 3) and SeeBlue plus2 protein standard (lane 4).

environment. As seen in Fig. 3, the fluorescence emission, λ_{max}, of TBADH in all organic solvents investigated behaved similarly, and appeared to consist of three different regions; 0% to 30% organic solvent (region 1), 30% to 60% organic solvent (region 2) and 60% to 90% organic solvent (region 3). In region 1, λ_{max} is blue shifted (lowered) upon increasing the organic content from 0% to 30%, this is followed by a red shift up until between 45% and 60% (region 2) and a blue shift at even higher organic solvent contents (region 3).

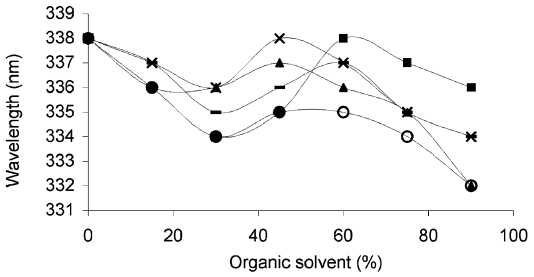

Fig. 3 Fluorescence λ_{max} of TBADH at 20 °C in methanol (■), ethanol (×), 1-propanol (▲), ACN (–) and THF (○).

The blue shift in region 1 is interpreted as arising from a decreased energy loss from excited Trp fluorophores to the reorienting solvent dipoles of the increasingly more hydrophobic surrounding media. One may well expect this trend to continue with increased hydrophobicity, but instead, a red shift is observed (region 2). Evidently some of the Trp residues suffer energy loss due to *increased* interaction with neighboring molecules on account of increased environmental polarity. Thus a dramatic change in Trp environment appears to take place in the transition from region 1 to region 2.

Previous fluorescence studies of enzymes exposed to mixtures of water and water-miscible organic solvents, using Trp as probes for the environmental polarity, showed that an increase of Trp exposure to the organic solvent–water mixture resulted in a fluorescence emission red shift which was correlated to protein denaturation and concomitant activity loss.[16–18] In this study, TBADH fluorescence red shifts can be correlated neither to protein denaturation nor to activity loss, as only a relatively small, linear, decrease in enzyme activity was observed in the organic solvents examined, see Fig. 1 and 3. However, the observed changes in Trp environment might be explained by disintegration of the TBADH tetramer.

X-Ray crystallographic studies[10] show that the TBADH tetramer may be represented as a dimer of two identical dimers composed of subunits A and B (dimer AB) and subunits C and D (dimer CD), Fig. 4. The largest areas of interaction in the tetramer exist between subunit A and B of dimer AB and the corresponding interface between subunits C and D; less intersubunit contact is present between the two dimers.[10,11,21]

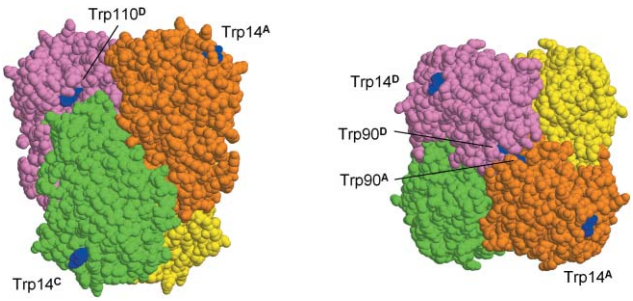

Fig. 4 TBADH tetramer side view (left) and from above (right). Trp's are coloured in blue and denoted with capital letters representing the subunit. Illustrations were created with RasTop 2.0.3 software[19,20] using the RSCB PDB molecular file 1BXZ.[10]

