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Drugs Against *Mycobacterium tuberculosis* 3-Isopropylmalate Dehydrogenase Can be Developed Using Homologous Enzymes as Surrogate Targets

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Abstract: 3-Isopropylmalate dehydrogenase (IPMDH) from *Mycobacterium tuberculosis* (*Mtb*) may be a target for specific drugs against this pathogenic bacterium. We have expressed and purified *Mtb* IPMDH and determined its physical-chemical and enzymological properties. Size-exclusion chromatography and dynamic light scattering measurements (DLS) suggest a tetrameric structure for *Mtb* IPMDH, in contrast to the dimeric structure of most IPMDHs. The kinetic properties (k_{cat} and K_m values) of *Mtb* IPMDH and the pH-dependence of k_{cat} are very similar to both *Escherichia coli* (*Ec*) and *Thermus thermophilus* (*Tt*) IPMDHs. The stability of *Mtb* IPMDH in 8 M urea is close to that of the mesophilic counterpart, *Ec* IPMDH, both of them being much less stable than the thermophilic (*Tt*) enzyme. Two known IPMDH inhibitors, O-methyl oxalohydroxamate and 3-methylmercaptomalate, have been synthesised. Their inhibitory effects were found to be independent of the origin of IPMDHs. Thus, experiments with either *Ec* or *Tt* IPMDH would be equally relevant for designing specific inhibitory drugs against *Mtb* IPMDH.

Keywords: Enzyme kinetics, expression, isopropylmalate dehydrogenase, inhibition, *Mycobacterium tuberculosis*, purification, physical-chemical characterization.

INTRODUCTION

3-Isopropylmalate dehydrogenase (IPMDH) is an essential enzyme in the leucine biosynthesis pathway of bacteria and plants, but it is absent in humans and other mammals. Various *in vivo* and *in vitro* studies demonstrate that those pathogenic bacterium strains that cannot synthesize leucine are unable to replicate inside their host cells [1-3]. The lack of the leucine biosynthetic pathway in humans (and generally in mammals) suggests that enzymes of this pathway, such as IPMDH, may serve as potential targets for inhibition by specific new drugs against pathogenic bacteria such as *Mycobacterium tuberculosis*. This bacterium is the major causative agent of tuberculosis, a potentially lethal disease that causes 2-3 million deaths per year, globally. Due to the frequent appearance of resistance against current antibiotics, there is an urgent need to find potential new drug targets and design more effective drugs. IPMDH, one of the catalysts of leucine biosynthesis, seems to be an appropriate target for inhibition by small-molecule drugs. Designing new inhibitory molecules against IPMDH, however, requires detailed knowledge of the structure and catalytic mechanism of this enzyme.

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IPMDH catalyzes the complex reaction of simultaneous oxidation and decarboxylation of the substrate 3-isopropylmalate (IPM) in the presence of NAD^+. For the catalytic reaction, the presence of a divalent metal ion (Mn^{2+} or Mg^{2+}) is also required. Our knowledge on the kinetics and mechanisms on IPMDH is limited [4-7]. Efforts have been mainly concentrated on revealing the molecular basis of thermostability [8-11]. There are also numerous crystallographic structural investigations of IPMDHs from various sources [12-19].

IPMDHs, in general, have a characteristic homodimer structure [20, 21] and each subunit can be divided into two domains. The substrate, MnIPM, located at the subunit-subunit interface, binds at the interface between the domains. Thus, IPM binding requires special attention with respect to regulation of both domain-domain and subunit-subunit interactions. In fact, the MnIPM-bound IPMDH exists in a presumably active, domain-closed conformation [14, 19]. A detailed atomic-level mechanism of domain closure has also been described for *Thermus thermophilus* (*Tt*) IPMDH from structural analysis [19]. The crystal structure of *M. tuberculosis* (*Mtb*) IPMDH is the only one in which the apo- (substrate-free) protein appears in a largely closed conformation [18]. As discussed previously, the different behaviour may be due to the specific crystallisation conditions used for structure determination or may be attributed to some species-specific differences in the structures of various IPMDHs.

