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Enhanced thermal stability of *Clostridium beijerinckii* alcohol dehydrogenase after strategic substitution of amino acid residues with prolines from the homologous thermophilic *Thermoanaerobacter brockii* alcohol dehydrogenase

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Abstract

A comparison of the three-dimensional structures of the closely related mesophilic *Clostridium beijerinckii* alcohol dehydrogenase (CBADH) and the hyperthermophilic *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH) suggested that extra proline residues in TBADH located in strategically important positions might contribute to the extreme thermal stability of TBADH. We used site-directed mutagenesis to replace eight complementary residue positions in CBADH, one residue at a time, with proline. All eight single-proline mutants and a double-proline mutant of CBADH were enzymatically active. The critical sites for increasing thermostability parameters in CBADH were Leu-316 and Ser-24, and to a lesser degree, Ala-347. Substituting proline for His-222, Leu-275, and Thr-149, however, reduced thermal stability parameters. Our results show that the thermal stability of the mesophilic CBADH can be moderately enhanced by substituting proline at strategic positions analogous to nonconserved prolines in the homologous thermophilic TBADH. The proline residues that appear to be crucial for the increased thermal stability of CBADH are located at a β-turn and a terminating external loop in the polypeptide chain. Positioning proline at the N-caps of α-helices in CBADH led to adverse effects on thermostability, whereas single-proline mutations in other positions in the polypeptide had varying effects on thermal parameters. The finding presented here support the idea that at least two of the eight extra prolines in TBADH contribute to its thermal stability.

Keywords: alcohol dehydrogenase; *Clostridium beijerinckii*; *Entamoeba histolytica*; mutagenesis; proline; *Thermoanaerobacter brockii*; thermostability

The key to understanding molecular adaptation of proteins to extreme temperatures can be found in the differences between the primary, secondary, tertiary, and quaternary structures of mesophilic and thermophilic members of homologous protein families. The issue has been the subject of numerous theoretical and experimental studies in the last two decades (reviewed in Russell & Taylor, 1995; Querol et al., 1996; Vieille & Zeikus, 1966; Vogt et al., 1997). Some studies were based on comparisons of the amino acid sequences and the three-dimensional structures of functionally related thermophilic and mesophilic enzymes (e.g., Perutz & Raidt, 1975; Menéndez-Arias & Argos, 1989; Warren & Petsko, 1995). Other studies employed largely site-directed mutagenesis, pioneered by Matthews (1993) and Fersht (Fersht & Serrano, 1993), that was utilized also in protein engineering.

To define the mechanisms governing the differences in thermostability, the enzyme family members to be studied should have a high degree of amino acid sequence identity and similar three-dimensional structures. Comparisons of the crystal structures of mesophilic and thermophilic proteins have pointed to almost every

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Abbreviations: ADH, alcohol dehydrogenase; DSC, differential scanning calorimetry; HLADH, horse liver ADH; TBADH, *Thermoanaerobacter brockii* ADH; CBADH, *Clostridium beijerinckii* ADH; EHADH, *Entamoeba histolytica* ADH; DTT, *d,l* dithiothreitol; Gdn-HCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

possible type of protein interaction as a possible structural determinant of thermostability. Examples include the stabilization of structure and dipole moments of α-helices (Hol et al., 1978; Davies et al., 1993); salt bridges (Perutz & Raidt, 1975; Walker et al., 1980; Hennig et al., 1995); increase in the number of interactions between charged side chains and neutral partners ("charged-neutral" hydrogen bonds, Tanner et al., 1996); improved hydrophobic interactions and oligomeric rearrangements that fill existing cavities and lead to tighter molecular packing (Kirino et al., 1994; Delboni et al., 1995); and the presence of proline, which reduces flexibility and increases thermostability (Watanabe et al., 1994). Salt-bridge networks that are energetically more favorable than isolated ion pairs (Horovitz et al., 1990) have been recorded as the most prominent structural feature that could explain the unusual properties of hyperthermophiles (Hennig et al., 1995; Yip et al., 1995).

Site-directed mutagenesis has been used to examine some effects of the various structural determinants discussed above on the thermal stability of several individual proteins (Tomschy et al., 1994; Moriyama et al., 1995), but no generalization to explain the underlying principles of stabilization has emerged.

