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Sequence and homology model of 3-isopropylmalate dehydrogenase from the psychrotrophic bacterium *Vibrio* sp. I5 suggest reasons for thermal instability

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The *leuB* gene from the psychrotrophic strain *Vibrio* sp. I5 has been cloned and sequenced. The gene codes for 3-isopropylmalate dehydrogenase, a 360-residue, dimeric enzyme involved in the biosynthesis of leucine. Three recently solved homologous isopropylmalate dehydrogenase (IPMDH) crystal structures from thermophilic and mesophilic organisms have been used to build a homology model for the psychrotrophic IPMDH and to deduce the possible structural reasons for its decreased thermostability. According to our model the psychrotrophic IPMDH contains fewer stabilizing interactions than its mesophilic and thermophilic counterparts. Elements that have been identified as destabilizing in the comparison of the psychrotrophic, mesophilic and thermophilic IPMDHs are a smaller number of salt-bridges, a reduction in aromatic–aromatic interactions, fewer proline residues and longer surface loops. In addition, there are a number of substitutions of otherwise strictly conserved residues that can be linked to thermostability.

Keywords: cold adaptation/homology model/isopropylmalate dehydrogenase/psychrotroph/sequence

Introduction

The analysis of the structure–stability relationship of proteins from extremophiles and the comparison with their mesophilic counterparts has provided insights into the factors governing thermal stability. In recent years several structures of thermophilic enzymes have been solved and have been compared with their mesophilic counterparts (Fujinaga *et al.*, 1993; Russell *et al.*, 1994; Hennig *et al.*, 1995; Korndörfer *et al.*, 1995; Yip *et al.*, 1995). So far, the other end of the spectrum, enzymes from psychrophilic organisms, has been largely neglected. This is mainly due to the instability of the psychrophilic enzymes and in some cases the lack of isogeny to their mesophilic counterparts. The X-ray structure of salmon trypsin, a phoikilotherm, i.e. a cold-adapting organism, is the only structure of a cold-adapted enzyme solved to date (Smalas *et al.*, 1994). The authors attributed the lower thermostability of the salmon trypsin to weaker interdomain interactions, to a decrease in the size of hydrophobic amino acids in the core and to a smaller number of interactions of the C-terminal helix with the rest of the molecule. Sequence comparison and structure prediction have been performed on α-amylase [from *Alteromonas haloplanctis* (Feller *et al.*, 1992)], subtilisin [from *Bacillus* TA41 (Davail *et al.*, 1994)] and triosephosphate isomerase [from *Moraxella* sp. TA137 (Rentier-Delrue *et al.*, 1993)].

Psychrophilic enzymes are found to be heat-labile and have a higher specific activity than their mesophilic counterparts due to either a higher turnover number (k_{cat}) or an increase in substrate affinity (i.e. lower K_M) at temperatures higher than their usual working temperatures (Asgeirsson *et al.*, 1989). It has been argued that an increase in conformational flexibility may lead to higher catalytic efficiency because this can reduce the activation energy barrier for the chemical reaction. This increase in flexibility may be the mechanism by which psychrophilic organisms compensate for the decrease in catalytic rates at low temperatures.

We have cloned and sequenced the *leuB* gene from *Vibrio* sp. I5, a psychrotrophic organism from arctic seawater (Wiebe *et al.*, 1992). The product of the *leuB* gene is 3-isopropylmalate dehydrogenase (IPMDH), which catalyzes the penultimate step in leucine biosynthesis. The enzyme is a dimer of identical subunits and uses NAD^+ and a divalent cation, preferably Mn^{2+}, as cofactors. IPMDH has been sequenced from a large number of microorganisms and plants. So far, sequences of IPMDHs originating from three thermophiles and 12 mesophiles, but no psychrophiles, are available. In addition, the crystal structures of two mesophilic [*Escherichia coli*, EcIPMDH, and *Salmonella typhimurium*, StIPMDH (Wallon *et al.*, 1996)] and a thermophilic [*Thermus thermophilus*, TtIPMDH (Imada *et al.*, 1991)] IPMDH have been determined. (The optimum growth temperature of the thermophile *Thermus thermophilus* is 75°C, that of the mesophiles *Escherichia coli* and *Salmonella typhimurium* is 37°C and that of *Vibrio* sp. I5 is around 20°C.) We have compared the IPMDHs from the thermophilic and mesophilic organisms and have identified some of the characteristics that lead to the stabilization of the thermophilic enzyme. That study suggests that the higher stability of the thermophilic enzyme is due to a larger number of hydrogen bonds and ionic interactions, shortening of surface loops, decrease of main-chain flexibility through additional prolines and a highly hydrophobic interface. We want to investigate whether the principles learned in that comparison will hold true over the whole spectrum of thermostability in IPMDH. The three available crystal structures provide a good background for modeling the structure of the *Vibrio* sp. I5 IPMDH (VibIPMDH).

We present here a three-dimensional model of the psychrotrophic IPMDH based on the crystallographic structures of IPMDHs available to date (TtIPMDH, EcIPMDH and StIPMDH). The model allows us to speculate on the reasons for the decreased thermostabilty of the psychrotrophic enzyme and to design point mutations to test our observations.

