Extremely thermostable L(+)-lactate dehydrogenase from *Thermotoga maritima*: Cloning, characterization, and crystallization of the recombinant enzyme in its tetrameric and octameric state

RALF OSTENDORP, GÜNTHER AUERBACH, AND RAINER JAENICKE

Institute of Biophysics and Physical Biochemistry, University of Regensburg, D-93040 Regensburg, Germany

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Abstract

L(+)-lactate dehydrogenase (LDH; E.C.1.1.1.27) from the hyperthermophilic bacterium *Thermotoga maritima* has been shown to represent the most stable LDH isolated so far (Wrba A, Jaenicke R, Huber R, Stetter KO, 1990, *Eur J Biochem* 188:195–201). In order to obtain the enzyme in amounts sufficient for physical characterization, and to analyze the molecular basis of its intrinsic stability, the gene was cloned and expressed functionally in *Escherichia coli*. Growth of the cells and purification of the enzyme were performed aerobically at 26 °C, i.e., ca. 60° below the optimal growth temperature of *Thermotoga*. Two enzyme species with LDH activity were purified to homogeneity. Crystals of the enzyme obtained at 4 °C show satisfactory diffraction suitable for X-ray analysis up to a resolution of 2.8 Å. As shown by gel-permeation chromatography, chemical crosslinking, light scattering, analytical ultracentrifugation, and electron microscopy, the two LDH species represent homotetramers and homooctamers (i.e., dimers of tetramers), with a common subunit molecular mass of 35 kDa. The spectroscopic characteristics (UV absorption, fluorescence emission, near- and far-UV CD) of the two species are indistinguishable. The calculated α -helix content is 45%, in accordance with the result of homology modeling. Compared to the tetrameric enzyme, the octamer exhibits reduced specific activity, whereas K_M is unaltered. The extreme intrinsic stability of the protein is reflected by its unaltered catalytic activity over 4 h at 85 °C; irreversible thermal denaturation becomes significant at ~95 °C. The anomalous resistance toward chemical denaturation using guanidinium chloride and urea confirms this observation. Both the high optimal temperature and the pH optimum of the catalytic activity correspond to the growth conditions of *T. maritima* in its natural habitat.

Keywords: association; lactate dehydrogenase; thermophilism; thermostability; *Thermotoga maritima*

Thermotoga maritima represents the most thermophilic organotroph ($T_{max} = 90$ °C) within the bacterial branch of the phylogenetic tree that has been isolated so far (Huber et al., 1986; Ravot et al., 1995). A number of glycolytic enzymes from the bacterium have been isolated and characterized in the past in an

attempt to elucidate adaptive mechanisms of hyperthermophiles in their hostile environment (for a review, see Jaenicke et al., 1996).

In the case of L(+)-lactate dehydrogenases, homologues from a great variety of organisms, except hyperthermophiles, have been studied in the past (Holbrook et al., 1975; Garvie, 1980; Clarke et al., 1989; Wigley et al., 1992; Iwata et al., 1994). The enzyme catalyzes the last step in anaerobic glycolysis, the conversion of pyruvate to lactate, with the concomitant oxidation of NADH. In general, with few exceptions, the protein has been found to be a homotetramer with a subunit molecular mass of ~35 kDa. In analyzing the correlation of the structure and stability of proteins, LDH has been a paradigm (Zuber, 1988; Menéndez-Arias & Argos, 1989; Züllig et al., 1991; Kotik &

Reprint requests to: Rainer Jaenicke, Institute of Biophysics and Physical Biochemistry, University of Regensburg, D-93040 Regensburg, Germany; e-mail: jaenicke.vax1.rz.uni-regensburg.d400.de.

Abbreviations: BCA, bicinchoninic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GdmCl, guanidinium chloride; HEPES, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; LDH, L(+)-lactate dehydrogenase; PGK-TIM, phosphoglycerate kinase-triosephosphate isomerase; Bl, *Bifidobacterium longum*; Bs, *Bacillus stearothermophilus*; Sa, *Squalus acanthias*; Ss, *Sus scrofa*; Tm, *Thermotoga maritima*.

Zuber, 1993). However, so far, any attempts to assign increased thermal stability to specific exchanges at the protein or DNA level have failed (Jaenicke, 1991; Böhm & Jaenicke, 1994).

Because of the low expression level of TmLDH under standard growth conditions (Wrba et al., 1990a), a detailed characterization of the enzyme was not possible until the gene was cloned by complementation in a mesophilic host (Ostendorp et al., 1993). Here we report the cloning and functional expression of the gene in *Escherichia coli*. Together with the cloning, the expression, purification, physicochemical characterization, and crystallization of TmLDH are described. The enzyme is found to be the most thermostable LDH isolated so far. Surprisingly, apart from the major tetrameric fraction, about 10% of the recombinant protein occurs as a stable homooctamer.

Results

Cloning the *ldh* gene

The *ldh* gene was cloned as part of a 4.5-kb fragment of the *T. maritima* genome (pTLDH25); its nucleotide sequence was determined as described previously (Ostendorp et al., 1993). In order to obtain recombinant LDH in sufficiently high amounts, the *ldh* segment was subcloned and overexpressed. Amplification by PCR from pTLDH25 made use of oligonucleotide primers based on the published *ldh* sequence; the PCR product was cloned into the expression plasmid pKM1. The gene was sequenced and found to be identical with the source plasmid. The sequence data are available under accession number X74302 (*T. maritima ldh* gene) at the EMBL gene library.