We have been studying two closely related members of the alcohol dehydrogenase family in anaerobic bacteria: the hyperthermophilic *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH), first isolated from the Yellowstone Park hot springs (Lamed & Zeikus, 1981), and its mesophilic counterpart *Clostridium beijerinckii* alcohol dehydrogenase (CBADH) (Ismaiel et al., 1993). We have isolated and characterized TBADH (Peretz & Burstein, 1989; Peretz et al., 1993, 1997b; Bogin et al., 1997), and cloned, sequenced, and overexpressed both *T. brockii* and *C. beijerinckii adh* genes (Peretz et al., 1997a). Although they have a 75% sequence identity, TBADH and CBADH differ greatly in thermostability: TBADH reversibly catalyzes the oxidation of secondary alcohols to the corresponding ketones with a $T_{1/2}^{60\,\mathrm{min}}$ (temperature at which 50% of the enzymatic activity is lost after 60 min) of 93.8 °C, whereas in CBADH the $T_{1/2}^{60\,\mathrm{min}}$ was 63.8 °C (under more stringent conditions used in the present study). We crystallized both TBADH (Zhang et al., 1993; Korkhin et al., 1996b) and CBADH (Korkhin et al., 1996b) and determined their respective structures at resolutions of 2.5 and 2.05 Å (Korkhin et al., 1996c). Comparing the three-dimensional structures of these enzymes suggested that among several structural determinants that might contribute to the high thermal stability of TBADH, eight extra proline residues (Peretz et al., 1997a), some in apparently critical sites, could be potential thermostability factors.

Delboni et al. claim that a high number of prolines augments the high thermostability of trisphosphate isomerase from the thermophile *Bacillus stearothermophilus* (Delboni et al., 1995). One explanation for this effect is that proline enhances thermostability by decreasing the entropy of the unfolded state (Watanabe et al., 1991; Nicholson et al., 1992; Hardy et al., 1993). The observation that proline residues, especially in such strategically important position as loops, β-turns, α-helices, and coils within external loops (Matthews et al., 1987; Watanabe et al., 1991, 1994, 1996, 1997), is related to the thermal stability of certain thermophilic enzymes, provided the rationale to explore the possibility that one or more of the eight extra prolines in TBADH is important for thermophily.

In this report, we describe the enhancement of thermostability in the mesophilic CBADH after performing site-directed mutagenesis, one residue at a time, to substitute proline for each of the eight residues in CBADH analogous to the extra prolines the thermophilic counterpart TBADH. Preliminary results of this study have been reported elsewhere (Korkhin et al., 1996a).

Results and discussion

Mutagenesis

The amino acid sequences of TBADH and CBADH are presented in Figure 1. For comparison, the amino acid sequence of the human pathogen *Entamoeba histolytica* ADH (EHADH) is included as well. A definite pattern of high-sequence conservation among the three members of this enzyme family can be seen: 75% identity between TBADH and CBADH, 66% identity between EHADH and TBADH, and 62% identity between EHADH and CBADH. The identity between the proline residues of these enzymes is even higher. All 13 proline residues of CBADH are preserved in TBADH (100% identity), of which nine are preserved in EHADH (69% identity). Twelve of the 15 prolines of EHADH are also preserved in TBADH (75% identity) (Figs. 1, 2). Because amino acid residues that are vital for the geometry of a protein or its active site, as well as residues participating in catalytic or regulatory activities, must be conserved in protein families, the nine proline residues common to the three ADHs are the most likely to be essential for maintaining the structure and function of the enzymes. In addition to the highly conserved proline residues, the thermophilic TBADH (352 total residues) contains eight more prolines than the mesophilic CBADH (351 total residues). The eight extra proline residues, constituting 9% of the total difference between the sequences of these enzymes, are distributed throughout the structure. Of the eight extra prolines, five (prolines 22, 149, 222, 275, and 316) are unique to TBADH, and three (prolines 24, 177, and 347) are present in both TBADH and EHADH (Figs. 1, 2).

Using site-directed mutagenesis, we substituted proline, one residue at a time, for the amino acid at each of the eight analogous residue positions in CBADH (residue positions 22, 24, 149, 177, 222, 275, 316, and 347). The recombinant proline mutants of CBADH were overexpressed, purified, and characterized. The electrophoretic mobilities in SDS-PAGE of all CBADH proline mutants were similar to that of the wild-type enzyme (data not shown).

Table 1 shows the enzymatic kinetic properties of TBADH, CBADH, and the proline mutants of CBADH at 40 °C and at 60 °C (partial), using 2-propanol as substrate. As anticipated, the enzymatic activity of the mesophilic CBADH was higher at 40 °C than at 60 °C, whereas that of the thermophilic TBADH was higher at 60 °C. The K_m values of both CBADH and TBADH changed very little at these temperatures.