We have determined stability and activity of VibIPMDH and established its psychrotrophic characteristics as compared with the mesophilic and thermophilic enzymes.

Materials and methods

Cloning of the Vibrio sp. I5 leuB gene

A culture of *Vibrio* sp. I5 was grown at 16°C in LB medium over a period of 24 h. Chromosomal DNA was isolated and partially digested with *Sau3*AI (New England Biolab). The fragments were sized in a sucrose gradient. Fragments of 3–6 kb were selected and cloned into *Bam*HI-digested and dephosphorylated pUC18 vector (Pharmacia). The clones were transformed into *E.coli* Xl1-blue supercompetent cells (Stratagene); 80–90% of the transformants were estimated to contain an insert. DNA was isolated from a pool of 3.2×10^4 individual colonies, which represents a library which is about threefold overdetermined for the purpose of screening for a gene of 1.1 kb. This library was amplified, plasmid DNA was isolated and transformed into *E.coli* OM17 (Δ*leuB*, a derivative of JM105; T.Oshima, personal communication). The transformants were grown on minimal medium without leucine to select for clones that are able to functionally complement a *leuB* deficiency.

Sequencing

Primers were synthesized using a Millipore DNA Synthesizer, five primers for the coding strand and four for the complementary strand (Figure 1a). The plasmid DNA was sequenced by PCR reaction using the PRISM DyeDeoxy Terminator reaction mix (Applied Biosystems). The sequencing reactions were analyzed with the Applied Biosystems 373A DNA Sequencer. The data from the ABI Sequencer were processed using the software Sequencher 2.1.1 for the Macintosh computer. Each nucleotide within the coding sequence was at least threefold oversequenced.

Thermal stability

The temperature sensitivity of the *Vibrio* sp. I5 *leuB* product was tested *in vivo* by complementation in OM17.

Thermal stability was measured in a DASM-4M differential scanning microcalorimeter in KPO_4, pH 7.6, 0.3 M KCl (data not shown). Additionally, residual activity was measured after incubation at 62°C in KPO_4, pH 7.6, 0.3 M KCl for increasing time periods (Figure 2).

Model building

The structure of VibIPMDH was modeled by homology-modeling using the structures of Tt-, Ec- and StIPMDH as templates (the PDB accession code for TtIPMDH is 1IPD). The structural alignment of the templates was prepared by combining automatic least-squares superposition and visual inspection. The sequences of Tt-, Ec-, St- and VibIPMDH were aligned with the Needleman–Wunsch algorithm. The alignment and the template structures were used to calculate ten 3D models for VibIPMDH using the program MODELLER (Sali and Blundell, 1993), version 3. In order to obtain reliable coordinates for the side chains at the subunit interface the dimeric form of the enzymes was used as a template. Three optimization cycles were carried out on the models and the model with the best stereochemical quality was selected as the representative model. The model was evaluated by the 3D profile method (Bowie *et al.*, 1991) using the program ProsaII (Sippl, 1993).

Results and discussion

Isolation of the psychrotrophic leuB gene and thermostability of VibIPMDH

The *leuB* gene from *Vibrio* sp. I5 was isolated based on its ability to complement leucine auxotrophy in JA221. About 1.5×10^5 colonies were screened at 30°C. Approximately 300 colonies grew under these restrictive conditions. Fourteen colonies were selected for further investigation. Retransformation confirmed the phenotype and restriction analysis showed that the inserts were 2.8–3.9 kb in size. The smallest clone was selected for sequencing. The clone carries the complete *leuB* gene and parts of the *leuC* gene upstream of *leuB*. The gene consists of an open reading frame (ORF) of 1080 bp, starting with the initiation codon ATG and ending with the termination codon TAA, coding for a protein of 360 amino acids (Figure 1b). The sequence is relatively G + C-rich with 58% G + C content. The sequence of VibIPMDH is 51% identical with TtIPMDH and 54% identical with St- and EcIPMDH, which indicates that it is not more closely related to either one or the other gene.

The enzyme supported growth of the Δ*leuB* cells at 30 and 37°C but not at 42°C. Measurements in a differential scanning calorimeter indicate that the VibIPMDH T_m is 8°C below that of its mesophilic counterpart from *E.coli* (data not shown). In our hands, the T_m of TtIPMDH is 90°C, that of EcIPMDH is 73°C and that of VibIPMDH is 66°C. The comparison of the residual activity of Vib- and EcIPMDH at 62°C clearly demonstrates that the psychrotrophic VibIPMDH is much less thermostable than its mesophilic counterpart (Figure 2).

Model building and refinement

IPMDH is a dimeric enzyme that is divided into two domains, an N- and C-terminal domain and a subunit interface forming domain. The two domains are connected by a 10-stranded β-sheet (Figure 3). The subunit interface of IPMDH is made up of helices g and h from each subunit, forming a four-helix bundle and the arm-like region that interacts with the same region from the other subunit forming a continuous antiparallel β-sheet (β-strands K and L). The active site is in a cleft between the two domains and is made up by residues from both monomers. The active site residues implicated in substrate and cofactor binding are strictly conserved between Ec-, St- and VibIPMDH.