The four tryptophans present in each TBADH monomer can be divided into three categories, each with a different degree of exposure to surrounding media: (a) Trp14 and Trp110, which are partially exposed on the surface of both the monomer and in the tetrameric structure, (Fig. 4), hence, their exposure to solvent will not change upon tetramer dissociation. (b) Trp90, which is almost completely solvent protected in the tetramer, being buried in the subunit–subunit interface between subunit A and D and between subunit B and C, (Fig. 4, right). Nevertheless, Trp90 becomes freely exposed to the solvent upon separating the tetramer into dimers, (Fig. 5), left. (c) Trp281, which is almost completely buried in the dimer, but becomes freely accessible to the solvent upon monomerisation, (Fig. 5, right). The local subunit environments of Trp14 and Trp110 remain unaffected despite tetramer disruption to dimers or monomers. Thus, the fluorescence red shift in region 2 can be concluded to arise from changes in the environments of Trp90 and/or Trp281 upon transition from the more hydrophobic interior of the TBADH tetramer to the less hydrophobic organic solvent–water mixture. In the less polar solvents (methanol, ACN), intermediate red shifts appear at 45% organic content, probably indicating the disruption of tetramers into dimers, also exposing Trp90. At 60% (methanol, ACN) the red shifts are fully developed indicating the disruption of dimers into monomers, a process leading to the exposure of Trp281. The red shifts of λ_{max} of the ethanol, propanol, and THF series occur more abruptly, providing only a narrow concentration range in which the dimeric form is present before disrupting into monomers. The blue shift in region 3, which is continuous, can thus be rationalized as a result from the decreasing polarity of the medium, which influences all of the Trp residues as all are now exposed to the solvent. The red shifts (region 2) observed in ethanol, 1-propanol and THF, occur at lower organic content (45%) than for the less hydrophobic solvents, ACN and MeOH, (60%). Furthermore, the magnitude of the red shift in THF is lower in THF due to its significantly lower polarity, see Table 1.

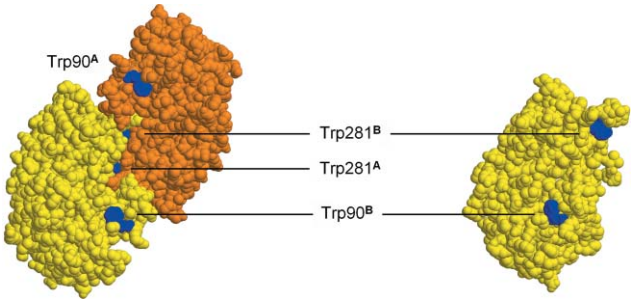

Fig. 5 TBADH dimer (left) and monomer (right).

In order to achieve the fluorescence emission λ_{max} of "totally denatured" TBADH, treatment with GuHCl was performed which resulted in a fluorescence emission λ_{max} of 352 nm. Thus, since the largest red shift of the fluorescence emission λ_{max} reached only 338 nm (though slightly suppressed by the organic media), see Fig. 3, substantial TBADH structure should be retained in all organic solvents and concentrations.

Quenching of TBADH fluorescence

To validate the basis for the observed changes in Trp fluorescence, fluorescence quenching studies were conducted to establish the extent of Trp exposure. Dynamic quenching (K_{sv}) and static quenching (V) both arise from physical contact between the quencher (acrylamide) and the fluorophore (the excited indole ring of Trp). Dynamic quenching is a result of collisional encounters between the fluorophore and the quencher while static quenching occurs when the fluorophore is more accessible to the quencher, resulting in complex formation between the two. The presence of static quenching, which is seen as upward curvature of a Stern–Volmer plot, Fig. 6, is quantified by the term V, Table 2.