The enzymatic efficiency (K_{cat}/K_m) and the K_m values of five of the single proline mutants of CBADH, namely A22P-, S24P-, A177P-, L275P-, and L316P-CBADH, and the two double proline muants A22P/S24P- and S24P/L316P-CBADH, were similar to those of the wild-type enzyme, indicating that neither binding nor catalytic efficiency of the enzyme was affected by the mutations (Table 1). Indeed, all those mutations are far from the active site of the enzyme (Korkhin et al., 1996a) (Fig. 3), and any structural changes resulting from these mutations do not appear to perturb the active site. Nevertheless, one of the mutations, H222P-CBADH (Fig. 3), also located at the far end of the molecule, did not affect K_m but reduced the K_{cat} of CBADH. This result strengthens the notion that a conformational change caused by a mutation located at one end of a protein molecule may induce a conformational

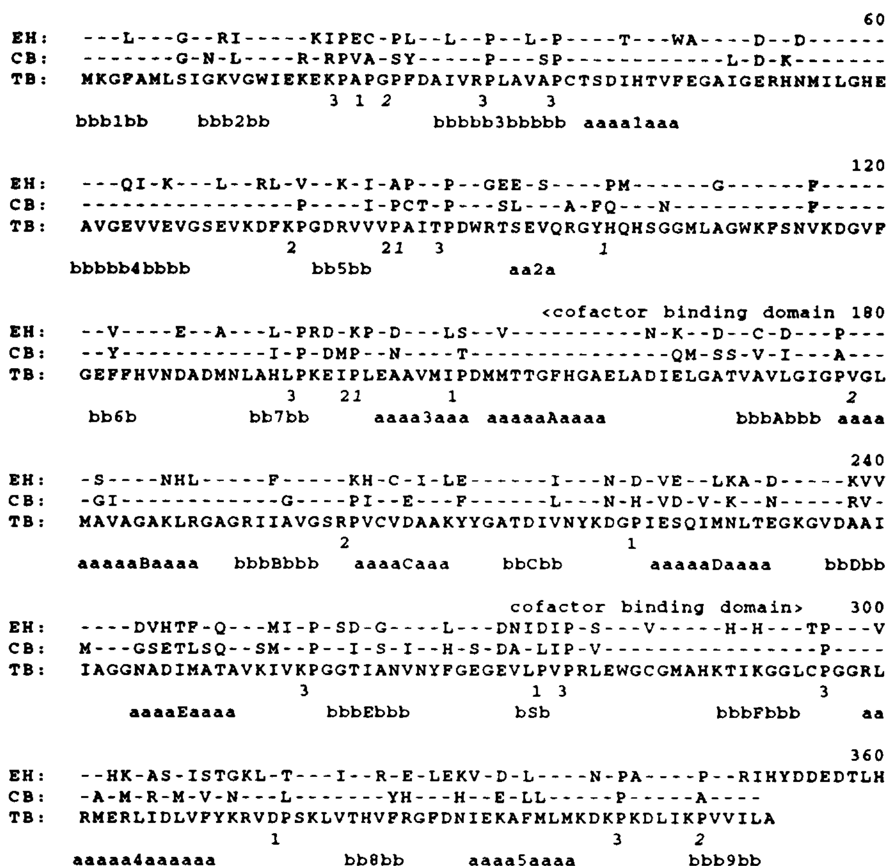

Fig. 1. Amino acid sequence alignment of alcohol dehydrogenases from *Thermoanaerobacter brockii* (TBADH) (Peretz & Burstein, 1989), *Clostridium beijerinckii* (CBADH) (Peretz et al., 1997a), and *Entamoeba histolytica* (EHADH) (Samuelson et al., 1992). The coenzyme binding domain is marked. Amino acid residues of TBADH are in the one-letter code, and identical residues in the other ADHs are marked with dashes. Prolines are marked **P**. The number of proline occurrences is given under the primary sequences: **3**: proline is present in all three ADHs; **2**: proline is present in both TBADH and CBADH, *2*: proline is present in TBADH and EHADH, **1**: proline is unique to TBADH, and *1*: proline is present only in EHADH. Secondary structure elements of β-strand (**bbbbb**) and α-helix (**aaaaa**) of CBADH are shown below the aligned sequences. Data from Korkhin et al. (1996c, 1998).

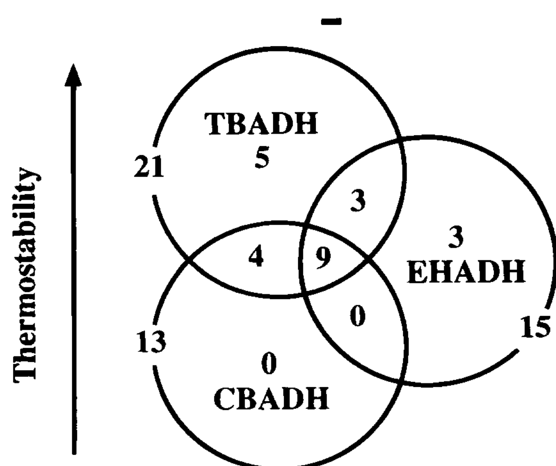

Fig. 2. Classification of the proline residues of TBADH, CBADH, and EHADH. Each circle represents one enzyme, and the vertical arrow shows the increase in thermostability parameters. The numbers shown within the regions created by the circles indicate the number of prolines shared by the ADHs. The total number of prolines in each ADH is shown on the circle.

change at another end of the molecule, thus affecting properties of the protein.