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Therefore, in the present work, we aimed to produce *Mtb* IPMDH in pure homogeneous form in order to characterize its enzymological and physical-chemical properties, and furthermore, to compare it with its better-characterised counterparts, the thermophilic *T. thermophilus* (*Tt*) and the mesophilic *Escherichia coli* (*Ec*) IPMDHs. Although sequence comparisons indicate extensive differences, we found that these differences are not associated with significantly different enzyme kinetic and physical-chemical properties.

MATERIALS AND METHODS

Chemicals and Enzymes

The substrate, 3-isopropylmalate (IPM) or (2*R*,3*S*)-2-hydroxy-3-(propan-2-yl) butanedioic acid was purchased in the racemic DL-*threo* form from Wako Biochemicals (Japan), NAD^+ and NADH were Sigma products. All other chemicals were commercially available as high purity reagent grade products.

The inhibitory compounds, O-methyl oxalohydroxamate and 3-methylmercaptomalate or (2*S*,3*S*)-(-)-2-hydroxy-3-sulfanylbutanedioic acid (in the form of the racemic mixture of trans-2-hydroxy-3-sulfanylbutanedioic acid) were synthesized chemically by using the methods of Pirrung [22] and Nango *et al.* [23], respectively. The purity of the synthesized products was at least 90% as determined by NMR measurements and elementary analysis.

Wild-type *Tt* and *Ec* IPMDHs were expressed in the *Ec* BL21 (DE3) pLysS strain, purified and stored as described previously [9, 10]. Their concentration (in molarity of the monomers) was calculated using ε_{280} values of 23950 and 27390 $M^{-1}cm^{-1}$ for *Tt* and *Ec* IPMDHs, respectively. *Mtb* IPMDH was overexpressed in *Ec* BL21 cc3 cells [24], transformed with pETM-11-*leuB* vector [18], in two different ways. First, *Mtb* IPMDH was expressed using the usual method of batch techniques. This method, however, resulted in mostly insoluble IPMDH in the form of inclusion bodies, from which the active enzyme could not be renaturated. The amount of soluble IPMDH protein in the supernatant was negligible. Finally, a fermentation procedure allowing control of cultivation parameters has been used for protein expression. The cells from an overnight pre-culture containing the plasmid pETM-11 with *Mtb* IPMDH gene were grown in minimal medium containing 13.3 g/L KH_2PO_4, 1.2 g/L $MgSO_4 \cdot 7\ H_2O$, 30 g/L glycerol, 1.7 g/L citric acid, 4 g/L $(NH_4)_2HPO_4$, 8.4 mg/L EDTA, trace metal ions and antibiotics (17 mg/L chloramphenicol, 50 mg/L kanamycin, 50 mg/L spectinomycin) at 30 °C, pH=7, stirring speed 950 rpm and 1.5 L/min airflow. After the initial amount of carbon source was consumed, a feed-batch phase followed for 4 h. The feeding solution (1000 g/L glycerol, 20 g/L $MgSO_4 \cdot 7\ H_2O$ and trace metal ions) was added at a rate to keep the dissolved oxygen levels at ~20%. Just before the end of this fed-batch phase, the temperature was lowered to 20 °C and the expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (1.4 mM final concentration) concomitantly by addition of a yeast extract solution (2 g/L final concentration). The induction procedure was repeated after 1.5 h. After the first induction step, the culture was incubated for about 3 h and then harvested. The cells were lysed using sonication in a buffer containing 10 mM imidazole, 50 mM NaH_2PO_4, pH=8.0 and 300 mM NaCl for 20 minutes with 25 second pulses on ice. The cell debris was pelleted by centrifugation for 30 min at 4 °C and 18000 rpm. The supernatant was loaded onto a Ni-NTA column (8 mL volume) equilibrated with 50 mM imidazole, 50 mM NaH_2PO_4, pH=8.0, 300 mM NaCl buffer. The protein was eluted by a linear gradient from 50 mM to 500 mM imidazole buffer in a total volume of 100 mL. The peak fractions were dialyzed overnight against 25 mM MOPS-KOH buffer pH=7.6 and concentrated to ~5 mg/mL. The protein concentration has been determined by using an apparent molar absorption coefficient of ε_{280}(app)=16941 M^{-1} cm^{-1} which was obtained by correction of the theoretically calculated value of ε_{280}=15930 M^{-1} cm^{-1} for the monomer [25] by the additional absorbance of a bound nucleotide (cf. Results). The yield of *Mtb* IPMDH from 1.5 L cell culture was ~8-10 mg. The purity of the protein (at least 95 %) was estimated by SDS-PAGE.