We used the X-ray structures of Ec-, St- and TtIPMDH to model the structure of the psychrotrophic enzyme. In the sequences of these four enzymes 144 residues are identical and an additional 86 are identical with either the Ec-, St- or TtIPMDH. The evaluation of the quality of the model was performed by 3D profile analysis. The energy diagram does not point to any gross errors in the model structure. The relative reliability of different regions of the model was evaluated by calculating the variance in corresponding Cα positions among the 10 models generated by MODELLER (Sali and Blundell 1993). This calculation shows that the least reliable parts are in the arm-like region and a variable surface

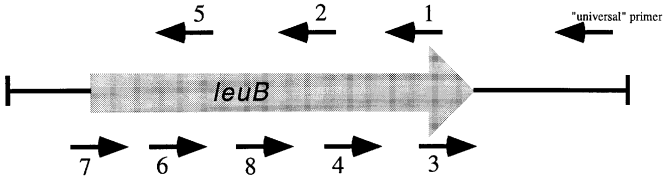

Fig. 1. (**a**) Location of primers used in sequencing the *Vibrio* sp. I5 *leuB* gene and surrounding sequence. Arrows show the direction of sequencing. (**b**) Nucleotide sequence and predicted protein primary structure of the *Vibrio* sp. I5 *leuB* gene. The Shine–Dalgarno sequence (S/D) and the stop-codon (*) are indicated. There is a good promoter upstream of the *leuB* gene, the gene coding for aspartate semialdehyde dehydrogenase follows downstream.

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                                                    ↓BsaAI
  1 TTTGACCTTGAAAGACACGATTGACCCAAAACCTTTGAAAGCACGTATCAGGCAGCGCACCCTTGTCTGTTTGCCGCCAT 80
                          -35                       -10
 81 CAAGTAATCAGTGGGCGAGTGCATCGGTCATTCCCCGCTTAGTGAGTTGAGAAGAAC ATG TCC AAC CAA ATT    152
  1                                        S/D                M   S   N   Q   I     5

153 CTG ATT CTC CCC GGT GAC GGT ATT GGC CCG GAA ATC ATG GCC GAA GCG GTC AAA GTG CTG  212
  6 L   I   L   P   G   D   G   I   G   P   E   I   M   A   E   A   V   K   V   L   25

213 AAC CTG GCC AAC GAA AAG TAC AGT CTG GGT TTT GAG TTG AGC TTT GAT GAC CTC GGT GGT  272
 26 N   L   A   N   E   K   Y   S   L   G   F   E   L   S   F   D   D   L   G   G   45

273 GCA GCC ATC GAC CGT TAC GGT GTG CCG TTG GCT GAC GAA ACC TTG GCC CGT GCT AAG GCC  332
 46 A   A   I   D   R   Y   G   V   P   L   A   D   E   T   L   A   R   A   K   A   65

333 GCT GAT GCC ATT CTG CTG GGT GCC GTA GGC GGT CCG AAA TGG GAT GCC ATC GAC CCA GCT  392
 66 A   D   A   I   L   L   G   A   V   G   G   P   K   W   D   A   I   D   P   A   85

393 ATT CGT CCA GAG CGC GGT CTG CTG AAG ATT CGT TCG CAG CTG GGT CTG TTT GGC AAT CTG  452
 86 I   R   P   E   R   G   L   L   K   I   R   S   Q   L   G   L   F   G   N   L  105

453 CGT CCG GCC ATC CTC TAT CCG CAA CTG GCC GAT GCG TCT AGC CTC AAG CGC GAA ATC GTT  512
106 R   P   A   I   L   Y   P   Q   L   A   D   A   S   S   L   K   R   E   I   V  125

513 GCC GGC TTG GAC ATT CTC ATC GTC CGC GAA CTG ACC GGC GGC ATC TAT TTT GGT CAG CCG  572
126 A   G   L   D   I   L   I   V   R   E   L   T   G   G   I   Y   F   G   Q   P  145

573 CGT GAA AGC AAA GTG CTG GAG AGC GGC GAG CGG ATG GCC TAT GAC ACC TTG CCT TAC AGC  632
146 R   E   S   K   V   L   E   S   G   E   R   M   A   Y   D   T   L   P   Y   S  165

633 GAG AGT GAA ATC CGC AGG ATT GCT CGT GTT GGT TTT GAC ATG GCC CGT GTG CGC GGC AAG  692
166 E   S   E   I   R   R   I   A   R   V   G   F   D   M   A   R   V   R   G   K  185

693 AAG CTT TGC TCG GTG GAC AAG GCC AAC GTA CTG GCG TCC AGC CAA CTG TGG CGC ACC GTG  752
186 K   L   C   S   V   D   K   A   N   V   L   A   S   S   Q   L   W   R   T   V  205