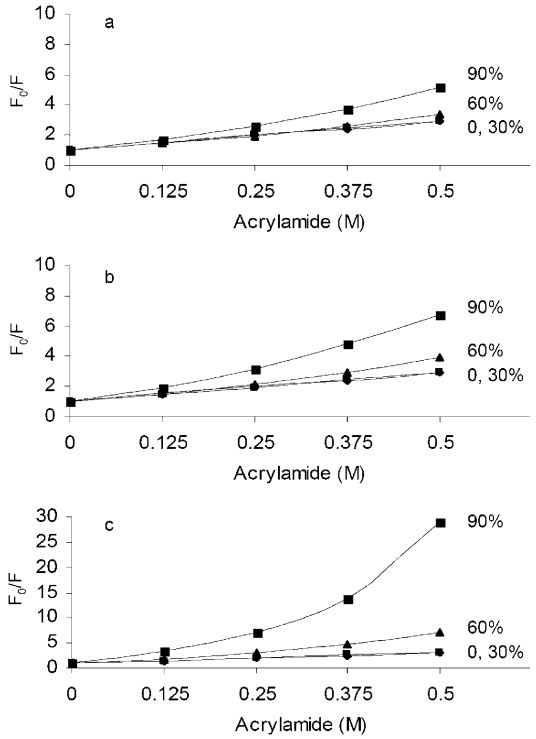

Fig. 6 Stern–Volmer plots showing fluorescence quenching by acrylamide of Trp of TBADH in (a) MeOH, (b) ACN and (c) THF. Total organic content is indicated by percentages in the figure. Trp fluorescence in H_2O (F_0), divided by fluorescence at λ_{max} for the respective organic solvent concentration (F), is plotted against quencher concentration, [Q]. Note the difference in scale on the y-axis in (a, b) *versus* (c).

Fig. 6 shows the acrylamide quenching of Trp residues of TBADH in (a) methanol, (b) ACN and (c) THF. The quenching patterns of the homologous alcohol series represented by methanol (a) are very similar, as judged by dynamic quenching (K_{sv}) and static quenching (V) in Table 2.

The K_{sv} for TBADH in water is 3.8 while V is 0, indicating almost solely the presence of collisional quenching. Thus, the indole rings of the partially exposed Trp110 and Trp14 on the surface of the TBADH tetramer, appear to be sufficiently protected from solvent to prevent complex formation with the quencher. Upon exposure to 30% of the alcohols or ACN no significant alteration in K_{sv} or V occurs, indicating that the Trp residues are equally inaccessible to acrylamide in water or 30% of these organic solvents.

This is in agreement with the proposed tetrameric structure in region 1, Fig. 3. The presence of static quenching, V, at 60% of the alcohols or ACN suggests that the TBADH tetramer dissolves into monomers causing exposure of the indole rings

Table 2 Quenching of Trp fluorescence by acrylamide

	0%		30%		60%		90%	
Solvent	K_{SV}	V	K_{SV}	V	K_{SV}	V	K_{SV}	V
Methanol	3.8	0	3.9	0	2.3	0.9	3.5	1.2
Ethanol	3.8	0	3.4	0	2.5	0.9	3.9	1.3
1-Propanol	3.8	0	3.5	0	2.4	1.0	2.6	1.6
ACN	3.8	0	3.8	0	2.8	1.0	5.2	1.3
THF	3.8	0	2.4	0.7	3.1	2.0	6.1	3.9

Acrylamide (0–0.5 M final concentration) was added to TBADH pre-incubated in mixtures of water and organic solvents. Fluorescence in the absence of acrylamide/fluorescence in the presence of acrylamide (F_0/F), at λ_{max} for each organic solvent concentration, was plotted against acrylamide concentration (Stern–Volmer plot, see Experimental section). Dynamic quenching constants K_{SV} and static quenching constants V were extracted from the plots using eqn. (1). In cases of $V < 0.5$, the plots were considered essentially linear and V was set at 0. K_{SV} values were then determined using the simplified linear equation [eqn. (2)], which fits the tangents of the curves at 0 M acrylamide.

of Trp90 and Trp281 to the quencher, in agreement with the interpretation of the fluorescence behaviour of TBADH in region 2, Fig. 3. The slightly increased static quenching at 90% organic content might indicate structural changes within the monomers, causing further exposure of Trp14 and Trp110 to the quencher. In terms of the influence of THF, static quenching occurs at lower concentration (30%) and is more pronounced at higher concentrations, which suggests an earlier induction of tetramer disruption and more pronounced changes in monomer structure resulting in increased exposure of the Trp indole rings. The possibility of protein aggregation being responsible for the reduced enzymatic activity upon increasing the organic content is excluded since a) aggregation would lead to a decrease in Trp exposure and thereby lower the static quenching (V), not increasing it b) aggregation would keep Trp90 and Trp281 buried within the aggregates and increasing the organic content would only affect Trp14 and Trp110 on the surface of the aggregates, thus producing a blue shift instead of a red shift.