Expression and isolation of the enzyme

The hyperthermophilic enzyme was expressed in *E. coli* at 26 °C and purified aerobically at room temperature. *E. coli* strain BL21DE3 (Studier & Moffatt, 1986) was transformed via electroporation with the new LDH expression plasmid pTmLDH1. Growth of the cells was optimized with respect to temperature, induction conditions, and incubation time. Surprisingly, highest yields of the enzyme were obtained without induction with isopropyl β -D-thiogalactoside, in agreement with previous studies on the expression of recombinant GAPDH from *T. maritima* (Tomschy et al., 1993). A possible explanation might be a mutation in the promoter region. After 20 h at 26 °C, the cell-free extracts showed maximum LDH activity in the standard assay at 55 °C; under this condition, the interference of *E. coli* enzymes is excluded. The given growth conditions in the mesophilic host differ from the in vivo conditions in *T. maritima* by ca. 60 °C, confirming earlier observations for GAPDH and the PGK-TIM fusion protein (Tomschy et al., 1993; Beaucamp et al., 1995). Considering the subtle balance of weak interactions involved in the stabilization of proteins (Jaenicke, 1991), the fact that the temperature in the given range does not affect the authenticity of the native enzyme is striking. It becomes even more surprising in denaturation/renaturation experiments in which folding and association have been found to be highly efficient over the whole range of temperatures between 4 and 95 °C (R. Ostendorp, unpubl. results).

The purification made use of the high thermal stability of the recombinant enzyme compared with the protein inventory of its mesophilic host; ca. 40 mg protein are obtained within a few

days starting from 2 L *E. coli* culture (Table 1). Nucleic acids and *E. coli* proteins were removed by protamine sulfate and high-temperature precipitation, without significant loss of the recombinant enzyme (Fig. 1). In order to prevent coprecipitation of *E. coli* proteins and recombinant LDH (produced as ca. 10% of total cell protein), the cell extract was diluted fivefold. The subsequent purification made use of anion-exchange and gel-permeation chromatography, as described in the Materials and methods. From the Q-Sepharose column, LDH-activity eluted as a single peak. However, including gel-permeation chromatography as an additional purification step, two peaks with LDH activity were observed: a high molecular weight component (ca. 5–10%) with an estimated molecular mass of 280 kDa, and the expected 140-kDa tetramer (Fig. 2). Rechromatography at room temperature and at varying protein concentration did not change the result, suggesting multiple forms of the enzyme. In accordance with other LDHs, an earlier estimate of the quaternary structure of the main component indicated that TmLDH is a tetramer (Wrba et al., 1990a). That the octamer represents an assembly product of the tetramer caused by the high expression level in *E. coli* can be excluded based on the result of preliminary reconstitution experiments at low protein concentrations (Dams, 1995). Using the present purification protocol, a total of ~22,000 units of enzyme activity (measured at 40 °C and pH 7) were recovered in 160 mL volume (Fig. 1).

Physical characterization

Molecular mass and states of association

LDH from *T. maritima* has been estimated previously to be a 140-kDa homotetramer (Wrba et al., 1990a), in accordance with the known average molecular masses of LDHs from mesophilic and thermophilic sources, and the monomer molecular weight of 34,994, calculated from the amino acid sequence (Ostendorp et al., 1993). In order to prove the authenticity of the recombinant enzyme and to confirm the tetrameric and octameric states of association, the molecular masses and the subunit compositions of the purified protein fractions were determined by SDS-PAGE, gel-permeation chromatography, chemical crosslinking, and analytical ultracentrifugation. As shown in Figure 1, SDS-PAGE reveals the same molecular

Table 1. Purification scheme of recombinant LDH from *T. maritima*

Fraction	Volume (mL)	Total protein ^a (mg)	Total units ^b (U)	Specific activity (U/mg)	Recovery (%)
Crude extract	230	1,104	63,731	58	100
Protamine sulfate precipitation	232	650	56,161	86	88
Heat precipitation	1,105	141	47,319	336	74
Q-Sepharose HP	80	80	45,092	564	71
Superdex 200 pg					
Tetrameric enzyme	96	32.3	20,582	637	32
Octameric enzyme	64	3.6	1,554	432	2

^a According to the BCA protein assay.

^b Measured at 40 °C and pH 7.0.

Fig. 1. SDS-PAGE of the purification steps of tetrameric and octameric LDH from *T. maritima*. Samples have been incubated for 10 min at 100 °C in the presence of 1.25% SDS and 2.5% β -mercaptoethanol. Lane 1, molecular mass standard; phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa); lane 2, crude extract; lanes 3,4, soluble supernatants after protamine sulfate and heat precipitation, respectively; lane 5, pooled fractions of LDH after anion exchange chromatography; lanes 6,7, combined fractions of tetrameric (M_4) and octameric (M_8) LDH after gel-permeation chromatography, respectively.

weight (~35,000) for both the tetramer and octamer, with no other bands detectable. Native gel-permeation chromatography provides clear evidence for the tetramer and octamer (Fig. 2A). The result is confirmed by chemical crosslinking of protein solutions with LDH concentrations down to 1 μ g/mL. Because the crosslinking reaction commonly does not go to completion, subsequent SDS-PAGE yielded monomeric, dimeric, tetrameric, and octameric species, all corresponding to the calibrated M -values (Fig. 2B). In the case of the native octamer, no monomeric and dimeric species were detectable.

As shown by dynamic light scattering experiments, the difference in the molecular masses between the homotetramer and homo-octamer is reflected by a large difference in the hydrodynamic volumes (Table 2). The estimated hydrodynamic radii of the octamer and tetramer correspond to the values observed for standard proteins in the 140- and 280-kDa molecular weight range. The results also prove homogeneity of both the tetramer and octamer, even after long-time incubation (Ott, 1996).