SDS-, Native- and Urea Gel Electrophoresis

SDS gel electrophoresis was carried out using the method of Laemmli [26]. Native gel electrophoresis was performed according to Ornstein [27]. The resolving and stacking gel was 10% and 5%, respectively. IPMDH (3-5 μg) was loaded into the gel, and the electrophoresis was carried out at 180 V for 1 h. The urea gel electrophoresis was performed similarly to the native gel electrophoresis, but all solutions contained 8 M urea and the samples of IPMDH also were denatured in 8 M urea. The gels were stained with Coomassie Brilliant Blue G-250.

Gel Filtration Chromatography

A Superose 12 HR 10/30 column from Pharmacia LKB Biotechnology was used to determine the molecular weight of native *Tt*, *Ec* and *Mtb* IPMDHs. The column was equilibrated with 25 mM MOPS-KOH pH=7.6 buffer and the flow rate was set to 0.5 mL/min. One hundred μL of the IPMDH samples (concentration 1.2 mg/mL) were injected into the column and the eluted proteins were detected at 280 and 260 nm.

Dynamic Light Scattering (DLS) Measurements

The molecular sizes of the various IPMDHs were tested with a DynaPro Titan Temperature Controlled Micro Sampler (Wyatt Technology Corporation, U.S.) instrument equipped with a laser (power 2%) operating at 830 nm. Samples of IPMDHs with final protein concentration of 8.3 mg/mL were prepared in 25 mM MOPS-KOH buffer, pH=7.6 and filtered through a Millipore filter (pore size 0.1 μm). A quartz microcell (12 μL) with a 1.5 mm light path was used and the scattered light intensities were collected at an angle of 90°. Each data set was collected for 20 s and repeated 25 times at 21 °C. The data were evaluated by the aid of Dynamics V6 (tm) Version 6.10.0.10 software using the Isotropic Spheres Model and the Regularization Fit. The size distribution of all the investigated samples were effectively monomodal, i.e., the larger aggregates were insignificant. In addition, the samples were found to be monodisperse.

Enzyme Activity Measurements

Activity of IPMDH (6-12 μg/mL, i.e., 0.16-0.32 μM monomer) was assayed in the presence of the substrates IPM, $MnCl_2$ and NAD^+ as well as 10 mM DTT in 25 mM MOPS-KOH buffer (pH=7.6). In each series of experiments the concentration of one of the substrates was varied and the other ones were kept constant, close to the saturating value (0.5 mM IPM, 0.5 mM $MnCl_2$ or 3 mM NAD^+). Alternatively, the substrate IPM was replaced by the analogue 3-methylmercaptomalate, named as (*2S,3S*)-(-)-2-hydroxy-3-sulfanylbutanedioic acid according to the IUPAC nomenclature. In the inhibitory studies activity was measured at different (in each case constant) concentrations of inhibitor and at varying concentration of the substrate, IPM. Formation of NADH was recorded spectrophotometrically at 340 nm (ε_{340} = 6220 M^{-1} cm^{-1}), at 20 °C, using a Jasco (Tokyo, Japan) V-550 spectrophotometer equipped with a Grant Y6 thermostat. The experimental activity values of the substrate saturation curves have been fitted to the Michaelis-Menten equation and yielded the V_{max} and the K_m values. From the V_{max} values the molar activities of *Tt*, *Ec* and *Mtb* IPMDH subunits at pH=7.6 have been derived to be 238 ± 30, 700 ± 80 and 200 ± 20 min^{-1}, respectively. When $MgCl_2$ (2 mM) replaced $MnCl_2$ the molar activities of all IPMDHs were reduced by about 50%. The results of the inhibitory studies have been evaluated in a double reciprocal Lineweaver-Burk plot.