753 GTC ATT GAG GTA GCC AAG GAT TAT CCG GAC GTC GAA CTC AGC CAC ATG TAC GTT GAT AAC  812
206 V   I   E   V   A   K   D   Y   P   D   V   E   L   S   H   M   Y   V   D   N  225

813 GCT GCG ATG CAG CTG GTG CGC GCG CCC AAG CAG TTC GAT GTA ATG GTC ACC GAC AAT CTG  872
226 A   A   M   Q   L   V   R   A   P   K   Q   F   D   V   M   V   T   D   N   L  245

873 TTT GGC GAC ATT TTG TCG GAT GAG GCT TCC ATG CTT ACT GGT TCC ATC GGC ATG CTG CCG  932
246 F   G   D   I   L   S   D   E   A   S   M   L   T   G   S   I   G   M   L   P  265

933 TCC GCT TCG CTG GAT GCC AAC AAC AAA GGC ATG TAC GAA CCG TGC CAC GGC AGC GCG CCG  992
266 S   A   S   L   D   A   N   N   K   G   M   Y   E   P   C   H   G   S   A   P  285

993 GAT ATC GCC GGA CAG GGC ATT GCC AAC CCG TTG GCG ACC ATC CTG TCG GTG TCG ATG ATG 1052
286 D   I   A   G   Q   G   I   A   N   P   L   A   T   I   L   S   V   S   M   M  305

1053 TTG CGT TAC AGC TTC AAC CAG ACG GCT GCA GCC GAT GCG ATC GAG CTG GCG GTG AGC AAC 1112
 306 L   R   Y   S   F   N   Q   T   A   A   A   D   A   I   E   L   A   V   S   N  325

1113 GTG CTG GAT CAG GGC CTG CGT ACC GGT GAT ATT TAC AGC GCC GGT ACG ACC AAG GTC GGC 1172
 326 V   L   D   Q   G   L   R   T   G   D   I   Y   S   A   G   T   T   K   V   G  345

1173 ACC ACA GCA ATG GGC GAT GCA GTA GTC GAA GCG TTA CGC AGT CTT TAA TCTTCCTGGCCCGTCG 1236
 346 T   T   A   M   G   D   A   V   V   E   A   L   R   S   L   *                  361

1237 GACGGTCTCCGGCGGTCAGCTTATATAGGTGTAGTGGTT ATG AAA CGT GTA GGT CTG ATC GGT TGG CGT 1305
   1                            S/D           M   K   R   V   G   L   I   G   W   R    10
1306 GGC ATG GTC GGT TCC GTG CTG ATG CAA CGC ATG CTG GAA GAG CAG GAT TTC GAC TTG GAT 1365
  11 G   M   V   G   S   V   L   M   Q   R   M   L   E   E   Q   D   F   D   L   D   30
1366 CGA ACC GGT ATT CTT CAC CAC CTC CAA TGT TGG CGG TCA AGG GCC GGC GGA TTG GTA AGG 1425
  31 R   T   G   I   L   H   H   L   Q   C   W   R   S   R   A   G   G   L   V   R   50
1426 AAA CGG CTG CGC TAA AAG ATG CCT ACA GAT TGA CGAGCTGAAAAGCCTCGACGTGATCCTCTAGAGTCG 1494
  51 K   R   L   R
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Fig. 1. continued

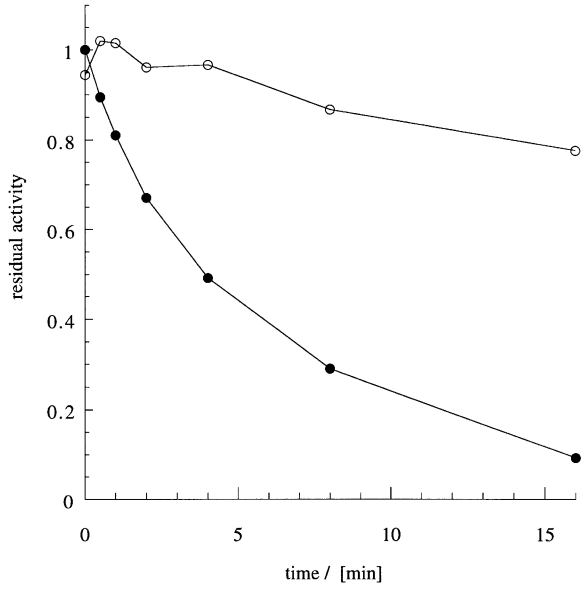

Fig. 2. Residual activity of VibIPMDH and EcIPMDH after incubation at 62°C in KPO_4, pH 7.6, 0.3 M KCl. The activity of the psychrotrophic enzyme declines much faster than that of its mesophilic counterpart. After 4 min, VibIPMDH has only 50% residual activity, whereas EcIPMDH is still almost 100% active.

