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# A Better Enzyme to Cope with Cold

## COMPARATIVE FLEXIBILITY STUDIES ON PSYCHROTROPHIC, MESOPHILIC, AND THERMOPHILIC IPMDHS*

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**3-Isopropylmalate dehydrogenase (IPMDH) from the psychrotrophic bacterium *Vibrio* sp. I5 has been expressed in *Escherichia coli* and purified. This cold-adapted enzyme is highly homologous with IPMDHs from other organisms, including mesophilic *E. coli* and thermophilic *Thermus thermophilus* bacteria. Its molecular properties are similar to these counterparts. Whereas the *E. coli* and *T. thermophilus* enzymes are hardly active at room temperature, the *Vibrio* IPMDH has reasonable activity below room temperature. The thermal stabilities, conformational flexibilities (hydrogen-deuterium exchange), and kinetic parameters of these enzymes were compared. The temperature dependence of the catalytic parameters of the three enzymes show similar but shifted profiles. The *Vibrio* IPMDH is a much better enzyme at 25 °C than its counterparts. With decreasing temperature *i.e.* with decreasing conformational flexibility, the specific activity reduces, as well; however, in the case of the *Vibrio* enzyme, the residual activity is still high enough for normal physiological operation of the organism. The cold-adaptation strategy in this case is achieved by creation of an extremely efficient enzyme, which has reduced but still sufficient activity at low temperature.**

The study of enzymes from extremophiles (1) is an increasingly important field in both research and industry. A wealth of information regarding thermophilic enzymes has been accumulated (2, 3), and from the numerous three-dimensional structures, several structural features important for thermal stability have been identified by comparisons with mesophilic counterparts (4–6). Environmental adaptation of proteins to low temperature, however, is much less understood (7, 8). A few psychrophilic or psychrotrophic structures have recently been obtained either by x-ray crystallography (9–12) or by homology modeling (13–17). A general conclusion is that these enzymes are thermolabile because of the weakening of intramolecular interactions, and that they exhibit high catalytic activity at low temperatures (8).

In this work, variants of 3-isopropylmalate dehydrogenase (IPMDH;[1] EC 1.1.1.85) from three bacteria, the thermophilic *Thermus thermophilus*, the mesophilic *Escherichia coli*, and the psychrotrophic *Vibrio* sp. I5 (an organism isolated from arctic sea water) (18), were used to study the mechanism of thermal adaptation at the level of proteins. IPMDH, a member of the oxidoreductase family, catalyzes the conversion of 3-isopropylmalate into 2-ketoisocaproate, which is the penultimate step of leucine biosynthesis. The enzyme is a homodimer and uses $NAD^+$ and a divalent cation ($Mg^{2+}$ or $Mn^{2+}$) as cofactor. IPMDH from *T. thermophilus* has been extensively studied (19), its crystal structure has been solved (20), and several mutants have been constructed (21, 22). The crystal structure of *E. coli* IPMDH has also been solved (23). IPMDH from *Vibrio* sp. I5 has not been studied in detail; however, its amino acid sequence is known, and a homology model for its three-dimensional structure was constructed (15). In a previous study, we performed hydrogen-deuterium (H/D) exchange experiments to characterize the dynamic behavior of *E. coli* and *T. thermophilus* IPMDHs (24). The conclusion was that the thermophilic enzyme is much more rigid at room temperature than the mesophilic IPMDH, whereas the enzymes have nearly identical flexibilities under their respective optimal working conditions. Thus, this observation suggested a close relationship between conformational flexibility and enzyme function.

The aim of the present study was to compare IPMDH from the psychrotrophic *Vibrio* sp. I5 with its mesophilic and thermophilic counterparts to reveal the mechanism of cold adaptation used by this psychrotrophic enzyme (15). Comparative measurements of thermal stabilities, conformational flexibilities, and the kinetic parameters of the three enzymes were carried out. To our knowledge this is the first study on a set of selected enzymes isolated from microorganisms covering a range of physiological temperatures from −1.5 °C to 80 °C.

### EXPERIMENTAL PROCEDURES

*Materials*—Threo-DL-3-isopropylmalic acid was purchased from Wako Chemicals, and NAD was from Roche Molecular Biochemicals. Chromatography media were obtained from Pharmacia Fine Chemicals. $D_2O$ (99.95% purity) was obtained from Merck. Other chemicals (high purity grade) were products of Merck, Reanal (Budapest, Hungary), and Sigma.