The activity was also tested at different pH-s using the buffers 25 mM MOPS (pH 6.0-8.0), 10 mM HEPES (pH 6.8-8.1), 50 mM Tris-HCl (pH 7.1-9.0), 50 mM diethanolamine (pH 7.7-10.8), 25 mM 1,2-diaminoethane (pH 9.0-11.0). The overlapping pH-ranges of the particular buffers assured elimination of any influence of the specific buffer components on the enzyme activity. In each case the activity was measured at two different substrate concentrations close to saturation and these values were averaged. The experimental activity values as a function of pH were fitted according to the Henderson-Hasselbach equation of a simple deprotonation dissociation curve:

$$\nu_{measured} = \frac{\nu_{extrapolated}}{1+10^{-(pH-pK)}} \quad (1)$$

where $v_{measured}$ is the activity value measured at a given pH;

$v_{extrapolated}$ is the maximal activity value extrapolated to the high pH-range;

pK is characteristic of a catalytic dissociating side-chain.

UV Absorbance, CD and Fluorescence Spectral Measurements

UV absorbance was recorded in the range of 240 and 350 nm using a Jasco V-550 (Tokyo, Japan) spectrophotometer equipped with a Grant Y6 thermostat. The absorbance of proteins was measured in a cuvette with 1 mm path length and at 2 nm bandwidths.

CD measurements were performed with a Jasco J-720 spectropolarimeter equipped with a Neslab RTE 111 computer-controlled thermostat. For recording the far-UV CD spectra, the cuvette with 1 mm path length was used at a protein concentration of 0.6 mg/mL (15 μM monomer).

The protein fluorescence spectra were recorded using a SPEX (Edison, NJ) Fluoromax-3 spectrofluorimeter equipped with a Peltier thermostat. The samples were excited at 295 nm, and the emission was monitored between 300 and 400 nm using the cuvette with a 10 mm path length. Slits were 2 nm and 4 nm wide for excitation and emission, respectively. All measurements were carried out at 20 °C.

FRET Experiments

The Förster resonance energy transfer (FRET) between the Trp(s) of IPMDH and the bound NADH was recorded at 20 °C in the presence of Mg^{2+} and IPM as reported by Dean and Dvorak [4] using a SPEX Fluoromax-3 spectrofluorimeter equipped with a Peltier thermostat (Edison, NJ). The usual mixture contained 12 μg/mL (0.32 μM monomer) IPMDH, 12.5 μM NADH, 3 mM $MgCl_2$ and 0.5 mM IPM. Alternatively, IPM was replaced by the substrate analogue (*2S,3S*)-(-)-3-methylmercaptomalate. The protein was excited at 295 nm and the emission by the bound NADH was recorded between 300 and 540 nm in a cuvette with 10 mm path length. The slits of 2 and 4 nm were applied for excitation and emission, respectively.

IPM or analogue binding to the complex of native IPMDHs (*Tt* and *Mtb*) with NADH and Mg^{2+} was detected by fluorimetric titration using the signal of FRET occurring between the protein Trp(s) and the bound NADH, upon addition of increasing concentrations of the substrate/analogue. The equation used for calculation of K_d of the IPM/analogue-binding:

$$I_{measured} = I_{max} * [S]/(K_d + [S]) \quad (2)$$

where $I_{measured}$ is the fluorescence intensity recorded at different substrate concentrations;

I_{max} is the fluorescence intensity extrapolated to infinite substrate concentrations;

[S] is the molar concentration of the substrate;

K_d is the dissociation constant.

Denaturation of IPMDH Monitored by Protein Fluorescence Measurements

Denaturation of *Mtb* IPMDH was initiated by dilution into a solution containing 8 M urea, 25 mM MOPS-KOH buffer (pH=7.6). Time courses of unfolding were followed at 20 °C by monitoring the changes in protein fluorescence. The protein concentration in the denaturation experiments was 12 μg/mL (0.32 μM, monomer). Experiments were carried out both in the absence of substrates and in the presence of 0.6 mM IPM and 0.6 mM $MnCl_2$. For fluorescence the samples were excited at 295 nm, and the emission spectra were recorded in 5 min intervals between 300 and 400 nm using a cuvette with 10 mm path length. The slits were set to 2-4 nm for excitation and emission. The fluorescence intensities at 335 nm and the λ_{max} (the wavelengths at maximum protein fluorescence intensity) were plotted at different time intervals during denaturation. The time-dependent curves of

protein denaturation were fitted by a single exponential equation.