Analytical ultracentrifugation of the tetrameric and octameric fractions of the enzyme under the same conditions shows that both states are stable over a wide range of concentration. Sedimentation velocity experiments yield $s_{20,w}^0 = 7.0 \pm 0.2$ S and 10.2 ± 0.3 S, respectively. The result of high-speed sedimentation equilibria at protein concentrations <4 mg/mL is 136.6 ± 3.6 and 280.9 ± 4.8 kDa; the corresponding meniscus depletion experiments, with a lower concentration limit of ~40 μ g/mL, yield 132.8 ± 7.2 and 269.3 ± 8.4 kDa. In 4 M guanidinium chloride, the monomer is the only species observed under high-speed meniscus-depletion conditions; correcting for the solvent effect on the partial specific volume (Durchschlag & Jaenicke, 1982), the limiting value is 44.7 ± 4.5 kDa. As indicated by electron-microscopical image reconstruction, the tetramers and octamers are well-defined structural entities (T. Dams, R. Ostendorp, M. Ott, K. Rutkat, & R. Jaenicke, manuscript submitted; cf. inserts in Fig. 2A). There is no indication for a tetramer-of-dimers structure, as has been described previously for the octameric *Thermotoga* enolase (Schurig et al., 1995). Instead, a dumbbell-shaped dimer-of-tetramers is observed. Whether both species are present under normal growth conditions in *Thermotoga* needs further exploration.

Spectroscopic characteristics

Because of the low expression level of LDH in *T. maritima*, previous studies did not allow the spectral analysis of the natural enzyme. Applying UV absorption, fluorescence emission, and CD spectroscopy to the recombinant enzyme, the spectral characteristics of tetrameric and octameric TmLDH are found to be indistinguishable; evidently, the association of the tetramer has no conformational effects. Even the near-UV CD, which is considered a most sensitive fingerprint of protein conformation, does not show significant differences. The UV absorption spectrum has its maximum at 278 nm, with a shoulder at 283 nm (Fig. 3A), reflecting the dominance of the nine tyrosines over the single tryptophan residue; the 11 phenylalanine residues contribute mainly to the absorption in the 250–260-nm region. The A_{280}/A_{260} ratio for the purified recombinant enzyme is 1.9; the extinction coefficients for the tetrameric and octameric species (calculated according to Gill & von Hippel, 1989) are $71,240 \text{ M}^{-1} \text{ cm}^{-1}$ and $142,480 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.

The fluorescence emission spectrum with its maximum at 336 nm ($\lambda_{exc} = 280$ nm) is dominated by the single tryptophan residue (Fig. 3B). Upon denaturation in 6 M GdmCl, it is exposed to the solvent, leading to a drastic decrease in intensity and a red shift to 350 nm. A second peak at 305 nm may be attributed to the exposure of tyrosine residues. With increasing temperature, a blue-shift of the fluorescence maximum is observed, suggesting that hydrophobic interactions in the environment of aromatic residues become more pronounced. The same effect has been reported for glyceraldehyde 3-phosphate dehydrogenase from *T. maritima* (Wrba et al., 1990b). It supports the hypothesis that enhanced thermal stability is correlated with increased hydrophobic interactions within the core of the protein.

The near-UV and far-UV CD spectra closely resemble those observed for other LDHs (Rudolph & Jaenicke, 1977) (Fig. 3C,D). The far-UV region is typical for an α -helical protein with a minimum at 222 nm, corresponding to a calculated α -helix content of ~45% (Chen et al., 1972). This value exceeds the helicity of other LDHs, in accordance with the modeled structure of the

Fig. 2. Molecular masses of the different states of association of TmLDH. **A:** Gel-permeation chromatography on Superdex 200 HR in 20 mM sodium phosphate buffer, pH 6.9, in the presence of 1 mM EDTA and 0.1 M NaCl. Left: Linearization of the elution volumes of standard proteins and TmLDH. Markers ($\blacktriangle$): thyroglobulin (669 kDa), ferritin (450 kDa), catalase (232 kDa), aldolase (158 kDa), IgG from mouse (150 kDa), enolase from rabbit (88 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), cytochrome *c* (12.3 kDa). ($\circ$) represents the tetrameric and octameric LDH from *T. maritima*. Inserts: Electron-microscopical images of the two LDH species after negative staining (with 3% uranyl acetate) and image processing. Right: Elution profiles of the tetrameric (M_4) and octameric (M_8) TmLDH from the Superdex 200 HR column. **B:** Chemical crosslinking with subsequent SDS-PAGE on 5–25% gradient gels. Left: Linearization of the migration fronts of standard proteins and TmLDH on the gel. Markers ($\blacktriangle$): myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa). ($\circ$) represents the monomeric, as well as the crosslinked dimeric, tetrameric, and octameric LDH from *T. maritima*. Right: 5–25% SDS-PAGE. Lane 1, molecular mass standard (see above); lane 2, monomeric LDH subunit (not crosslinked); lane 3, crosslinked tetrameric enzyme (with a dimeric fraction below); lane 4, crosslinked octameric enzyme.

enzyme (see below). Upon denaturation in >4 M GdmCl at 40°C for 2 h, both the tetramer and the octamer are dissociated and denatured to the monomer in its fully unfolded form. Again, neither the far-UV nor the near-UV CD spectra reveal significant differences between the tetramer and the octamer.

The spectroscopic results corroborate the above observation that in the octameric form two tetramers must be connected as structurally unaltered entities; obviously, they do so via nonaromatic surface sites. No energy transfer and no change in helicity can be observed.

Catalytic properties and stability

In contrast to the close structural similarity between the tetramer and the octamer, the catalytic properties of the two entities

are different: the specific activity and $V_{\max}$ of the octamer are reduced compared to the tetramer; the apparent K_M values are unaltered and identical with the value obtained for the natural protein (Table 2). Based on the similarity of the three-dimensional structure (see below), we may assume that the binding sites for both the substrate and the coenzyme are located on the surface of the tetramer. Thus, alterations of the catalytic properties of the octamer compared to the tetramer may be attributed to either blocking of active sites or reduced flexibility of the assembly.