Circular dichroism

Circular dichroism (CD) studies of TBADH secondary structure were performed in order to examine the additional possibility of structural changes within the monomers. In order to achieve accurate CD signals a significantly higher TBADH concentration was used which in some cases induced protein aggregation at higher concentrations of organic solvents. This is seen in the methanol series, Fig. 7(a), by the presence of suppressed positive ellipticity at 192 nm (absorptive flattening) in conjunction with increased organic content (60% and 90%), which previously has been attributed to the shielding of some chromophores when present in large aggregates.[22]

Importantly, the CD spectrum of TBADH in 30% methanol resembles the spectrum of native TBADH in water. In 60% and 90% methanol, the CD spectra suggest substantial structural alterations despite the fact that the enzyme retained substantial activity in 60% and 90% methanol. This further supports that protein aggregation is responsible for these unexpectedly large distortions. CD spectra of TBADH in the primary alcohols methanol, ethanol and 1-propanol show the same trends (though with accentuated effects for 1-propanol, results not shown). The effect of aggregation is more pronounced in THF, Fig. 7(b) already appearing at 30% THF, causing severe distortion of the CD spectrum at 90% THF. Nevertheless, Fig. 7(c) shows that the CD spectra of TBADH in water, 30% ACN or 60% ACN are quite similar in shape; and that TBADH apparently retains its structure and aggregation seems absent.

Thus, TBADH structure is well preserved in 60% ACN, and since no major decrease in activity occurs in conjunction with increasing the ACN concentration to 90%, its secondary structure should remain intact, leaving aggregation as the cause for the distorted CD spectrum at 90% ACN. Furthermore, since both fluorescence, fluorescence quenching, and the activity behaviour of TBADH in methanol, ethanol and ACN are very similar, it is reasonable to expect that a native-like monomer structure is present in the presence of various ranges of concentrations of water-miscible organic solvents.

General discussion

When enzymatic reactions are performed in neat organic solvents the water deficiency prevents the protein from unfolding since it is kinetically trapped in its native conformation.[3] However, the decreased protein flexibility also lowers enzyme performance. In more polar solvents (methanol, ethanol, ACN), the main factor reducing TBADH activity would probably be water stripping, since polar organic solvents are capable of solubilisation and removal of essential water from the enzyme surface[23,24] and also affect the active site polarity.[25] TBADH denaturation appears to be relatively modest according to CD measurements, but it is probably more pronounced for the less polar solvents 1-propanol and THF (these solvents being more effective in disrupting protein hydrogen bonding and hydrophobic interactions)[26] according to their induction of increased static quenching.

It has been argued that protein stability in TBADH is enhanced by several factors, amongst them intersubunit interactions.[10,21] However, upon exposure to the polar organic