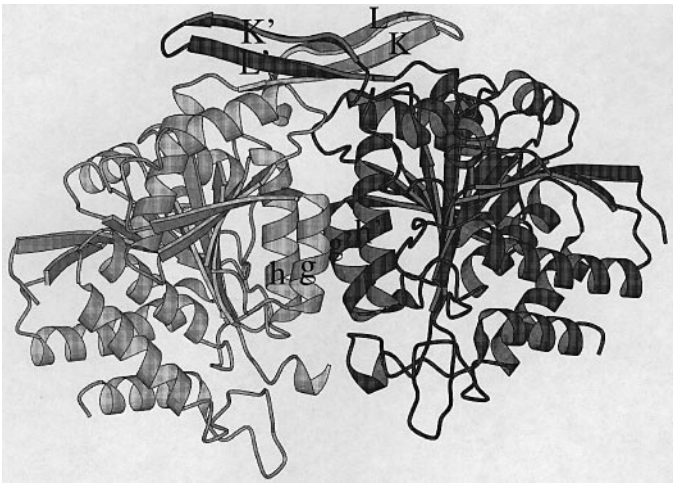

Fig. 3. Ribbon diagram of the IPMDH dimer. The subunit interface is composed of a four-helix-bundle, formed by helices g and h from both monomers and an arm-like region that forms an extended β-sheet (β-strands K and L) with the same region of the other monomer.

loop (residues 148–154 and 337–340), probably due to insertions in these regions.

Comparison based on sequence analysis

Proline is believed to decrease the backbone entropy of unfolding by constraining the main-chain flexibility. (Matthews *et al.*, 1987; Herning *et al.*, 1992; Nicholson *et al.*, 1992), i.e. the pyrrolidine ring severely restricts the possible conformations of the preceding residue. The number of proline residues in the psychrotrophic enzyme is smaller than that in either its mesophilic or its thermophilic counterparts with 16 (VibIPMDH), 19 (EcIPMDH) and 25 (TtIPMDH) prolines, respectively. In fact, this is the smallest number of proline residues found throughout the 12 sequences of IPMDH that were compared.

To estimate the importance of hydrophobic interactions in the stabilization of VibIPMDH, we calculated the buried polar and apolar surface areas of three IPMDHs (Table I). Interestingly the ratio of buried polar to apolar surface area of VibIPMDH is close to that of TtIPMDH, both of which are smaller than that of EcIPMDH. This indicates that the core of VibIPMDH is more hydrophobic than that of EcIPMDH, but makes no statement about the density of packing in these enzymes. Without a measure of packing density it is not evident that the hydrophobic core of VibIPMDH is more stablilized by hydrophobic interactions than that of its mesophilic counterpart. Another explanation for the lower ratio of polar to apolar surface area in the psychrotrophic enzyme might lie in the temperature dependence of hydrophobic interactions. Hydrophobic interactions become weaker with decreasing temperature and will therefore be less stabilizing at the lower operating temperature of the VibIPMDH.

Table I. Buried polar to apolar surface areas and their ratios in Vib-, Ec- and TtIPMDH

	VibIPMDH	EcIPMDH	TtIPMDH
Buried polar surface area (Å^2)	12011	13395	11612
Buried apolar surface area (Å^2)	26075	26266	25740
Ratio of buried polar to apolar surface area	0.46	0.51	0.45

Table II. Residues unique to the psychrotrophic IPMDH

	Residue in VibIPMDH	Residues in IPMDH family
Flexibility substitutions	Ala56	Pro
	Ser119	Pro
Packing substitutions	Ala28	Val, Leu, Ile
	Gly103	Ala, Ser
Hydrophilicity substitutions	Ser97	Lys
	T204	Glu, Lys

The comparison of 12 sequences of IPMDH from different organisms (Figure 4) reveals several potentially interesting substitutions in the psychrotrophic enzyme. These substitutions are divided into three classes: flexibility substitutions, packing substitutions and hydrophilicity substitutions (Table II). The substitution of a proline is defined as a flexibility substitution. This type of substitution is found at positions 56 and 119 (Vib-numbering), where the prolines which are conserved throughout the rest of the IPMDH family are replaced by alanine and serine in VibIPMDH, respectively. These flexibility substitutions are both located in loop regions at the surface of the molecule. The loop around position 56 is highly flexible in the mesophilic enzyme as judged by temperature factors (Wallon *et al.*, 1996). This loop is located in the vicinity of the active site loop. The loop around residue 119 is not implicated to be flexible, it is located following the β-strand connecting the two domains.

Packing substitutions change the packing density in the hydrophobic core. We looked mainly for hydrophobic side chains that are replaced by smaller ones in the psychrotrophic enzyme. Examples are Ala28 for valine, leucine or isoleucine and Gly103 for alanine and serine in the comparison group. The model implies that the packing substitutions create small cavities in the hydrophobic core. Recent studies on T4 lysozyme (Erikson *et al.*, 1992) and chymotrypsin inhibitor 2 (Jackson *et al.*, 1993) have demonstrated that the removal of one or