*Enzyme Preparation*—Cloning of the *Vibrio* sp. I5 leuB gene was carried out as described elsewhere (15). The obtained plasmid, pGWII, was transformed into *E. coli* OM17 cells. *T. thermophilus* IPMDH was produced in *E. coli* BMH 71–18 cells, using a recombinant plasmid pUTL118 carrying the *leuB* gene from *T. thermophilus* (25). Mesophilic IPMDH was expressed in the *E. coli* strain RDK1782 (26) transformed with pWally, a derivative of pBluescript $KS^-$ carrying the *leuB* gene from *E. coli.* Plasmids pWally, pGWII, and pUTL118 were a kind gift from Gerlind Wallon (Brandeis University, Waltham, MA). Cells containing *leuB* genes of *T. thermophilus* and *Vibrio* sp. I5 were grown at 37 °C in the presence of 100 μg/ml ampicillin and induced with isopropyl-1-thio-β-D-galactopyranoside, whereas cells carrying the gene coding for the *E. coli* IPMDH were grown at 30 °C in the presence of both 100 μg/ml ampicillin and 50 μg/ml kanamycin, heat-induced (42 °C for 1 h) and grown further for 3 h at 37 °C. The purification procedures of all three enzymes were based on the method of Yamada *et al.* (19). Crude extracts were obtained by sonication. Active ammonium sulfate fractions were then subjected to Butyl-Sepharose and, after dialysis, to DEAE-Sephacell and Sepharose S-200 chromatography. The final products were observed as

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[1] The abbreviations used are: IPMDH(s), 3-isopropylmalate dehydrogenase(s); H/D, hydrogen/deuterium; IPM, threo-DL-isopropylmalic acid.

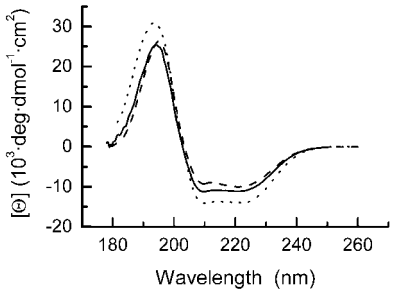


FIG. 1. **The far-UV CD spectra of *Vibrio* sp.** I5 (——), *E. coli* (- - - - -), and *T. thermophilus* (·····) IPMDHs are shown. Measurements were carried out in 20 mM potassium phosphate buffer, pH 7.6, using an enzyme concentration of 0.4 mg/ml or lower (below 200 nm). *deg*, degrees.

single bands on SDS-polyacrylamide gel electrophoresis gels stained with Coomassie Brilliant Blue.

*Enzyme Activity Measurements*—The activity of IPMDH was measured in 20 mM potassium phosphate buffer, pH 7.6, containing 25 $\mu$l of enzyme solution, 0.47 mM threo-DL-3-isopropylmalate, 0.93 mM NAD, 0.2 mM $MnCl_2$, and 0.3 M KCl in a final volume of 600 $\mu$l. Initial velocities were determined by monitoring the absorbance of NADH formed at 340 nm on a Jasco V-500 spectrophotometer, whereas temperature was controlled by a Grant thermostat. Optimal working temperature ranges of the enzymes were defined as the temperature ranges where enzyme activities were higher than 85% of their maxima.

*Circular Dichroism Measurements*—CD measurements were carried out on a JASCO J-720 spectropolarimeter equipped with a Neslab RTE-100 computer-controlled thermostat. All cells were cylindrical, water-jacketed quartz cells. Protein concentrations were set to 0.4 mg/ml in 20 mM potassium phosphate buffer (pH 7.6) containing 0.3 M KCl. Cells of 0.1- and 0.01-cm pathlength were used for recording far-UV spectra. For heat denaturation studies a cell of 0.1 cm was applied. Unfolding was monitored at 221 nm with a heating rate of 50 °C/h. Transition temperatures were determined after a slight smoothing using the first derivatives of the transition curves.

*Kinetic Analysis*—Apparent Michaelis constants ($K_m$) and catalytic constants ($k_{cat}$) were determined in steady state kinetic experiments at different temperatures in 20 mM potassium phosphate buffer (pH 7.6) containing 0.3 M KCl, 0.2 mM $MnCl_2$, the enzyme, and various concentrations of NAD and IPM. The initial rates were measured by monitoring the absorbance of NADH formed at 340 nm on a Jasco V-500 spectrophotometer equipped with a Grant thermostat. Enzyme concentrations were set to about 0.3 $\mu$M at each temperature.