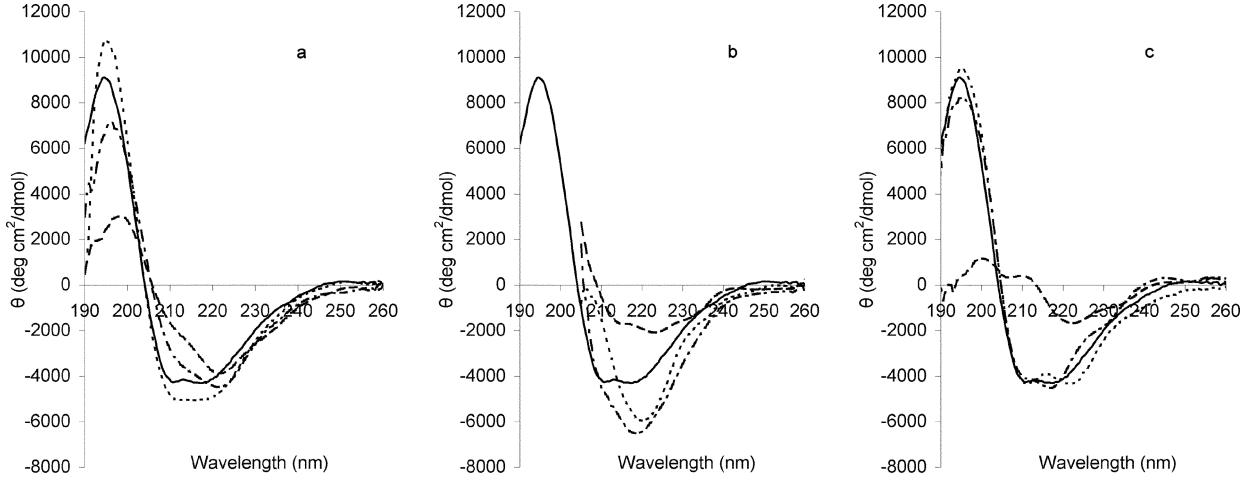

Fig. 7 CD spectra of TBADH in (a) methanol, (b) THF, (c) ACN. TBADH in 30% organic solvent (---), 60% organic solvent (- -), 90% organic solvent (– –) and water (—). Measurements in THF only possible at 205–260 nm due to high absorbance at lower wavelengths.

solvents employed in this study, disruption of the tetramer into its subunits cannot be correlated to any decrease in enzyme activity. That the enzyme is active in its monomeric form is not particularly surprising since all proteins in a thermophilic organisms must be heat resistant,[10] *i.e.* of high stability. This does not contradict that oligomeric protein thermostability may be enhanced by intersubunit interactions. Interestingly, the active site-channel of each monomer is in contact with two amino acid residues from the neighbouring monomer,[10] an interaction which is lost upon monomerisation, though without causing any obvious decline in enzyme activity.

Conclusion

The influence of various water-miscible organic solvents on the yield and enantioselectivity of the TBADH catalysed reaction of 2-pentanone to (*R*)- and (*S*)-2-pentanol has been investigated. Significant activity in up to 87% methanol, ethanol and ACN was observed. The structural basis for the perturbation of enzyme activity has been studied using a combination of fluorescence and CD studies. Collectively, the spectroscopic studies indicate that the enzyme is resilient to simple alcohols and some aprotic media (ACN). Furthermore, this study has shown that the oligomeric structure is not critical for catalytic activity since the disruption of the tetramer into its subunits cannot be correlated to any decrease in enzyme activity.

Experimental

General

TBADH (EC 1.1.1.2, Lot 81H40692) and NADPH (Lot 41K7049), SIGMA (USA). All other solvents and reagents were of analytical grade purchased from commercial suppliers and used without further purification.

Enzymatic reduction of 2-pentanone

Reductions were performed in triplicate in a range of mixtures of glycine buffer (20 mM, pH 7,8) and various organic solvents (methanol, ethanol, 1-propanol, ACN, THF). To a series of Eppendorf tubes was added, in the following order: glycine buffer (0, 300, 500, 800, 1250 μL, respectively), organic solvent (1300, 950, 750, 450, 150, 0 μL, respectively), 2-pentanone (38.5 μL), 2-propanol (385 μL), 2-mercaptoethanol (25 μL, 234 mM in glycine buffer), NADPH (25 μL, 3.8 mM in glycine buffer), commercial TBADH (200 μL, 10 U mL^{-1} in glycine buffer). The reaction mixtures were placed on a rocking table for 20 h at 20 °C, and reactions were interrupted by addition of 0.5 g $(NH_4)_2SO_4$. Samples were centrifuged at 4500*g*, 2 min and supernatants were separated from pellets. To each pellet, 200 μL glycine buffer was added, then shaken and centrifuged at 4500*g*, 5 min and the resulting supernatants were combined with the corresponding supernatants from the previous step. The combined supernatants were extracted with 4 × 1 mL CH_2Cl_2 and phases separated by centrifugation at 1200*g*, 2 min. Finally, 1 mL of each organic phase was placed in vials for GC-MS analysis. All procedures were performed at 20 °C.

GC-MS analysis of reduction products

Reduction substrate and products (1 μL) were analysed using a CP-Chirasil-Dex CB 25 m chiral capillary column (Chrompack) on a Shimatzu GC-17A equipped with a Shimatzu QP-5000 MS detector using the following analysis conditions: injector, 250 °C; interface, 200 °C; column pressure, 45 psi; gas flow, 74 mL min^{-1}; split ratio, 60. Column oven temperature program: 45 °C (13 min) to 75 °C, 10 °C min^{-1}. Baseline separation of 2-pentanone and (*R*)- and (*S*)-2-pentanol was achieved and the corresponding peak areas were integrated to calculate conversion and enantiomeric excess.