Introducing proline at residue 347 of CBADH, in the vicinity of the catalytic site (Korkhin et al., 1996c), reduced both K_m and K_{cat}. Furthermore, substituting proline for Thr-149, which is adjacent to the active-site zinc ligand Asp-150 (Fig. 1), increased the K_m and decreased the K_{cat}, resulting in a 20-fold reduction in K_{cat}/K_m of CBADH (Table 1).

Thermostability and structural considerations

We used two independent methods to determine the thermostability of TBADH, EHADH, CBADH, and the CBADH proline mutants. The temperature of 50% inactivation ($T_{1/2}$) was determined by exposing the enzyme to temperatures of 30–100 °C for 60 min, and measuring the residual enzymatic activity. $T_{1/2}^{60\,min}$ is the temperature at which 50% of the enzymatic activity is lost after 1 h of incubation, as judged from the plot of residual activity vs. temperature (see Fig. 4). Thermal unfolding transition(s) (T_m) of the enzymes was determined by differential scanning microcalorimetry (DSC) (see Fig. 5). The thermal denaturation of the three

Table 1. *Kinetic parameters of CBADH and its proline mutants*[a]

	K_m (mM)		K_{cat} (min^{-1})		K_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	
ADH	40 °C	60 °C	40 °C	60 °C	40 °C	60 °C
CBADH	7.2	9.2	8,703	5,388	1,216	586
TBADH	5.8	5.7	2,619	12,567	451	2,193
CBADH proline mutants						
A22P-	6.9	7.4	9,148	5,582	1,322	751
S24P-	7.7	8.9	12,405	3,392	1,618	381
T149P-	13.4	nd	868	nd	65	nd
A177P-	6.1	nd	6,471	nd	1,057	nd
H222P-	7.2	nd	4,528	nd	631	nd
L275P-	5.8	8.9	6,159	2,112	1,058	238
L316P-	4.4	7.8	6,071	4,772	1,400	609
A347P-	3.7	4.3	2,177	2,861	588	658
A22P/S24P-	6.6	7.0	10,474	4,920	1,586	701
S24P/L316P-	5.7	7.3	11,599	8,261	2,039	1,127

[a]Enzymatic activity was measured by following the formation of NADPH at 340 nm. K_m values for 2-propanol were determined with varying concentrations of 2-propanol (0.1–100 mM), enzyme (5–120 nM), and 0.5 mM NADP in 100 mM Tris-HCl (pH 8.8) in a total volume of 1 mL, in triplicate. Values are the averages of three experiments and the individual measurements were within 10% of the quoted mean.

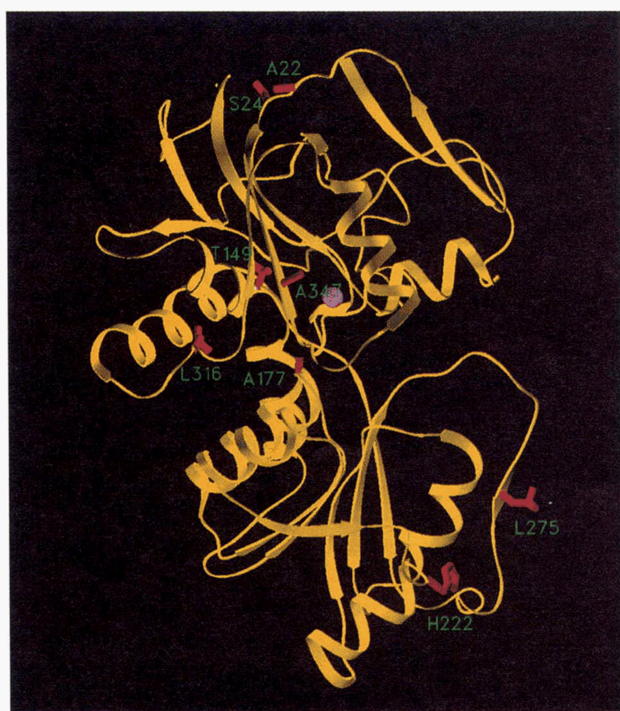

Fig. 3. The residue positions of proline substitutions in *C. beijerinckii* ADH. The model of the monomer is according to Korkhin et al. (1996c, 1998). The amino acid residues substituted by proline are indicated by residue position number and the one-letter code.