Determination of the $k_{cat}$ values was carried out by global analysis of the saturation curves for IPM and NAD. For non-linear regression fitting the Nonlinear Statistical Regression Program, version 3.4 (Phillip H. Sherrod) was used on the basis of the following adapted form of the Michaelis-Menten equation.

$$\nu = \frac{V_{max} \cdot [NAD^+] \cdot [IPM]}{k_{NAD^+} \cdot k_{IPM} + k_{IPM} \cdot [NAD^+] + k_{NAD^+} \cdot [IPM] + [NAD^+] \cdot [IPM]} \quad \text{(Eq. 1)}$$

Catalytic constants ($k_{cat}$ values) were determined by measuring the activities in the form of 4 × 4 matrices of IPM and NAD concentrations. The 16-point data set was then fitted by Equation 1. In this set of measurements the substrate and coenzyme concentrations were as follows for *T. thermophilus* and *E. coli* IPMDH: NAD, 50, 200, 500, and 1000 $\mu$M; IPM, 40, 100, 300, and 600 $\mu$M. For *Vibrio* sp. I5 IPMDH, the concentrations were as follows: NAD, 50, 100, 200, and 400 $\mu$M; IPM, 20, 40, 100, and 200 $\mu$M. Substrate inhibition limits the highest IPM concentration to be used (particularly at lower temperatures). To increase accuracy at low and high temperatures in the case of *Vibrio* sp. I5 IPMDH, at 14 °C and 50 °C, we preformed measurements at the following additional substrate concentrations: NAD, 600 $\mu$M (50 °C); IPM, 10 $\mu$M (14 °C).

*Hydrogen/Deuterium Exchange*—H/D exchange of peptide protons was followed by a Bruker IFS 28 Fourier-transformed infrared spectrophotometer. Temperature was controlled with a Techne TU 16D temperature controller, and the actual temperature was measured by a sensor attached directly to the $CaF_2$ cell window. Lyophilized samples were dissolved within a few seconds in $D_2O$ to give solutions of pD 8.15 in 20 mM potassium phosphate, containing 0.3 M KCl, and then filled into the cell. The IR spectra (400–4000 $cm^{-1}$ region) were recorded starting 30–50 s after complete dissolution. Absorbances of

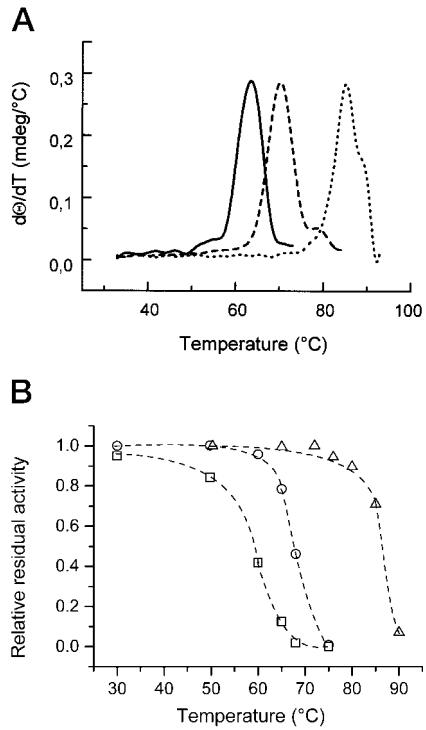


FIG. 2. **Heat denaturation of *Vibrio* sp.** I5 (——), *E. coli* (- - - - -), and *T. thermophilus* (·····) IPMDHs in 20 mM potassium phosphate buffer, pH 7.6, are shown. *A*, heat denaturation as followed by CD spectroscopy on 221 nm at a heating rate of 50 °C/h. The melting temperatures are as follows: 63.5 °C, *Vibrio* sp. I5 IPMDH; 70.1 °C, *E. coli* IPMDH; and 85.2 °C, *T. thermophilus* IPMDH. $d\Theta/dT$, derivative of ellipticity by temperature; *mdeg*, millidegree. *B*, residual activities of the *Vibrio* sp. I5 (□), *E. coli* (○), and *T. thermophilus* (△) IPMDHs after a 10-min incubation at different temperatures.