Renaturation of IPMDH Followed by ANS Fluorescence and by Enzyme Activity Measurements

The concentration of ANS was determined using ε_{350} = 4954 M^{-1} cm^{-1} [28] in water. For renaturation studies, denatured *Mtb* IPMDH were prepared at protein concentrations of 600 μg/mL (8.15 μM) upon incubation for 1 h in 25 mM MOPS-KOH buffer, pH=7.6, containing 8 M urea. Refolding experiments were initiated by 100-fold dilution of the denatured protein into the MOPS buffer containing ANS that was present in the renaturation mixture in a 200-fold molar excess over IPMDH. The excitation wavelength was 350 nm, and refolding kinetics was followed at 480 nm. The slits for both excitation and emission were 4 nm.

Refolding followed by enzyme activity measurements was initiated by direct dilution of the denatured protein into the activity assay solution. During the reactivation process formation of NADH was continuously recorded at 340 nm. The activity values at various time intervals of renaturation have been obtained by derivation of this curve at various times.

Sequence Comparison and Alignment

Protein sequences of various bacterial IPMDHs were downloaded from the Uniprot database (www.uniprot.org/uniprot). The sequences were compared using the algorithm downloaded from http://www.ebi.ac.uk/Tools/psa/emboss_needle.

The alignment of IPMDH sequences from various origins was first carried out by the software BioEdit. These alignments were checked using the molecular graphics software Insight II 95.0 (Biosym/MSI, San Diego, CA, USA) and were corrected manually, if required.

Comparison of Some Structural Features of the Investigated IPMDHs

Identification of the atomic contacts of the non-conserved amino-acid residues of *Mtb* IPMDH with the conserved ones were carried out using the X-ray coordinates of the crystal structure (pdb: 1W0D) with Insight II, as described earlier [19].

The possible binding modes of the substrates, Mn^{2+}-complex of IPM and NAD^+, to *Mtb* IPMDH have been determined by molecular modelling based on the known crystal structures of *Tt* IPMDH (pdb codes 2Y41 and 2Y42) [19].

The extent of hydrophobic surface area of the three investigated IPMDHs was calculated from their pdb coordinates using the software ALPHASURF [29].

Prediction of the energetically stable oligomeric states of the three IPMDHs was carried out using the PISA server [30].

The isoelectric points and the molecular charges of the three investigated IPMDHs was estimated by the PROPKA software [31].

RESULTS AND DISCUSSION

Expression of *Mtb* IPMDH by Fermentation and its Molecular Size

Figure **1A** illustrates various stages of expression of *Mtb* IPMDH by SDS-PAGE. The last sample is the purified enzyme obtained after the Ni-affinity chromatography. This run clearly confirms the purity (at least 95%) of the produced *Mtb* IPMDH. The yield was 8-10 mg protein from 1.5 L of cell culture.