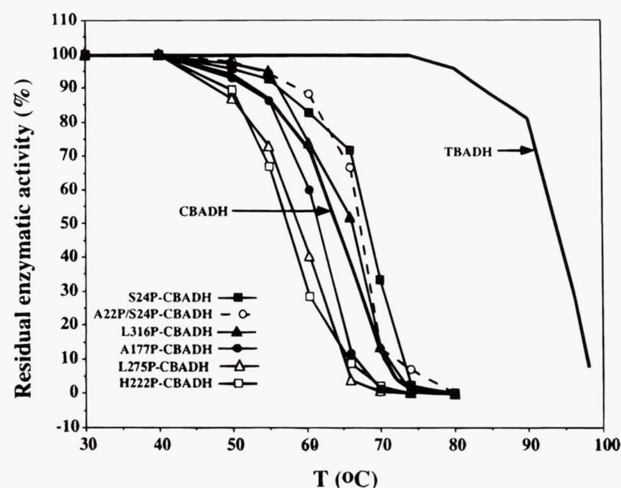

Fig. 4. The effect of proline mutations on the thermal stability of *C. beijerinckii* ADH. The thermal stability of the enzymes was determined by monitoring their residual enzymatic activity after 60 min incubation at varying temperatures. Enzymatic activity was assayed by spectrophotometric analysis of NADPH formation at 340 nm (see legend to Table 1). Values are expressed as the averages of at least three experiments. Thermal transitions of TBADH and CBADH (no symbols) and of S24P- (filled squares), A22P/S24P- (open circles), L316P- (filled triangle), A177P- (filled circles), L275P- (open triangles), and H222P-CBADH (open squares) are shown.

wild-type ADHs and of all proline mutants of CBADH was an irreversible process at neutral and slightly alkaline pH, and the asymmetry of the endotherms (in Fig. 5 and others not shown) indicated multistate unfolding.

The thermal stabilities of the proline mutants of CBADH differed from that of the wild-type enzyme. Table 2 shows the changes in $T_{1/2}^{60\,min}$ and T_m for each proline substitution. For the three wild-type enzymes, the $T_{1/2}^{60\,min}$ values are somewhat lower (by 2–7 °C) than the T_m values, indicating that the loss of enzymatic activity preceded the "melting" or unfolding of the enzyme molecule (see Table 2).

Table 2 also shows that the proline content of the three native ADHs is strongly correlated with their thermal stability: the higher the proline content, the greater the thermal stability. The individual

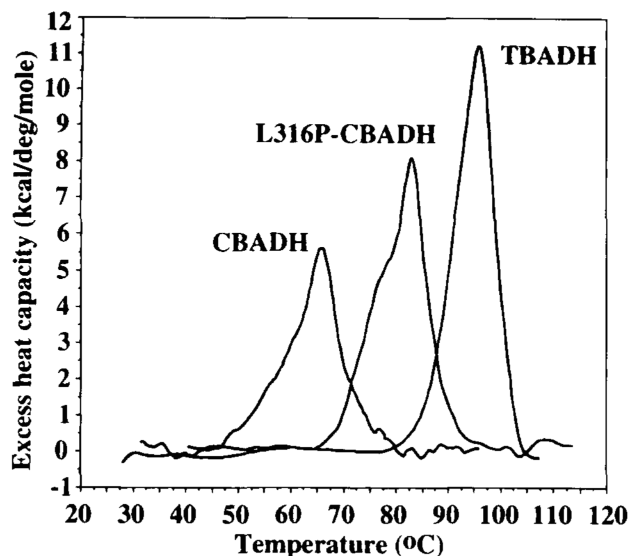

Fig. 5. Thermal denaturation monitored by high sensitivity Differential Scanning Microcalorimetry. Experiments were performed under constant pressure (2 atm pressure of nitrogen gas) at a scan rate of 1 °C/min with protein samples (1–2 mg/mL) in 25 mM sodium phosphate buffer containing 1 M NaCl (pH 8.25). T_m values were defined as the maximum of the excess heat capacity functions.

contributions of each additional proline, however, were not identical and were not necessarily additive.

In general, an amino acid contributing to the stability of a protein in one sequence context may cause no change or may even reduce stability in another, depending on the amino acids at other positions. This is illustrated in TBADH, where three alternating proline residues, in residue positions 20, 22, and 24, participate in an external loop between β-strands 2 and 3 (Figs. 1, 3). The same loop in CBADH contains only one proline residue at position No. 20. Replacing Ala-22 of CBADH with proline had very little effect on its thermal stability (<1 °C, Table 2). Replacing Ser-24 with proline, however, increased the thermal stability of CBADH by ~4 °C. This difference may be because residue 24 is positioned in a β-turn of the external loop, connecting it to β-strand No. 3. A proline residue at this location stabilizes the β-turn far better than does a serine residue. In the case of EHADH, both Pro-20 and Pro-24 are present and the apparently ineffective Pro-22 is missing (Fig. 1). $T_{1/2}^{60\,\text{min}}$ (77.5 °C) and T_m (84.5 °) of EHADH are intermediate between those of CBADH and TBADH. Replacing both residues 22 and 24 with prolines (Table 2, double proline mutant A22P/S24P-CBADH), so that the enzyme contained proline at all three positions as in TBADH, increased the T_m of CBADH by ca. +6 °C, suggesting that the flexibility of the external loop was further restrained.