The “thermophilic properties” of the tetrameric enzyme in terms of its stability and catalytic efficiency are illustrated in Figure 4. The origin of the protein from a hyperthermophile is reflected by its extreme thermal stability, which exceeds the upper temperature limit of growth (85°C). This is in contrast to LDHs from mesophiles and moderate thermophiles, which have been

Table 2. Physicochemical characterization of recombinant LDH from *T. maritima*

	Tetramer fraction	Octamer fraction
Molecular mass (kDa) ^a		
Amino acid sequence	35/140	35/280
SDS-PAGE	33/—	33/—
Gel-permeation chromatography	—/145	—/271
Chemical crosslinking	33/64/149	—/—/270
Analytical ultracentrifugation		
0 M GdmCl	137 ± 4	281 ± 5 (10.2S)
1.0 M GdmCl		250 ± 2 (9.0S)
2.5 M GdmCl		90 ± 15 (5.4S)
3.0 M GdmCl		≤70 (2.4S)
4.0 M GdmCl		35 ± 8 (1.0S)
Size of the molecule (Å) ^b		
Dynamic light scattering (hydrodynamic diameter)	84	122
Electron microscopy (diameter)	88 ± 6	175 ± 5/85 ± 10
UV-absorption spectroscopy		
ϵ_M (M ⁻¹ cm ⁻¹) _{denat.} ^c	71,240	142,480
$\epsilon_{280\text{nm}, 0.1\%, 1\text{cm}}$ (cm ² mg ⁻¹)	0.55	0.55
$A_{280\text{nm}}/A_{260\text{nm}}$	1.9	1.85
λ_{max} (nm) native	278	278
Fluorescence spectroscopy		
λ_{max} (nm) native (40 °C)	336	336
λ_{max} (nm) 6 M GdmCl	350	350
CD-spectroscopy		
$\theta_{222\text{nm}}$ (grad cm ² dmol ⁻¹) (25 °C)	16,094	16,094
α -helix content (%) ^d	45	45
Catalytic activity		
Specific activity (U/mg) (55 °C, pH 6)	800	634
K_M app. pyruvate (μM) (38 °C)	19 ± 2	18 ± 2

^a Molecular masses of different states of association (monomer/oligomer), determined from the gene (amino acid) sequence (Ostendorp et al., 1993), SDS-PAGE in the presence of 2.5% β -mercaptoethanol, gel-permeation chromatography under standard conditions, glutaraldehyde crosslinking, and analytical ultracentrifugation (weight-average molecular mass from high-speed sedimentation equilibrium; sedimentation coefficients, $s_{20,w}$, in parentheses).

^b Hydrodynamic volume determined by dynamic light scattering under standard conditions (Ott, 1996); electron microscopy data represent the length and height of the projections of up to 500 particles (T. Dams, R. Ostendorp, M. Ott, K. Rutkat, & R. Jaenicke, manuscript submitted).

^c Calculated according to Gill and von Hippel (1989).

^d Calculated according to Chen et al. (1972).

shown to exhibit optimum activities near their physiological temperatures. In the case of the *Thermotoga* enzyme, it is the instability of the coenzyme in the enzyme assay rather than the enzyme itself that limits the determination of the maximum temperature of catalytic activity. Thus, the tabulated specific activities (cf. Table 2) are only meant to illustrate the relatively high catalytic efficiency of the enzyme compared with its mesophilic homologues. Considering the high activation energy of 114 kJ mol⁻¹ calculated from the Arrhenius plot in Figure 4A, the turnover of the enzyme under optimal growth conditions is of the order of 4,000 U/mg. How metabolites and cofactors are stabilized at high temperature within the cell is unknown. In the case of *Thermotoga*, because of the strict anaerobic status of the cytosol, an increased rate of resynthesis may be assumed as a possible mechanism (Hensel & König, 1988; Leibrock et al., 1995).

There is no indication for cold denaturation of the enzyme, even after long incubation at 0 °C, i.e., more than 80° below the physiological temperature (Fig. 4A). As shown in kinetic exper-

iments at varying temperatures, the extreme heat stability is also reflected by the long-term stability at temperatures up to 85 °C (Fig. 4B). At denaturation temperatures between 100 and 108 °C, the activation energy of irreversible thermal unfolding is found to be ~800 kJ mol⁻¹. In order to monitor the thermal transition of the recombinant enzyme, the irreversible heat inactivation after 2 h incubation at varying temperature was determined. Up to 95 °C, the activity remains unchanged, whereas above this temperature, deactivation is enhanced, until, at 105 °C, the enzyme is fully deactivated. Excess NAD⁺ shifts the limiting temperature slightly, at the same time increasing the cooperativity of the transition (cf. Wrba et al., 1990a; Rehder & Jaenicke, 1992).

As has been shown for other proteins from hyperthermophiles, the anomalous thermal stability is paralleled by an increase in stability against other denaturants such as urea or guanidine. For example, comparing the half-concentration, ($c_{\text{GdmCl}}/2$), in the case of porcine and *Thermotoga* LDH, the midpoint of denaturation is found to be shifted from 0.5 M to

Fig. 3. Spectroscopic properties of TmLDH. **A:** UV-absorption spectrum at 25 °C, protein concentration 0.87 mg/mL, pathlength, 1 cm. **B:** Fluorescence emission spectrum at 40 °C in arbitrary units, protein concentration 5 μ g/mL, pathlength, 1 cm, λ_{exc} = 280 nm. Native enzyme (—) and enzyme denatured in 6 M GdmCl (---). **C:** Far-UV CD spectrum at 25 °C, protein concentration 0.45 mg/mL for the native enzyme and the enzyme denatured in 6 M GdmCl. Tetramer (—) and octamer (---). **D:** Near-UV CD spectrum of the tetrameric (—) and the octameric (---) enzyme at 25 °C, protein concentration 0.85 mg/mL.