the amide I and amide II bands were evaluated from the spectra at the wavenumbers of their maxima, *i.e.* at 1650 and at 1547.5 $cm^{-1}$, respectively. The values were corrected with the base-line absorbances measured at 1789 $cm^{-1}$. The fraction of unexchanged hydrogens ($X$) was calculated from the ratio of amide II and amide I bands considering the absorbances of the unexchanged proteins and of the completely deuterated ones as 100 and 0%, respectively. The details of these experiments and the method of evaluation were described previously (24). The results were presented in the form of relaxation spectra, *i.e.* $X$ *versus* log ($k_0 t$), where $k_0$ is the chemical exchange rate constant, which was calculated from the empirical form (24) as follows.

$$k_0 = (10^{-pHread} + 10^{pHread\ -\ 6}) 10^{0.05(T\ -\ 25)}\ s^{-1}. \quad \text{(Eq. 2)}$$

## RESULTS AND DISCUSSION

IPMDHs from *Vibrio* sp. I5, *E. coli*, and *T. thermophilus* were purified to homogeneity as confirmed by Coomassie Brilliant Blue-stained SDS-polyacrylamide gel electrophoresis gels. The N-terminal amino acid sequence of the recombinant *Vibrio* sp. I5 IPMDH (Met-Ser-Asn-Gln-Thr), which was checked by automated amino acid sequencing, corresponds to the amino acid sequence deduced from the nucleotide sequence (15).

Based on enzyme activities measured at different pH values and in the presence of various essential monovalent cations, the catalytic properties of psychrotrophic IPMDH seem to be similar to those reported for its mesophilic and thermophilic counterparts (19, 27), *i.e.* the enzyme is activated by $K^+$ and to a lesser degree by $NH_4^+$ ions, and the divalent cations $Mn^{2+}$ and $Mg^{2+}$ are appropriate cofactors.

The high similarity of the far-UV CD spectra (Fig. 1) suggests that the *Vibrio* IPMDH is structurally very similar to the other two enzymes. Unfolding experiments monitored by CD spectroscopy revealed that the apparent melting temper-

TABLE I

*Optimum growth temperature and growth temperature ranges for Vibrio sp. I5, E. coli, and T. thermophilus IPMDHs and optimal working temperature ranges of the respective IPMDHs*

The quantities were estimated as described under "Experimental Procedures" or, where indicated, obtained from literature sources.

| Organism | *Vibrio* sp. I5 | *E. coli* | *T. thermophilus* |
|---|---|---|---|
| Optimum growth temperature (15) | ~20 °C | ~37 °C | ~75 °C |
| Growth temperature range (18, 34, 25) | −1.5 –at least 30 °C[a] | 10 –45 °C | 55 –80 °C |
| Optimal working temperature range of IPMDHs | 46 –63 °C | 48 –65 °C | 66 –84 °C |

[a] Personal communication from Gerlind Wallon, Brandeis University, Waltham, MA.

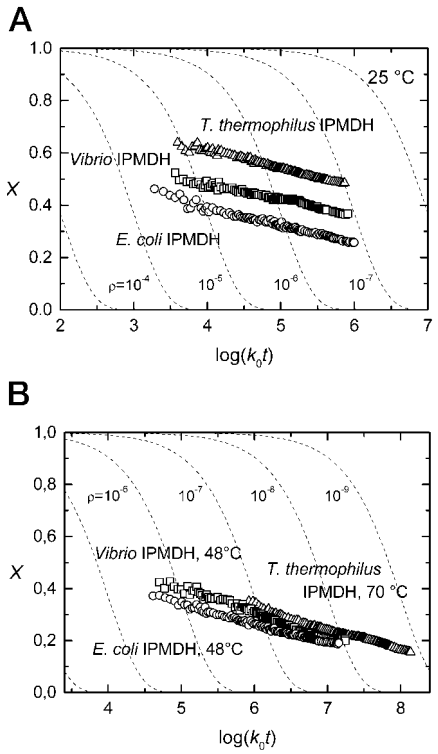


FIG. 3. *A*, hydrogen-deuterium exchange data, summarized in the form of relaxation spectra for *Vibrio* sp. I5 IPMDH (□), *E. coli* IPMDH (○), and *T. thermophilus* IPMDH (△) on pD 8.15 at 25 °C. *X* is the fraction of unexchanged peptide hydrogens, *t* is the time, and $k_0$ is the chemical exchange rate constant. The *dashed lines* represent the exchange rate curves for hypothetical polypeptides characterized by a given probability (the *p* values) of solvent exposure of the peptide groups (24). *Curves* indicate a more rigid structure for the thermophilic enzyme. *B*, hydrogen-deuterium exchange data of IPMDHs near to their temperature optima (*Vibrio* sp. I5 IPMDH, 48 °C (□); *E. coli* IPMDH, 48 °C (○); *T. thermophilus* IPMDH, 70 °C (△)). *Curves* obtained for the three enzymes reflect very similar flexibilities.