Comparative native gel electrophoresis of *Tt*, *Ec* and *Mtb* IPMDHs is shown in (Fig. **1B**). Surprisingly, the electrophoretic mobility of *Mtb* IPMDH was much lower compared to the other two IPMDHs. Considering the closely similar isoelectric points of the three IPMDHs as calculated using PROPKA [31] (pI=6.0 for both *Tt* and *Mtb*, pI=5.66 for *Ec* IPMDH) the significantly reduced mobility of *Mtb* IPMDH would not be expected. Therefore, this experiment raised the possibility that *Mtb* IPMDH has a larger molecular size (e.g. higher degree of association of its polypeptide chains). In order to test this assumption, we have repeated the electrophoresis assay in the presence of 8 M urea (Fig. **1D**). Under this condition, the polypeptide chains became dissociated and unfolded. However, their electrophoretic mobilities were still influenced by their charges at the pH of the running buffer. We have estimated using PROPKA [31] that the chains of *Tt*, *Mtb* and *Ec* possessed the following net charges at pH=9.0 (i.e., at the pH of the electrophoresis): -9.8, -14.4 and -20.3, respectively. Thus, the electrophoretic mobilities of the three enzymes in 8 M urea should follow the order of their charges. Indeed, as shown by (Fig. **1D**), the three enzymes with similar denatured states showed running rates, with the *Mtb* enzyme being between the *Tt* and *Ec* IPMDHs. Thus, the slight differences in the charges of the IPMDH molecules cannot explain the observed retarded electrophoretic mobility of the *Mtb* IPMDH on the native gel (Fig. **1B**). These observations are in accordance with the assumption of a different quaternary structure of *Mtb* IPMDH, possibly a higher oligomerization state. Therefore, we have carried out size exclusion chromatography under native conditions with the three investigated IPMDHs. As illustrated in (Fig. **1C**), the apparent molecular weights of *Tt*, *Ec* and *Mtb* IPMDHs were about 64 kDa, 66 kDa and 89 kDa, respectively. For *Tt* and *Ec* IPMDHs, dimeric structures have been confirmed [13, 20], and their molecular masses are estimated at 76 kDa, i.e., somewhat higher than the presently obtained experimental values (64-66 kDa). Thus, it is possible that the experimentally determined elution volumes were perturbed due to the non-spherical shape of the molecule. Therefore, the estimated increased molecular mass of *Mtb* IPMDH likely reflects a tetrameric structure, since a trimeric structure would be inconsistent with the symmetry properties of the molecule. A similar gel-filtration experiment with *Sulfolobus* sp. strain 7 IPMDH also suggested an oligomeric state only slightly higher than a dimer [32] and its tetrameric state has indeed been demonstrated later by analytical ultracentrifugation [33].

To confirm the oligomerization states of the IPMDHs used in the present work, we have also carried out comparative DLS studies. The predicted apparent hydrodynamic radii of *Tt*, *Ec* and *Mtb* IPMDHs were obtained to be 29.8±2,