Fig. 4. Thermal activation and inactivation of TmLDH. **A:** Temperature dependence of the catalytic activity, measured in 50 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 5 mM cysteamine; protein concentration 15 μ g/mL. Insert: Arrhenius plot. **B:** Kinetics of thermal inactivation at various temperatures (buffer as in A); protein concentration 15 μ g/mL; for details see text.

~2 M. On the other hand, decreasing the temperature from 80 °C (close to physiological) to 40 °C, $(c_{\text{GdmCl}})_{1/2}$ of TmLDH is found to be increased from 1.0 M to 2.6 M (Fig. 5A). Further lowering of the temperature down to 5 °C does not show any further shift of the equilibrium transition. Thus, even in the presence of the denaturant, no significant downward curvature of the temperature profile of $(c_{\text{GdmCl}})_{1/2}$ is detectable, in contrast to observations reported for thermophilic PGK and GAPDH (Nojima & Noda, 1979; Rehder & Jaenicke, 1992). Using urea as the denaturant reveals that, at 20 °C, the enzyme is still active in the presence of 9 M urea (data not shown). The pH dependence of the activity of both the tetrameric and octameric forms of the enzyme results in a similar profile as reported for LDH from mesophiles (Fig. 5B). As indicated by the maximum activity around pH 6, the enzyme is optimally adapted to its cellular environment.

Crystallization

As has been shown previously, neither the amino acid composition nor the primary structure allow clear-cut conclusions with respect to the molecular basis of the extraordinary thermal stability of TmLDH to be drawn (Ostendorp et al., 1993). A solution to this problem can only be provided by the high-resolution three-dimensional structure of the enzyme. In order to progress along this line, crystals of the enzyme were grown, making use of the hanging-drop method (Fig. 6). Crystal growth is found to be inhibited strongly at room temperature, whereas at 4 °C, the enzyme shows a strong tendency to form crystals. This observation is unexpected because, 60° below its physiological temperature, the low flexibility of the enzyme should favor crystallization. The most stable crystals were obtained from solutions containing 5 mg/mL protein in 50 mM HEPES buffer, pH 7.5, in the presence of 0.5 M sodium acetate and 25 mM cadmium sulfate and excess NADH, with and without the alloste-

ric effector FBP and the substrate analogue oxamate, over 1 mL of 0.1 M HEPES, pH 7.5, 1 M sodium acetate, and 50 mM cadmium sulfate in the well. After equilibration at 4 °C, crystals of about $150 \times 270 \mu\text{m}$ were formed within a few days. Evaluation of the diffraction pattern is under way; the 2.8-Å resolution needs still to be improved in order to allow a detailed analysis of relevant structural differences between the known structures of LDHs from mesophilic and psychrophilic sources and the enzyme from *Thermotoga*.

Homology modeling

Because, at present, no X-ray structure is at hand, an attempt was made to model the three-dimensional structure based on known high-resolution data of mesophilic and thermophilic LDHs and the known amino acid sequence of the *Thermotoga* enzyme. The aim was not primarily to predict the structure with respect to high accuracy in the side-chain angles. Rather, it was intended to determine whether structural comparisons allow significant differences to be detected between TmLDH, on the one hand, and its less stable counterparts with known high-resolution structures, on the other. Such differences could then serve as a rational basis for site-directed mutations. LDHs from *Bacillus stearothermophilus* (Wigley et al., 1992), *Bifidobacterium longum* (Iwata & Ohta, 1993), and *Squalus acanthias* (Abad-Zapatero et al., 1987) with 2.5-, 2.0-, and 2.1-Å resolution, respectively, were used to model the unknown structure of TmLDH. Their sequences show between 42 and 51% identical amino acids, the RMS deviation of their C_α atoms in the subunit backbone is ≤ 2.4 Å. Because of the functional similarity of TmLDH and BILDH, the latter enzyme was used as a template following the method of Taylor (1988). Using the program COMPOSER, implemented in the software package SYBYL 6.1 (Tripos Associates, St. Louis, Missouri), the model was constructed by means of a structurally defined framework. The

Fig. 5. Guanidine- and pH-dependent denaturation of TmLDH. A: GdmCl-induced equilibrium unfolding transitions at 5 °C (◆), 40 °C (●), 60 °C (□), 70 °C (+), and 80 °C (▲) in 50 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA, 5 mM cysteamine, as monitored by fluorescence emission at 328 nm ($\lambda_{\text{exc}} = 280$ nm) at an enzyme concentration of 10 $\mu\text{g/mL}$. Samples were incubated for at least 48 h at the respective temperatures prior to the measurements. Insert: Temperature dependence of $(c_{\text{GdmCl}})_{1/2}$. B: Effect of pH on the catalytic activity of tetrameric (●) and octameric (○) TmLDH ($c = 10 \mu\text{g/mL}$) at 55 °C. Samples were incubated for 48 h at the indicated pH values. The following buffers were used: glycine-HCl, pH 1.4–3.4; citrate-sodium phosphate, pH 2.4–7.4; Tris(hydroxymethyl)-aminomethane-HCl, pH 7.4–9.0; glycine-NaOH, pH 9.0–12.5. Buffers were titrated with NaCl to a constant ionic strength of 0.1 at the respective pH.