ature of *Vibrio* IPMDH is 7 °C below that of *E. coli* IPMDH; the experimental values are 63.5, 70., and 85.2 °C for the *Vibrio* sp. I5, *E. coli*, and *T. thermophilus* enzymes, respectively (Fig. 2*A*). The results of heat inactivation experiments (Fig. 2*B*) are in good correlation with these apparent melting temperature values, suggesting that temperature-induced unfolding is highly cooperative under these conditions. The unfolding temperature of the *Vibrio* enzyme is unexpectedly high, considering that the *Vibrio* sp. I5 is a psychrotrophic bacterium, and its optimum growth temperature is somewhat below room temperature (see Refs. 15 and 18 and Table I). Carrying out activity measurements at various temperatures, we have explored the optimal working temperature range for the three enzymes. The data are summarized in Table I. The *Vibrio* enzyme was found to be active up to surprisingly high temperatures, and its optimal temperature range proved to be largely overlapping with that of the *E. coli* IPMDH. Most of the cold-adapted enzymes studied so far

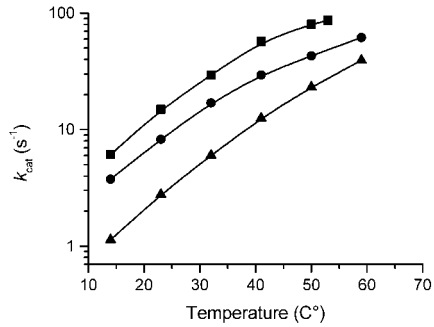


FIG. 4. **Catalytic constants ($k_{cat}$) at different temperatures of the *Vibrio* sp.** I5 IPMDH (□), *E. coli* IPMDH (○), and *T. thermophilus* IPMDH (△) are shown. Data, here plotted on a logarithmic scale, were obtained by measuring the activities in the form of 4 × 4 matrices of IPM and NAD concentrations in 20 mM potassium phosphate buffer (pH 7.6) containing 0.3 M KCl, 0.2 mM $MnCl_2$, the enzyme, and various concentrations of NAD and IPM. The initial rates were measured by monitoring the absorbance of NADH formed at 340 nm on a Jasco V-500 spectrophotometer equipped with a Grant thermostat. Enzyme concentrations were set to about 0.3 μM at each temperature.

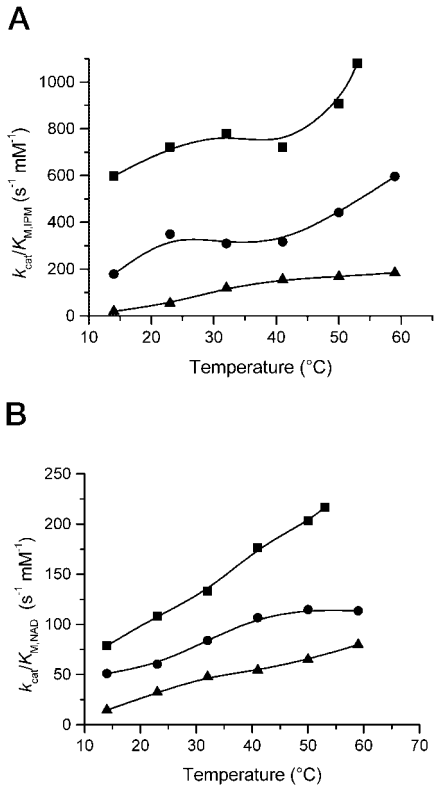


FIG. 5. **Specificity constants ($k_{cat}/K_{m,IPM}$ and $k_{cat}/K_{m,NAD}$) of the *Vibrio* sp.** I5 IPMDH (□), *E. coli* IPMDH (○), and *T. thermophilus* IPMDH (△) are shown for IPM (*A*) and NAD (*B*). Michaelis constants were calculated from the data set used for the $k_{cat}$ values (Fig. 4).

have been obtained from obligate psychrophiles and usually proved to be thermally labile (7, 8, 28).

It was suggested (1) and experimentally supported (24)

that the mutative adaptation of enzymes to changing environmental temperature tends to maintain optimum conformational flexibility at the physiological temperature. Therefore, psychrophilic enzymes are expected to have increased conformational flexibilities (7, 8, 28).