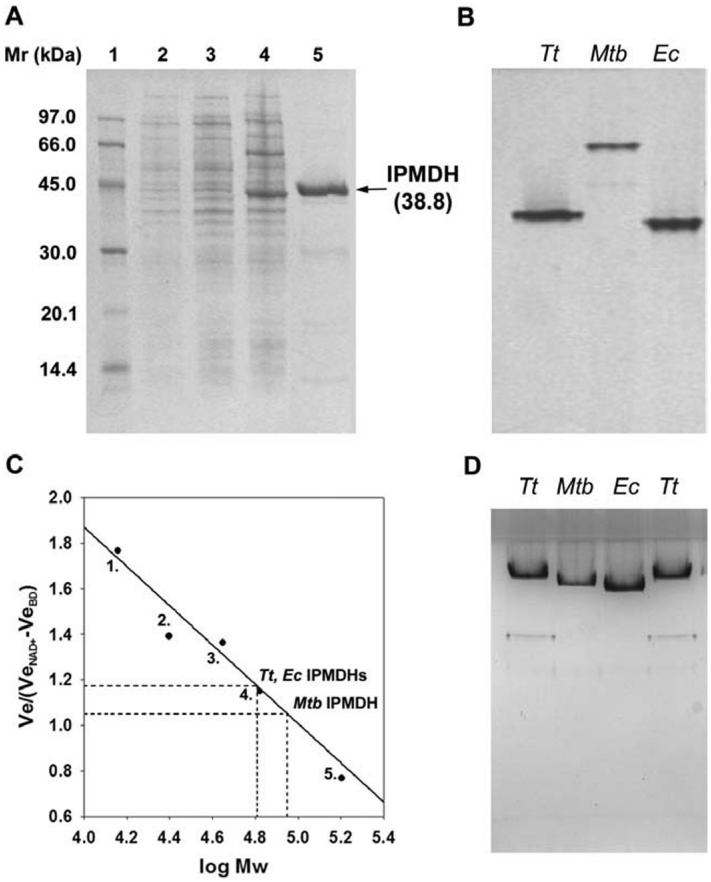

Figure 1. Gel electrophoresis and gel filtration of *Mtb* IPMDH-containing samples. In (**A**) samples for the SDS-PAGE were taken from the fermentor (intact cells) before the fed-batch phase (2), before the induction (3) and 3 hours after the induction (4). The samples were diluted 5 times and 3 µL was loaded into the gel. A sample of 5 µg of purified *Mtb* IPMDH (5) also was loaded into the gel. In (**B**) native gel electrophoresis of samples of *Tt*, *Mtb* and *Ec* IPMDHs were run as described in Materials and Methods. In (**C**) the results of the Superose 12 gel filtrations are shown. The following protein samples were used for calibration: 1. lysozime (14.4 kDa), 2. chymotrypsinogen A (25 kDa), 3. phosphoglycerate kinase (44.5 kDa), 4. bovine serum albumin (66.4 kDa) and 5. aldolase (160 kDa). The apparent molecular weights of *Tt*, *Ec* and *Mtb* IPMDHs were determined to be 63.6 kDa, 65.5 kDa and 88.6 kDa, respectively. In (**D**) gel electrophoresis of denaturated *Tt*, *Mtb* and *Ec* IPMDHs is illustrated in the presence of 8 M urea. The *Tt* sample was run both before and after the other samples in order to detect differences, if any, in their running rates, due to any inherent error of the electrophoretic run. A small fraction of the remaining native part of the *Tt* IPMDH is also observable.

31.5±1 and 43±2 Å, in the same order. The value for the *Tt* enzyme agrees well with the gyration radius of 28.7±0.3 Å determined earlier by small angle X-ray scattering [6]. These results are in agreement with the assumed higher oligomerization state of *Mtb* IPMDH. Using DLS we have also found that various nucleotides (dNTP mixture, NAD^+, ATP), possibly present during bacterial expression, do not affect the values of hydrodynamic radii.

Using the crystallographic structure of *Mtb* IPMDH (PDB entry 1W0D) we predicted the likely oligomerization state of the protein using the PISA server [30] which generates all possible oligomerization states by symmetry operations and selects the most likely state by calculating buried surfaces and estimating dissociation free energies. Based on these calculations, the most likely oligomerisation state was predicted to be a tetramer (Fig. **2**). This tetramer appears to be a weakly associated dimer of two more strongly associated dimers, with an estimated dissociation free energy of 12.4 kcal/mol for the dissociation of the tetramer into two dimers and 40.6 kcal/mol for the dissociation of each dimer into monomers. The interface between the two dimers is also smaller (2685 $Å^2$) and less hydrophobic (~60% apolar) than the interfaces between the monomers in the dimers (~4300 $Å^2$ each, ~68% apolar) as calculated by the program ALPHASURF [29]. Identical modelling for the *Tt* IPMDH predicted even smaller (2062 $Å^2$) and less hydrophobic (~54%) hypothetical interfaces between the dimers (the available crystal structure of the dimeric *Ec* IPMDH that exhibits a different conformation, however, did not allow to carry out similar modelling studies).

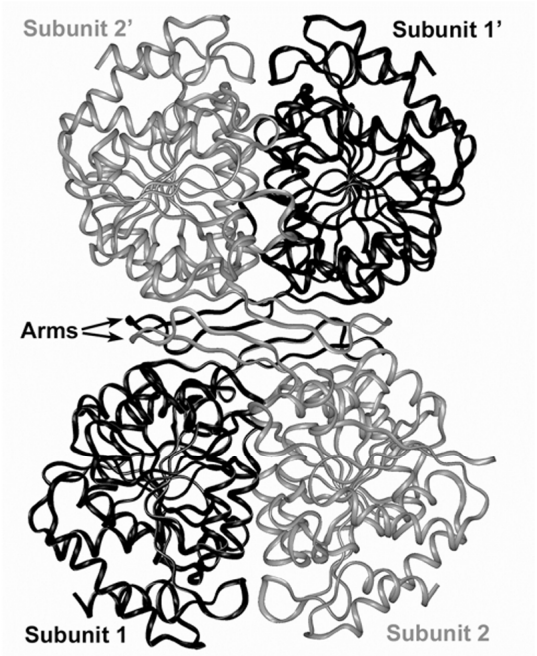

Figure 2. Modelling of the tetrameric structure of *Mtb* IPMDH. The oligomeric structure of *Mtb* IPMDH was predicted using the X-ray coordinates (pdb 1W0D, [18]) by the aid of PISA server [30]. The subunits within each dimer are illustrated by black and grey ribbon diagrams, respectively. The interacting arms of the subunits are indicated by arrows.