Fig. 6. Crystals of tetrameric LDH from *T. maritima* grown at 4°C. Making use of the hanging drop method, the enzyme crystallized in 50 mM HEPES, pH 7.5, supplemented with 0.5 M sodium acetate and 25 mM cadmium sulfate in the presence of excess NADH, FBP, and oxamate. Bar: 100 μ m.

framework of structurally conserved regions was determined by superimposing the three homologous structures, applying the program INSIGHT 2.3.5 (Biosym Technologies, San Diego, California) in order to define topologically equivalent residues with a cut-off distance of 3.5 Å. Sequentially conserved amino acid residues were defined by multiple sequence alignment of the known prokaryotic LDH sequences, as shown in Figure 7. What is obvious from the sequence alignment is the anomalously short N-terminus and, more important, the C-terminal extension of TmLDH. Because there is no homologous sequence in the

C-terminal region, modeling was only performed to residue A309. Secondary structure prediction for the last 10 amino acid residues suggests an extension of helix α H (Fig. 8). The standard Needleman and Wunsch (1970) algorithm was used with a gap penalty of 8, based on the MDM 78-PAM 250 mutation probability index (Dayhoff et al., 1983). Secondary structural elements were determined using the program DSSP (Kabsch & Sander, 1983). The reliability of the final structure was demonstrated by a PROCHECK analysis (Laskowski et al., 1993). To refine the geometry of the model, energy minimization was performed making use of the program DISCOVER (version 2.96), with an all-atom force field and the algorithm steepest descents, followed by conjugate gradients minimization. The percentage of the template structures with respect to the model is as follows: 23.3% for BsLDH, 50.5% for BILDH, and 26.2% for SaLDH.

The predicted structure of TmLDH shows a close similarity to the known high-resolution structures reported by Rossmann et al. (1975). The NAD-binding domain (M1 to G139) shows the classical twisted β -sheet topology. Insertions and deletions are observed exclusively in loops and turns. The substrate binding site, with Q79, R85, D145, R148, and H172, is fully conserved. The predicted structure does not contain any residue in disallowed regions of the Ramachandran diagram: 85.7% of the residues are accumulated in the most favored regions, 12.9% in additional allowed regions, and 1.4% in "generously allowed regions." Thus, the model is fully compatible with the reference crystal structures at 2 Å resolution. Interpreting the Ramachandran diagram in terms of elements of secondary structure, there are 46% α -helices and 18.8% β -sheet, in agreement with both CD spectra and secondary structure predictions (Rost & Sander, 1994), which yield 45.5% α -helices and 15.5% β -sheet. Comparing this result to other LDHs, the secondary structure content of TmLDH seems to be increased slightly (Holbrook et al., 1975).

Fig. 7. Structure-based sequence alignment of amino acid sequences of LDHs from *T. maritima* (Tm), *B. stearotherophilus* (Bs), *B. longum* (Bl), and *S. acanthias* (Sa). Bold, homologous sequences whose three-dimensional structures were used for the modeling of the TmLDH structure. Shaded, amino acid residues identical with respect to the TmLDH sequence. α , β , secondary structure elements predicted for TmLDH. SCR, structural conserved regions in all structures used for the modeling procedure. LDH structures from the following PDB-files were used: 1LDN, 1LLD, and 1LDM for BsLDH, BILDH, and SaLDH, respectively.

Fig. 8. MOLSCRIPT (Kraulis, 1991) schematic representation of the structure-based model of the TmLDH monomer. Compared to known high-resolution structures, the N-terminus (preceding βA) is shortened to one amino acid residue (Met 1), whereas the C-terminal helix αH is proposed to be extended to Lys 313 (see also Fig. 7) and might fit into a groove on the surface of the molecule. The topology of the NAD-binding domain (βA - βF) and the residues responsible for substrate binding are strictly conserved in the hyperthermophilic enzyme.

Applying the criteria worked out by Böhm and Jaenicke (1994) and Hilbert et al. (1993), the predicted structure has to be considered with caution: given a sequence identity of <50%, homology modeling cannot make unequivocal structure predictions in diverging loop regions. Therefore, the possible contribution of helix-dipole stabilization or detailed information with respect to stabilizing hydrophobic interactions can only be derived from the high-resolution structure. Results that are unchallenged, apart from the minimum strain of the complete polypeptide chain in the ϕ/ψ space of the Ramachandran diagram, are summarized in Table 3.

Discussion

The molecular basis of thermophilic adaptation has been in the center of protein research for more than two decades. In recent years, 16S rRNA sequencing of a wide variety of bacteria and archaea led to the conclusion that hyperthermophiles represent the root of the phylogenetic tree, thus suggesting temperature-sensitive mutations as the major mechanism of evolution. How-

ever, the reason for increased or decreased thermal stability of proteins still remains enigmatic. One promising approach to come closer to the solution of the problem has been the analysis of variations in the solvent properties and the structures of homologous proteins regarding their intrinsic stability. In this context, hyperthermophilic bacteria are especially valuable. So far, only a few proteins have been analyzed to the level of resolution allowing tentative conclusions to be drawn. They belong, to a large extent, to NAD-dependent dehydrogenases.

In the present work, an attempt was made to extend previous studies on lactate dehydrogenases from psychro-, meso-, and thermophiles (Zuber, 1988; Jaenicke, 1991) to a hyperthermophilic bacterium, *T. maritima*, this way extending the temperature range of stability close to the upper limit of viability in the biosphere. The recombinant enzyme was expressed in *E. coli* in its authentic form, in spite of the large difference between the physiological temperatures of the guest and the host. However, there is a remarkable difference in that, at the anomalously high expression level, an octameric form of the enzyme is found in addition to the tetrameric main component. As taken from the electron-microscopical and spectral analyses, the contact surface of the octamer must be limited to a small area: there are no conformational differences detectable between the two states of association.

Comparisons of homologous lactate dehydrogenases, based on their amino acid composition, have no predictive value (Jaenicke, 1981, 1991). Similarly, sequence alignments of the primary structures of the various homologous LDHs so far did not yield unequivocal "traffic rules of thermal stabilization" (Böhm & Jaenicke, 1994). Also at the DNA level, GC contents in the range 31–62% (observed for two representative hyperthermophiles, *Methanococcus igneus*, $T_{max} = 91^\circ\text{C}$ and *Pyrococcus furiosus*, $T_{max} = 110^\circ\text{C}$) are not conclusive. However, what is evident from the alignment of the primary structures is that (1) the overall structure of TmLDH must be closely similar to its mesophilic counterparts, and (2) the catalytic mechanism is obviously conserved (Ostendorp et al., 1993).