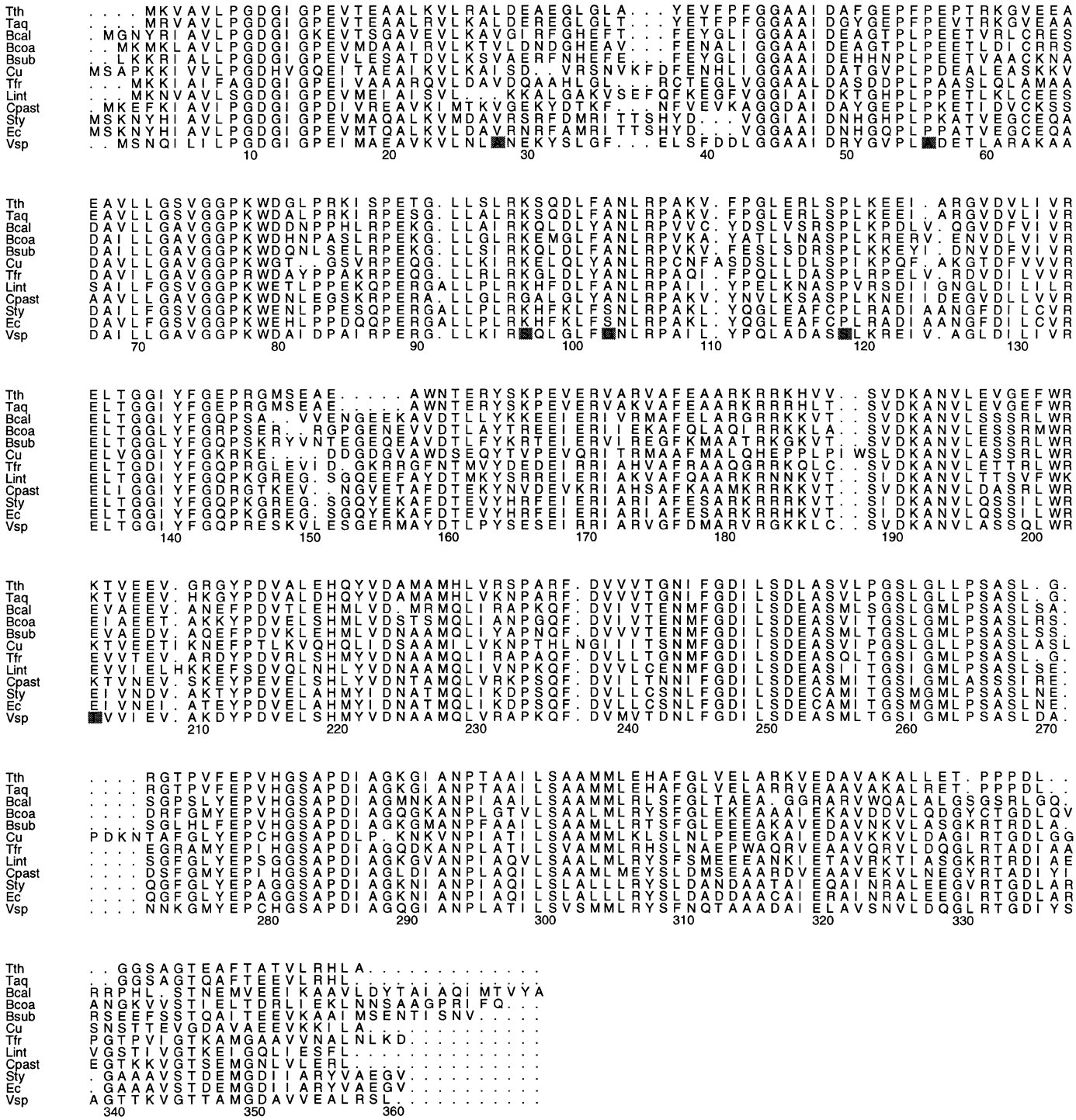

Fig. 4. Amino acid sequence alignment of 12 bacterial and fungal IPMDHs. Residues that are unique to the *Vibrio* sp. I5 IPMDH (Vsp) and are believed to contribute to its decreased thermostability are shaded in light gray, residues that are insertions with respect to the Ec- and StIPMDH are shaded in dark gray. The top three sequences are from thermophilic organisms (Tth, *Thermus thermophilus*; Taq, *Thermus aquaticus*; Bcal, *Bacillus caldotenax*), the others are from mesophiles (Bcoa, *Bacillus coagulans*; Bsub, *Bacillus subtilis*; Cu, *Candida utilis*; Tfr, *Thiobacillus ferrooxidans*; Lint, *Leptospira integralis*; Cpast, *Clostridium pasteurianum*; Sty, *Salmonella typhimurium*; Ec, *Escherichia coli*).

several methylene groups in the protein core can destabilize a protein if they lead to the formation of a cavity. By the same token, Kotsuka *et al.* (1996) stabilized TtIPMDH by filling a cavity in the thermophilic enzyme via an alanine to valine substitution. In contrast to these results, we have not been able to detect a decrease in thermostability by creating a cavity in the hydrophobic core of TtIPMDH (Wallon *et al.*, 1996). The cavity at position 28 in VibIPMDH may be even larger than can be expected from the removal of two methylene groups, since the hydrophobic residues surrounding the alanine are also smaller in the *Vibrio* enzyme, i.e. VibIPMDH has Leu, Ile, Val and Gln, whereas Ec- and TtIPMDH have Leu, Leu,