Purification of commercial TBADH for spectroscopic studies

Commercial TBADH was purified according to a procedure adapted from Peretz *et al.*[27] Samples were loaded onto a Red Sepharose CL-6B XK 26/40 column (Pharmacia), pre-equilibrated with buffer A (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 10/30 column (Pharmacia), flow 0.1 mL min^{-1} with buffer A. TBADH fractions were concentrated by ultrafiltration with a Vivaspin concentrator (Vivascience), 10 000 MW cut-off at 2300 g, 4 °C, 40 min. TBADH monomer size and purity was confirmed by using NuPAGE 4–12% Bis-Tris pre-cast gel kit with MES buffer, Coomassie staining and SeeBlue plus2 pre-stained protein standard (Invitrogen Life Technologies), activity was confirmed using the assay described above, and concentration was determined by Bio-Rad protein assay (Bio-Rad Laboratories) with IgG as standard on Ultrospec 3000 Pharmacia Biotech (data not shown).

Intrinsic tryptophan fluorescence of TBADH

Purified TBADH (35 μg mL^{-1} final concentration) was exposed to mixtures of water and organic solvents (methanol, ethanol, 1-propanol, ACN, THF) in a series of organic solvent concentrations (0–90%) for 30 min at 20 °C. Tryptophan emission spectra were recorded (290 to 400 nm) at 20 °C with a Hitachi F-2000 fluorescence spectrophotometer at an excitation wavelength of 295 nm to minimise interference from tyrosine[28] and acrylamide.[29] The excitation and emission bandpasses were set to 10 nm and the scan speed was set to 60 nm min^{-1}. Fluorescence emission maxima (λ_{max}) were extracted from the spectra and plotted against alcohol concentration.

Quenching of fluorescence of TBADH

Purified TBADH (46 μg mL^{-1} final concentration) was exposed to mixtures of water and organic solvents (methanol, ethanol, 1-propanol, ACN, THF) in a series of organic solvent concentrations (0–90%) for 30 min at 20 °C, followed by immediate addition of acrylamide (0–0.5 M final concentration). Acrylamide was prepared as 8 M stock solutions in respective organic solvents (0–90%). Tryptophan emission spectra were recorded as for tryptophan fluorescence measurements above. Fluorescence in absence of acrylamide at λ_{max} for respective alcohol concentration (F_0), divided by the fluorescence in presence of acrylamide (F), was plotted against acrylamide concentration, [Q]. The following equation,[29,30]

$$F_0/F = (1 + K_{SV}[Q])e^{V}/[Q] \quad (1)$$

was fitted to the plots to extract dynamic quenching constants K_{sv} and static quenching constants V by a least-squares procedure using the KaleidaGraph for Windows software package (Synergy Software, Reading PA, USA). In cases of V being very low (<0.5), the plots were considered linear and the simplified equation,

$$F_0/F = (1 + K_{SV}[Q]) \quad (2)$$

was used to determine K_{SV} from the linear regions of the plots (tangents at 0 M acrylamide).

Circular dichroism

Purified TBADH (0.2 mg mL^{-1} final concentration) was exposed to mixtures of water and organic solvents (methanol, ethanol, 1-propanol, ACN, THF) in a series of organic solvent concentrations (0–90%) for 30 min at 20 °C. CD spectra were recorded on a CD6 spectrodichrograph (Jobin-Yvon Instruments SA, France) at 20 °C with a path length of 0.5 mm. Data were collected in the wavelength range 190–260 nm (205–260 nm in THF due to high

absorbance) at 0.5 nm intervals with an integration time of 2 s. Each spectrum, both sample and background, was the average of 5 scans, separately intercompared before summation to detect anomalous alterations during the scanning period. Difference spectra were generated by subtracting the background spectrum from the corresponding sample spectrum.

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