The detailed three-dimensional structure requires further optimization of the crystallization conditions. As far as homology modeling allows a prediction of the spatial structure, the topology of the tetramer is to a high degree compatible with the known high-resolution structures of the LDHs from *B. stearotherophilus*, *B. longum*, and *S. acanthias*. Using the criteria worked out by Hilbert et al. (1993), the predicted structure shows an op-

Table 3. Homology modeling of LDH from *T. maritima*^a

Source	SaLDH	SsLDH	BsLDH	TmLDH
Number of polar residues	452	440	392	396
Number of apolar residues	576	596	588	564
Number of charged residues	288	288	284	316
Number of guanidinium groups	36	44	68	72
Number of salt bridges	160	173	202	238
Native "solvent-excluded volume" ($\text{\AA}^3$)	163,670	165,812	155,080	147,420
Packing density of the protein core region	0.658	0.646	0.667	0.701

^a Structural properties of mesophilic (Sa, *S. acanthias*; Ss, *S. scrofa*) and thermophilic (Bs, *B. stearotherophilus*) LDHs with known three-dimensional structures are compared with the characteristics of *T. maritima* LDH (TmLDH), calculated according to Böhm and Jaenicke (1992).

timum fit with the allowed regions in the Ramachandran diagram, thus indicating minimum strain in the native structure (Auerbach, 1994), in accordance with the close similarity of the physical properties of the enzymes under consideration.

As has been shown in the case of GAPDH from *T. maritima*, even at 2.5 Å resolution, no drastic structural changes can be detected if all available high-resolution structures of the enzyme are compared (Korndörfer et al., 1995). Significant differences refer to the increase in surface charges. They are assumed to participate in peripheral ion pairs, thus confirming the stabilization mechanism proposed by Perutz and Raidt (1975). Homology modeling of TmLDH points into the same direction (Table 3). Another contribution, taken from secondary structure predictions and homology modeling, is a slight increase in helicity in the case of the *Thermotoga* enzyme. Having in mind that the difference in free energy of stabilization between mesophilic and thermophilic proteins does not exceed 50–100 kJ mol⁻¹ (Jaenicke, 1991), this may contribute significantly to the increased intrinsic stability. Apart from these alterations, minor local changes or alterations in the binding of extrinsic factors may cause additional stabilization (Wigley et al., 1987; Züllig et al., 1990, 1991; Kallwass et al., 1992).

Because thermal stabilization of proteins is the cumulative effect of small improvements over the whole molecule, general strategies can hardly be expected. Contributions come from (1) peripheral ion pairs, (2) increased hydrophobicity or reduced hydrophobic surface area, and (3) increased packing density of the inner core of the protein. That these principles also hold for TmLDH is strongly supported by physicochemical data and molecular modeling, even without the detailed knowledge of the experimental high-resolution structure (cf. Tables 2, 3). The fact that, in the absence of the N-terminal "arm" the state of association remains unaltered at low protein concentration as well as high ionic strength, points to increased hydrophobic interactions in the subunit interfaces (Holbrook et al., 1975; Girg et al., 1983), similar to results reported for the enzyme from *B. stearothermophilus* (Müller et al., 1984). The hypothesis that the C-terminal extension might play the role of the N-terminal arm can be excluded based on mutational analyses of the truncated enzyme lacking the C-terminal residues 311–319 (R. Ostendorp & R. Jaenicke, manuscript submitted).

One additional feature of TmLDH, which has not been observed for other LDHs in the past, is the dimerization of the tetramer to form dumbbell-shaped octamers. They are found under normal growth conditions of *E. coli* when recombinant TmLDH is expressed in the mesophilic host. Whether they are significantly populated under physiological conditions in *Thermotoga* needs further experiments. As shown by analytical ultracentrifugation and electron microscopy, the octamer is the only stable species, apart from the tetrameric main component; there are no higher polymers detectable. Tetramers and octamers can be separated as homogeneous entities undergoing reassociation after preceding complete dissociation and denaturation, e.g., in 6 M guanidinium chloride. With these characteristics they differ from aggregates of BsLDH observed under pre-denaturation conditions at 55 °C (Kotik & Zuber, 1992). Similarly, they cannot be related to the concentration-dependent self-association of bovine liver glutamate dehydrogenase (Eisenberg et al., 1976). In contrast to these two systems, the anomalous quaternary structure of the recombinant TmLDH is independent of temperature, protein concentration, and effectors such as NADH

and FBP. Deeper insight into relevant structural differences are to be expected from the detailed electron microscopic and crystallographic analyses, which are in progress.

Materials and methods

Cloning and expression

Recombinant DNA techniques were based on Sambrook et al. (1989), using enzymes purchased from Boehringer Mannheim, New England Biolabs, and Stratagene. DNA sequencing was performed by the dideoxy-termination method of Sanger et al. (1977), using the T7-sequencing kit from Pharmacia. Oligonucleotides were supplied by MWG-Biotech (München).

The gene coding for LDH was amplified from the plasmid pTLDH25 by PCR, using oligonucleotide primers based on the published *ldh* sequence (Ostendorp et al., 1993). The amplified gene was cloned into the expression vector pKM1 (kindly provided by K. Maskos, ETH Zürich) via the restriction sites *Bam*H I and *Hind* III; pKM1 is a derivative of pASK40 (Skerra et al., 1991) with a T7 promoter replacing the lac promoter.

Cells of *E. coli* BL21DE3 (Studier & Moffatt, 1986), harboring the LDH expression plasmid (pTmLDH1), were grown at 26 °C for 20 h in 2 L LB-medium containing ampicillin (100 µg/mL).

Purification of the enzyme

All chromatographic steps were performed aerobically at room temperature. Chromatographic materials were purchased from Pharmacia. Unless indicated otherwise, the following standard buffer was used: 50 mM Tris/HCl, pH 7.7, containing 5 mM cysteamine and 1 mM EDTA (buffer A).