Table III. Parameters that might affect thermostability

Parameter	VibIPMDH (360 aa)	EcIPMDH (363 aa)	TtIPMDH (345 aa)	Effect on stability of VibIPMDH (compared with EcIPMDH)
Salt bridges	25	30	35	–
Aromatic–aromatic interactions	6	11	12	–
Subunit interface:				
4-Helix bundle	2 charged and polar residues	2 charged and polar residues	hydrophobic	0
Arm-like region (compared with TtIPMDH)	5 aa insertion	4 aa insertion	–	–
Proline content	16	19	25	–
Helix capping (stabilizing N-caps/C-caps)	5/5	4/4	7/4	+
N-terminus	–2	–	–4	+
C-terminus	–1	–	–3	+
Loop insertions	+13 (–1)[a]	+11	–	–

– Denotes an expected decrease in thermostability, + a positive effect on thermostability and 0 no expected effect on thermostability.
[a](–1) refers to the Ala94 insertion which is uniquely found in Ec, St and *Leptospira integralis*.

Ile and Met. The filling of the possible cavity by substituting the alanine with a larger hydrophobic side chain may increase the thermostability of the psychrotrophic enzyme.

Hydrophilicity substitutions change the hydrophilicity at the surface of the molecule. Three such substitutions were identified, namely Ser97, Gly100 and Thr204, which are substituted for charged residues that are not involved in the formation of salt bridges in the rest of the family. Hydrophilic residues on the surface will solvate the protein in aqueous solution and therefore aid in the stabilization of the folded state. Exposure of hydrophobic residues, on the other hand, will desolvate the protein. Both are mainly entropic effects, arising from the ordering of water around the protein.

Possible structural determinants of thermostability

We compared several structural aspects that are believed to contribute to protein stability, namely the numbers of conserved and potential salt bridges (Barlow *et al.*, 1983; Shortle 1992), aromatic interactions (Burley and Petsko, 1985) and helix caps (Serrano and Fersht, 1989) and the composition of the subunit interface (Kirino *et al.*, 1994). We chose not to compare hydrogen bonds, because energy minimization of a model structure will automatically maximize the number of possible hydrogen bonds within the protein and in the absence of an accurate model for protein–solvent interactions, this effect is likely to overestimate the number of internal hydrogen bonds.

Salt bridges have been implicated as part of the main stabilizing forces in protein structure. A salt bridge is defined as an ion pair with a distance of 2.5–4.0 Å between charged non-hydrogen atoms. Recent comparisons between hyperthermophilic enzymes from archea (Hennig *et al.*, 1995; Yip *et al.*, 1995) indicate extensive ion-pair networks as the largest contributor to the stabilization of those enzymes. Only 25 out of 30–35 salt bridges (St-, Ec-, TtIPMDH) are conserved as judged by the presence or absence of the salt bridge forming residues, indicating a possible reason for the lower thermostability of the psychrotrophic enzyme (Table III).

Burley and Petsko (1985) established that interacting aromatic residues in the core of the protein contribute to stability. An interacting pair is defined as one for which the distance between phenyl-ring centroids is less than 7 Å. The aromatic interactions found in Ec-, St- and TtIPMDH are not well conserved in the VibIPMDH; only six out of the 11/12 in St-, Ec-, TtIPMDH are observed in the model (Table III).

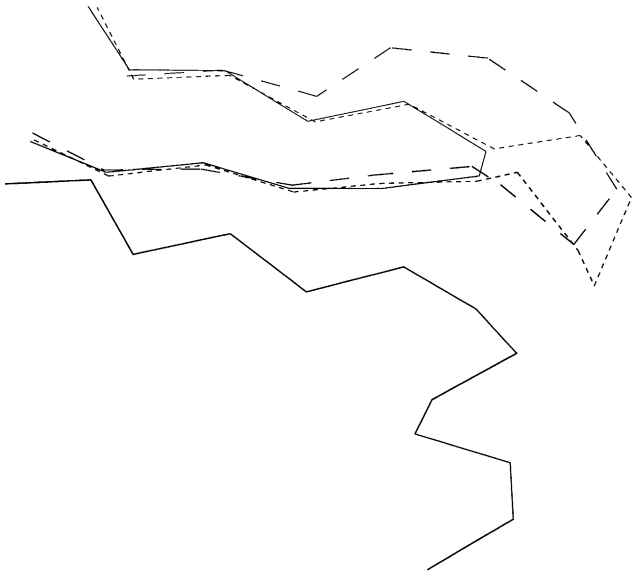

Fig. 5. Overlay of the Cα-trace of the arm-like region of Tt (——)-, Ec (– – –)- and Vib (- - -)-IPMDH. This loop is extended by four residues in EcIPMDH and five residues in VibIPMDH with respect to TtIPMDH. In EcIPMDH one additional main chain–main chain (mc–mc) hydrogen bond is formed between the monomers owing to this extension. No additional mc–mc hydrogen bonds can be formed by further extension of this loop.