To test the validity of this assumption for a psychrotrophic case, *i.e*. *Vibrio* IPMDH, flexibility measurements were performed by H/D exchange at room temperature and near to the temperature optimum of the enzyme (48 °C). The results were compared with those reported earlier (24) for the *E. coli* and *T. thermophilus* enzymes (Fig. 3, *A* and *B*). At room temperature, the H/D exchange curves of *Vibrio* IPMDH run between those of the mesophilic and thermophilic enzymes (Fig. 3*A*). This indicates that, in contrast to our expectation, the psychrophilic enzyme is more rigid at room temperature than its mesophilic homologue. However, if the comparison is made at the temperature optima of the enzymes (more precisely, at the lower end of the optimal temperature range to avoid thermal denaturation during the 3–4 h of H/D exchange experiments), *i.e*. at 48, 48, and 70 °C, the three curves run close to each other (Fig. 3*B*), reflecting similar degrees of flexibility.

We have to emphasize here that the exchange of solvent-exposed amide hydrogens is not monitored in these experiments (they are already replaced during the dead time of the experiments); therefore the data only report on the buried segments. Our findings show that at 25 °C, at least the buried segments of the *Vibrio* IPMDH are less flexible than those in its mesophilic counterpart (although more flexible than those of the thermophilic IPMDH). These results are in accordance with the conclusions from earlier calculations based on the homology model of the *Vibrio* IPMDH (15), which showed that in some relevant structural features, the *Vibrio* sp. I5 IPMDH resembles its thermophilic rather than its mesophilic counterpart. These features include the relatively high stability of helices and, more importantly, the increased hydrophobicity of the buried surfaces, *i.e*. a more hydrophobic core of the *Vibrio* sp. I5 IPMDH.

The fact that this psychrotrophic enzyme is stable and active in a broad temperature range prompted us to study the kinetic properties of the enzymes. A detailed kinetic study was performed by Dean and Dvorak (29) on the *T. thermophilus* IPMDH, and an uncompetitive pattern with a random binding of the substrates was suggested. Our study is restricted to compare the quality of the three enzymes. Plots of $k_{cat}$ values against temperature show an increased catalytic efficiency of the *Vibrio* sp. I5 IPMDH in comparison with the other two enzymes (Fig. 4) in the whole temperature range covered (6 to 52 °C). This is also reflected in the specificity constants ($k_{cat}/K_m$), whose values, compared with those of the mesophilic and thermophilic enzymes, are higher for both substrates at each temperature studied (Fig. 5). No such significant difference could be observed in the case of the $K_m$ values, though $K_{m,IPM}$ values of the psychrotrophic enzyme suggest a stronger substrate binding for IPM (data not shown).

The *Vibrio* sp. I5 IPMDH excels with its high specific activity among all IPMDHs described so far (15, 31–33). Enzymes from psychrophilic (not psychrotrophic) organisms have also been found to have high specific activities, though these enzymes lose their activities at 25–40 °C (13, 14, 30). In the present comparative study we revealed that the psychrotrophic *Vibrio* IPMDH, if compared with counterparts from organisms whose growth is restricted to warmer environments (25, 34), is a much better enzyme, having improved catalytic parameters in a broad temperature range. It is hard to pinpoint the structural background of this improvement; it might follow from a more precisely designed active site or a local increase in the flexibility of functionally important regions. A global flexibility increase, however, can be ruled out in light of the results of our H/D exchange measurements.

Life uses a diverse set of strategies to adapt enzymes to changing environmental temperatures (4, 35, 36). To retune the functionally relevant and highly temperature-dependent conformational flexibility pattern is one possible route. This strategy was identified in the case of IPMDH when *E. coli* and *T. thermophilus* enzymes were compared (24). If we compare with the mesophilic *E. coli* IPMDH, the thermophilic enzyme exhibits reduced flexibility, which is associated with increased thermal stability; however, its kinetic efficiency is about the same at its relevant (much higher) physiological temperature (24). The adaptation strategy of the psychrotrophic enzyme cannot be deduced by extrapolating the changes observed in the thermophile to mesophile transition. It involves the rearrangement of the hydrophobic core (15), which is reflected in the increased conformational rigidity. A fine readjustment of the active site results in an enzyme of increased specific activity in the whole temperature range available for the native conformation. The case of IPMDH presents us with the example that even in the same enzyme family the adaptation to the environment might be achieved by various strategies.

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