Replacing Thr-149 of CBADH, located in a sharp bend in a long α-helix and next to the active site zinc ligand Asp-150, with proline slightly lowered the T_m of CBADH (−1 °C, Table 2). The effect on $T_{1/2}^{60\,\text{min}}$ value, however, was much more pronounced (−6.5 °C, Table 2), suggesting that the mutation next to the active-site zinc ligand affected both the catalytic machinery and the thermal stability of CBADH. Indeed, the enzymatic activity of T149P-CBADH at 40 °C was the lowest of all proline mutants, and could not be detected at 60 °C, possibly due to the loss of zinc with increasing temperature (Table 1).

Both Ala-177 and His-222 of CBADH are at N-caps of α-helices. Replacing Ala-177 with proline scarcely affected its T_m, whereas introducing proline at residue position 222 destabilized the enzyme, lowering both T_m (−6.5 °C) and $T_{1/2}^{60\,\text{min}}$ (−6.3 °C) values of the wild-type enzyme. Whether the reduction in thermal stability due to the H222P mutation is related to a change in the catalytic machinery (Table 1) remains to be seen.

An amino acid change at one position can affect the "fitness" of amino acids at other positions, depending on the structural and functional links between the residues. In the present study, replacing Leu-275 of CBADH with proline lowered both T_m (−2.3 °C) and $T_{1/2}^{60\,\text{min}}$ (−5 °C) of the enzyme. This residue is located in the center of an extended stretch of residues involved in dimerization with a second monomer. The central residues of the loop (273–276) "embrace" the corresponding residues (276–273) of the anti-parallel loop of the second monomer. In TBADH, the fit of these interacting residues is better than that in CBADH, and it seems that replacing only one residue (Leu-275) is not adequate; a combination of at least two mutations in this loop might increase the thermal stability of CBADH.

Of the single-proline mutants in this study, Leu-316-Pro-CBADH showed the highest T_m (Table 2). Residue position 316 terminates an external loop between α-helix-4 and β-strand-8. A proline at this position stabilizes the turn at the end of the loop and the secondary structural elements in this area.

The sequence homology at the C-termini of CBADH and TBADH (residues 317–351) is very high: 80% identity and 97% similarity (allowing for six conservative replacements). Only residue 347 is different: alanine in CBADH and proline in TBADH (Fig. 1). Replacing Ala-347 with proline in CBADH increased its thermal stability (Table 2). Residue 347 is the second residue of β-strand-9 (Fig. 1). A proline residue in this position induces a slight twist to the β-strand that could restrict the flexibility of the otherwise loose end of the C-terminus.

Suzuki proline rule of thermostability and the ADHs

Suzuki's group studied oligo-1,6-glucosidases from several mesophilic and thermophilic *Bacilli* (Watanabe et al., 1991, 1994, 1996, 1997) and assigned to their proline residues fundamental roles in the thermostability of the enzymes. The Suzuki proline rule of thermostability (Watanabe et al., 1994) emerged from those findings and suggests that (1) proline residues that are critical for thermal stabilization are likely to be the second sites of β-turns or the first turns of α-helices; (2) most critical sites occur randomly over the protein surface; and (3) substituted prolines contribute independently (and additively) to thermal stabilization.

The results of our comparative study agree with Suzuki's observation that the proline residues critical for thermostability favor β-turns (Pro-24) or the termination (Pro-316) of external loops on the protein surface. By contrast, inserting proline residues at the N-caps of α-helices in CBADH either failed to enhance thermostability (Pro-177) or reduced both enzymatic activity and thermal parameters (Pro-222). On the other hand, introducing prolines into the middle of loops or polypeptide stretches, led to increases (Pro-22 and Pro-347) as well as decreases (Pro-149 and Pro-275) in the thermal stability of CBADH.