The N- and C-termini of an α-helix have unsatisfied main-chain hydrogen-bonding partners, which can be satisfied be either solvent or protein residues. The first and the last residue of a helix, the helix caps, may be able to provide the necessary hydrogen-bonding partners. The characteristics of the helix caps in VibIPMDH in terms of their stabilizing or destabilizing influence on an α-helix are similar to those found in the other enzymes (Table III).

The subunit interface in IPMDH is mainly composed of

two parts: (a) a four-helix bundle formed by two helices (g and h) from each monomer in the core of the dimer and (b) the arm-like region (β-strands K and L) that extends to the other monomer and interacts with the same region from the other subunit (Figure 3). In the thermophilic enzyme the residues that interact in the four-helix bundle are strictly hydrophobic. Ec-, St- and VibIPMDH, on the other hand, have three substitutions in this area: L246/E256/E253 [Tt/Ec(St)/Vib-numbering], A220/T230/T227 and V249/M259/M256, i.e. a charged, polar and hydrophobic residue, respectively. The glutamate and the threonine are located directly at the interface between the helices g and h and the methionine points slightly outward. In the Ec enzyme the glutamate forms a hydrogen bond with the main-chain carbonyl of the threonine, which indicates that this carboxyl group might be protonated. The burial of this charge at the hydrophobic subunit interface decreases the stabilization energy of the enzyme, as is shown by the results of Kirino *et al.* (1994). They demonstrated in point-mutational studies that the glutamate contributes significantly to the destabilization of the Ec dimer: the T_m, i.e. thermal melting temperature, increased by 7°C in the E256L/M259V mutant of the Ec enzyme.

The arm-like region is extended by one residue in the psychrotrophic enzyme with respect to the Ec- and St-enzymes, which in turn are extended by four residues with respect to the thermophilic one (Figure 5). Loops are believed to be important in protein stability as they might provide initiation points for unfolding (Leszczynski *et al.*, 1986; Daggett and Levitt, 1993; Hardy *et al.*, 1994). In EcIPMDH these additional residues in this surface loop are not believed to have a destabilizing effect, since two additional intermolecular hydrogen-bonds can be formed and the B-factors do not indicate that this region is significantly more flexible than the same area in TtIPMDH. It seems likely that the additional insertion in VibIPMDH will destabilize this loop, since no further intermolecular hydrogen-bonds are possible through elongation of this loop in this region.

There is another one-residue insertion with respect to EcIPMDH in a loop close to the C-terminus [Ala339(Vib)]. In EcIPMDH this loop is extended by three amino acids as compared with the TtIPMDH. This region is highly flexible in the mesophilic enzyme, as indicated by the absence of electron density, and can be expected to be more so in the VibIPMDH, owing to the addition of one residue.

Ec-, St- and *Leptospira integralis* IPMDHs have an additional residue in an α-helix at position A94(Ec/St); this residue is absent throughout the rest of the IPMDH family, including VibIPMDH. This insertion serves to elongate a flexible loop close to the active site. This active site loop is highly flexible in EcIPMDH (i.e. there is no electron density for parts of this loop in the crystal structure of EcIPMDH, which indicates that the region is very mobile) and has relatively high temperature factors in St- and TtIPMDH. It has been speculated that this loop has to move aside after catalysis to facilitate substrate or product release. It is difficult to evaluate the effect on thermostability of VibIPMDH since this amino acid is also absent in most of the other enzymes.

The N- and C-termini of the psychrotrophic IPMDH are shortened by two and one amino acid, respectively, compared with the Ec- and St-enzymes, but are two residues longer at both ends than TtIPMDH. The termini are highly flexible in the two mesophilic enzymes, protruding into the solvent and have fewer stabilizing interactions than the termini in TtIPMDH. The extended N- and C- termini are believed to destabilize the mesophilic enzymes by causing the ends to fray. (Hayashi-Iwasaki *et al.*, [year?]; Wallon *et al.*, 1996). The shorter termini of the VibIPMDH suggest that this effect may be less pronounced here.

Conclusion

We have cloned and sequenced the gene for a psychrotrophic IPMDH from *Vibrio* sp. I5 and have built a model for its three-dimensional structure based on the structures of homologs from mesophilic and thermophilic organisms. We have shown that the enzyme is less thermostable than its mesophilic and thermophilic counterparts *in vivo* and *in vitro*, as is expected from an enzyme from a psychrotrophic organism. The sequence alignment with its mesophilic and thermophilic counterparts and the comparison of the model with the available crystal structures reveal a number of potential determinants for this decreased thermostability (Table II and III). The model of the psychrotrophic enzyme is characterized by a smaller number of salt bridges, a reduction in aromatic–aromatic interactions, fewer proline residues and longer surface loops.

The putatively most destabilizing substitutions in the psychrotrophic enzyme are those listed in Table II. From the model we can deduce the position of those residues in the structure and speculate on their effect on protein stability. Mutations at those positions are expected to decrease flexibility, improve the packing of the hydrophobic core or increase the hydrophilicity of the surface.

This work will be followed by a thorough characterization, mutagenesis study and crystallization.

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