After determination of the absorbance at 584 nm, cells were harvested by centrifugation (4,000 × g, 20 min, 4 °C) and resuspended in buffer A to a calculated optical density of 50. Cell disruption was performed by two passages through an ice-cold French press cell at 15–20,000 psi. After addition of 30 µg/mL concentrated protamine sulfate solution (50 mg/mL), the suspension was centrifuged (30,000 × g, 60 min, 4 °C), and the supernatant diluted 1:5 with buffer A. The crude extract was then heated to 80 °C for 20 min and cleared of the aggregated material by centrifugation (48,000 × g, 90 min, 4 °C). After concentrating the solution by Amicon ultrafiltration (10-kDa cutoff), the sample was applied onto a 40-mL Q-Sepharose HP column (2.6 × 8 cm) equilibrated with buffer A. After elution with a linear salt gradient (0–0.4 M NaCl, total volume of 200 mL), the solution was concentrated by ultrafiltration and applied to a 120-mL Superdex 200-pg column (1.6 × 60 cm) preequilibrated with 0.1 M NaCl in buffer A. The active fractions were pooled and stored at 4 °C.

Protein concentration

The molar extinction coefficient of unfolded LDH was calculated from the amino acid sequence (Ostendorp et al., 1993), according to Gill and von Hippel (1989): $\epsilon_{280\text{nm}} = 71,240 \text{ M}^{-1} \text{ cm}^{-1}$. From the comparison of the absorbance of native and denatured LDH (6 M GdmCl) at 280 nm, the absorption coefficient for the native enzyme was determined: $A_{280\text{nm}, 0.1\%, 1\text{cm}} = 0.55$.

Concentration determinations of other proteins made use of the BCA protein assay (Pierce, Rockford) with bovine serum albumin as standard.

Enzyme assay

Unless noted otherwise, substrates, coenzymes, and buffer substances were purchased from Boehringer Mannheim or Pharmacia. LDH activity measurements made use of the conversion of pyruvate to lactate (oxidation of NADH). The standard assay was performed in 0.1 M imidazole/HCl buffer, pH 6.0, 1 mM EDTA, 5 mM cysteamine, supplemented with 0.6 mM sodium pyruvate, and 0.02 mM FBP at 55 °C. In order to exclude reactivation during the assay, trypsin (50 µg/mL) was included, altering the buffer conditions to pH 7.0 and 40 °C. After adding 0.27 mM NADH, the decrease in 366-nm absorbance was recorded, correcting for NADH autooxidation at high temperature and acidic pH. The temperature in the cuvette was checked by using a thermistor telethermometer.

Spectral analysis

UV absorption measurements were performed in a Cary 118C and a Perkin-Elmer 5 UV/VIS spectrophotometer, fluorescence emission in a Perkin-Elmer Fluoromax spectrophotometer (protein concentration 5–10 µg/mL in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 5 mM cysteamine at 40 °C). Equilibrium transitions were monitored by measuring the change in fluorescence emission at 333 nm, using a constant excitation wavelength of 280 nm. CD spectra were recorded in a Jasco J500A CD spectropolarimeter at 450–850 µg/mL protein concentrations, using 1-cm and 0.01-cm thermostated quartz cuvettes for the near-UV and far-UV region, respectively. The mean residue weight of 109.7 for LDH was calculated from the amino acid sequence. Dynamic light scattering made use of a Spectra Physics 165 Laser and an ALV 5000 Korrelator (ALV, Langen). The protein was diluted to a concentration of 0.4 mg/mL in 20 mM sodium phosphate buffer, pH 7.0, plus 2 mM EDTA and 5 mM cysteamine at 20 °C. Densitometric scans of immobilized proteins on SDS-polyacrylamide gels (stained with Coomassie blue) were performed on an Elscript 400 densitometer (Hirschmann, Unterhaching).

Molecular mass

The quaternary structure of the enzyme was determined by analytical ultracentrifugation and chemical crosslinking with glutaraldehyde (Hermann et al., 1981; Zettlmeissl et al., 1982). The procedure was optimized regarding glutaraldehyde concentration (1% w/v), protein concentration (1 µg/mL), and the subsequent SDS-PAGE (5–25% acrylamide gels).

Sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Model E ultracentrifuge equipped with a high-sensitivity light source and photoelectric scanning system, making use of double sector cells (12 mm) with sapphire windows in an An-G rotor. In order to detect possible concentration-dependent dissociation, the meniscus-depletion technique (Yphantis, 1964) was applied. Scanning wavelengths were 235 and 280 nm, respectively. Sedimentation coefficients were determined at 44,000 rev/min, plotting $\ln r$ versus time, and correcting for 20 °C and water viscosity. Sedimentation equilib-

ria at 8,000 and 11,000 rev/min were calculated from $\ln c$ versus r^2 plots, making use of a computer program developed by G. Böhm (University of Regensburg).

Stability

In order to monitor unfolding equilibrium transitions, the protein was incubated for at least 48 h in 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 5 mM cysteamine, at varying concentrations of guanidinium chloride or urea. Denaturant concentrations were calculated from the refractive indices of the solutions (Pace, 1986). The thermal stability was measured by incubating the enzyme solutions in 0.5-mL safe-lock Eppendorf-microtubes, overlaid with Nujol mineral oil (Perkin-Elmer), for 2 h at temperatures between 40 and 105 °C. Residual activities were determined at 40 °C in the presence of trypsin (Sigma) (Chan et al., 1973). The kinetics of thermal deactivation were followed by incubating the same samples at temperatures between 85 and 108 °C. Aliquots were taken at different times, and the residual activities measured as described before. pH-dependent activity measurements in the range between pH 1.4 and 12.5 were performed using different buffers adjusted to the same ionic strength and the temperature of the standard enzyme assay.

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