As to rule (3) above, that proline mutations contribute independently to thermal stabilization (Watanabe et al., 1994), we believe that such contributions are not necessarily additive in CBADH for the following reasons: the ΔT_m value of the double proline mutants

Table 2. *Thermal parameters of CBADH, EHADH, TBADH, and of CBADH proline mutants*

ADH	Proline content (mol %)	$T_{1/2}^{60\,min}$ (°C)[a]	$\Delta T_{1/2}^{60\,min}$ (°C)[b]	T_m (°C)[c]	ΔT_m (°C)[b]
CBADH	3.70	63.8	—	66.0	—
EHADH	4.17	77.5	+13.7	84.5	+18.5
TBADH	5.97	93.8	+30.0	98.5	+32.5
CBADH proline mutants					
A22P-		63.8	0	66.7	+0.7
S24P-		68.2	+4.4	69.9	+3.9
T149P-		57.3	−6.5	65.0	−1.0
A177P-		61.5	−2.3	66.5	+0.5
H222P-		57.5	−6.3	59.5	−6.5
L275P-		58.8	−5.0	63.7	−2.3
L316P-		66.0	+2.2	76.8	+10.8
A347P-		64.0	+0.2	68.6	+2.6
A22P/S24P-		67.1	+3.3	72.1	+6.1
A24P/L316P		66.1	+2.3	77.1	+11.1

[a] $T_{1/2}^{60\,min}$ was determined by monitoring the residual enzymatic activity after incubation for 60 min at different temperatures. Values are the averages of three experiments, and the individual measurements were within 1 °C of the quoted mean.
[b] Relative to CBADH.
[c] T_m is defined as the maximum of the excessive heat capacity function determined by differential scanning microcalorimetry. Values are the averages of two experiments, and the individual measurements were within 0.3 °C of the quoted mean.

A22P/S24P-CBADH was higher than the sum of the ΔT_m values contributed by the single-proline mutants, while that of S24P/L316P-CBADH was lower than the sum of the contributions of the single-proline mutants. On the other hand, the $\Delta T_{1/2}^{60\,min}$ values of both double proline mutants of CBADH were very close to the sums of the values contributed by the single proline mutants. Furthermore, although the single mutation L275P-CBADH destabilized the wild-type enzyme (Table 2), and another single mutation, A273V-CBADH, in the same external loop it had little effect on the thermal stability of the enzyme (O. Bogin, unpubl. obs.) the thermostability ($\Delta T_{1/2}^{60\,min}$) of the double mutant A273V/L275P-CBADH was enhanced by 7 °C (Bogin et al., 1996; Burstein et al., 1997).

Conclusions

Our results show that the thermal stability of the mesophilic CBADH can be moderately enhanced by substituting proline at strategic positions analogous to nonconserved prolines in the homologous thermophilic TBADH. The two proline residues that appear to be crucial for the increased thermal stability of CBADH are located at a β-turn and a terminating external loop in the polypeptide chain. We found that positioning proline at the N-caps of α-helices in CBADH had adverse effects on thermostability, whereas single-proline mutations in other positions in the polypeptide chain resulted in either slightly increased or decreased thermostability. The finding presented here support the idea that at least two of the eight extra prolines in TBADH contribute to its thermal stability.

Materials and methods

Materials

The enzymes for DNA cloning, sequencing, and amplification were from Amersham (Buckinghamshire, England), New England Biolabs (Beverley, MA), Fermentas ABI (Vilnius, Lithuania), or Promega (Madison, WI). [γ-^{32}P]ATP was from Amersham. Oligonucleotides for cloning, sequencing, and site-directed mutagenesis of the *T. brockii adh* and *C. beijerinckii adh* genes were synthesized by the Weizmann Institute of Science Chemical Synthesis Laboratory. All other chemicals were of analytical grade.

Isolation of DNA and mutagenesis

DNA was extracted by the alkaline lysis procedure (Sambrook et al., 1989), separated by agarose gel (1%, w/v) electrophoresis, and then purified to homogeneity using GenecleanII kit (Bio 101). Construction and expression of the genes encoding TBADH and CBADH was performed according to Peretz et al., as described elswhere (Peretz et al., 1997a). The plasmid pBS-P200^{CBADH} was used as the template to generate all recombinant plasmids coding for the A22P-, S24P-, T149P-, A177P-, H222P-, L275P-, L316P-, and A347P-CBADH proline mutants. Site-directed mutagenesis was performed according to Kunkel et al. (Kunkel et al., 1987), and the mutations were verified by DNA sequencing of the relevant plasmids. The oligonucleotides used for generating the mutants are listed below; the exchanged bases are underlined:

for A22P-CBADH: 5′-GCATCATACGAACCCGGAACTGGCC TTTC-3′

for S24P-CBADH: 5′-CGTACAATAGCATCATACGGACCCGCA ACTGGC-3′

for A22P/S24P-CBADH: 5′-CGTACAATAGCATCATACGGAC CCGCAACTGGC-3′

for T149P-CBADH: 5′-CCAGAAGTCATCATATCTGGTATCAT AACAGCA-3′

for A177P-CBADH: 5′-CCCATTAAGCCAACAGGTCCAATGC CAATTACC-3′

for H222P-CBADH: 5′-CTTGATCAACTATACGACCATTTTT ATAA-3′

for L275P-CBADH: 5′-CTACACGTGGTATTGGTAAAGCATCT CC-3′

for L316P-CBADH: 5′-GTAACTAATTTACTTGGATCAACACGA TTA-3′

for A347P-CBADH: 5′-ATAATATAACTACTGGTTTAATTAA GTCTTT-3′ (1)

Protein expression and purification

All recombinant plasmids harboring CBADH and its mutated variants were transformed into *Escherichia coli* strain TG-2 (Sambrook et al., 1989). The tetrameric recombinant proteins were purified according to a modification of the procedure described by Peretz and Burstein (1989). Briefly, the transformed cells were cultured in LB with Ampicillin (100 mg/L) for 14–17 h at 37 °C, harvested by centrifugation at 8,000 × *g* for 20 min at 4 °C, and resuspended in 25 mM Tris-HCl, 0.1 mM DTT, 0.1 mM EDTA, 1 mM benzamidine, 0.02% sodium azide (pH 7.3) (buffer A). Cells were disrupted for 5 min by pulsed sonication (Branson Sonifier 450) using a rosette cup within ice, followed by centrifugation at 23,000 × *g* for 15 min, to remove cell debris. The supernatant was then heat-treated for 3 min at 65 °C and centrifuged again for 15 min at 12,000 × *g*. The clear supernatant was applied onto a DEAE-52-cellulose column (13 × 3 cm) (Whatman Chemicals, England), pre-equilibrated with buffer A at 4 °C, and was extensively washed with the same buffer until no protein was eluted (using Bradford reagent). The recombinant proteins were eluted from the column with a solution of 0.1 M NaCl in buffer A. The enzymatically active fractions were pooled and dialyzed extensively against buffer A for 12 h, and then applied to a short Red-Sepharose column (7 × 3 cm) (Pharmacia, Sweden). The recombinant enzyme was eluted from the column with a linear gradient of NaCl (0.1–2 M) in buffer A. Fractions containing enzymatic activity were collected and concentrated by ultra filtration (Amicon YM-30), and stored at 4 °C. All the enzymes used in this study were purified to homogeneity as judged by SDS-PAGE.

Enzyme assay and kinetic experiments

Enzymatic activity of the ADHs was measured at 40 or 60 °C by following the reduction of $NADP^+$ (and monitoring the formation of NADPH) at 340 nm (ϵ_{340} = 6.2 mM^{-1} cm^{-1}). The standard assay mixture contained 150 mM 2-propanol and 0.5 mM $NADP^+$ and 150 mM Tris-HCl (pH 8.8) in a total volume of 1 mL. One unit of ADH is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of 2-propanol/min under initial velocity at the above-mentioned conditions. Kinetic parameters were measured and calculated using a Beckman DU-7500 spectrophotometer equipped with a Multicomponent/SCA/Kinetics Plus software package and a thermostated water circulating bath. K_m values for 2-propanol were determined with varying concentrations of the alcohol (0.1–100 mM) and enzyme (5–120 nM) and 0.5 mM NADP (above saturation concentration of the coenzyme) in 100 mM Tris-HCl (pH 8.8). Values reported are the averages of three experiments and the individual measurements were within 10% of the quoted mean.

Thermal inactivation ($T_{1/2}^{60\,min}$) analysis

The thermal stability of ADH was determined by monitoring the residual enzymatic activity after 60 min incubation in 25 mM Na/K phosphate buffer (pH 6.8) containing 1 M NaCl at different temperatures. Enzymatic activity was assayed by spectrophotometric analysis of NADPH formation at 340 nm, under the standard conditions described above. Values are expressed as the means of three experiments, and the individual measurements were within 10% of the quoted mean.

Differential scanning calorimetric analysis

Calorimetric measurements were made in a high sensitivity differential scanning microcalorimeter (MicroCal MCS instrument). Experiments were performed between 20 and 105 °C at a scan rate of 1 °C/min. The protein sample (1–2 mg/mL), dialyzed against a solution of 25 mM sodium phosphate buffer containing 1 M NaCl (pH 8.25), and the dialysis buffer were degassed immediately before loading into sample and reference cells. The solutions in the measuring cells were kept under 2 atm pressure of nitrogen gas to prevent any degassing during heating. The baseline for each run was determined in an identical experiment with buffer in both cells and was subtracted from each scan. T_m was taken to be the maximum of the excessive heat capacity function (Fig. 5).

Analytical procedures

DNA sequencing was performed using an Applied Biosystems Model 373A DNA sequencer by the dideoxy method and appropriate primers.

SDS-PAGE was performed on 12% slab gels and 5% stacking gels (Laemmli, 1970), using a Bio-Rad MiniProtean II system. The gels were stained with Coomassie brilliant blue.

Protein concentrations were determined according to the methods of Bradford and Lowry, with bovine serum albumin as standard.

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