In the predicted tetrameric structure of *Mtb* IPMDH, it is peculiar that the arms of each subunit are important not only for the stabilization of the dimers, but also for maintaining the tetrameric structure. It is also notable that the substrate binding sites (illustrated on Fig. **8**) are not perturbed or shielded by the oligomeric contacts. Thus, the catalytic efficiency of the enzyme is possibly not changed in the tetrameric state, i.e., the biological relevance of the enzyme is most probably not dependent on the dimer-tetramer transition.

Spectral Properties of *Mtb* IPMDH in Comparison with *Tt* and *Ec* IPMDHs

Since certain structural characteristics of proteins are often reflected by their spectral properties, we first recorded the UV spectrum of *Mtb* IPMDH and compared it to its *Tt* and *Ec* counterparts (Fig. **3A**). In contrast to the other two enzymes, the absorption maximum of *Mtb* IPMDH is not at 280 nm but somewhat blueshifted. Thus, in contrast to the ratio of A_{280}/A_{260}=1.6, characteristic of other IPMDHs, this ratio is only 0.8 in the case of *Mtb* IPMDH. We assumed that the latter enzyme binds some nucleotide that is most probably NAD^+. In order to test this possibility, we have added the other substrate IPM and Mn^{2+} (at closely saturating concentrations, cf. Methods) to the enzyme, but no NADH formation could be detected. Therefore, we can only conclude that some other unidentified nucleotide of the cell remains bound to the enzyme, in spite of the isolation procedure. The existence of a nucleotide in this solution was shown by the spectrum (grey solid line) with λ_{max}=260 nm obtained after removal of the protein by heat denaturation and centrifugation of the precipitate. When this spectrum of the nucleotide was subtracted from the original spectrum of the native *Mtb* IPMDH (dotted), the spectrum of the nucleotide-free *Mtb* IPMDH (black solid line) can be obtained. Taking into account the ratio of the A_{280} values of the spectra of dotted and black solid lines at 280 nm, the theoretical molar absorption coefficient of *Mtb* IPMDH (ε_{280}=15930 M^{-1} cm^{-1} for the monomer [25]) can be corrected and obtained to be ε_{280} (app.)=16941 $M^{-1}cm^{-1}$. Furthermore, the binding of the nucleotide to *Mtb* IPMDH is probably not very tight, as it can be removed from the protein by gel filtration (not shown).

The far-UV CD spectra of *Mtb*, *Tt*, and *Ec* IPMDHs are compared in (Fig. **3B**). As expected, the molar ellipticity values, characteristic of the three proteins are very similar to each other. Thus, the amounts and the qualities of the secondary structural elements in all three proteins are very similar to each other, as suggested by the similarities of their X-ray structures [13, 18, 19].

The protein fluorescence emission spectra of *Mtb*, *Ec* and *Tt* are shown in (Fig. **3C**). The emitted fluorescent intensities are seemingly proportional to the Trp content of the three proteins: *Mtb*, *Ec* and *Tt* IPMDHs contain 1, 2 and 3 Trp side-chains per mole subunits, respectively. However, the characteristic λ_{max} values of the spectra are slightly different, possibly due to the different chemical environments of the Trp side-chains in the three different proteins.

A special fluorescent phenomenon, FRET, exhibited by all the investigated IPMDHs is illustrated in (Fig. **3D**). The occurrence of this Förster (F) resonance (R) energy (E) transfer (T) has been observed with *Tt* IPMDH from its Trp side-chains to the nicotinamide ring of the bound NADH when the other substrate IPM is also bound [4]. It was shown that the presence of the metal ion Mn^{2+} or Mg^{2+} is also essential for this phenomenon [6]. It is reasonable to assume that the conformational changes caused by the binding of IPM (possibly associated with the domain closure) bring the NADH nicotinamide and the Trp indole rings in a relative orientation optimal for the occurrence of FRET. It is seen in (Fig. **3D**) that increasing fluorescent intensity of the bound NADH is accompanied by decreasing protein fluorescence intensity for all three enzymes. Thus, *Mtb* IPMDH exhibits the FRET phenomenon, similarly to the other two IPMDHs.

It may be notable that similar FRET spectra (although with somewhat lower intensities) were obtained when IPM was replaced by the substrate analogue (2*S*,3*S*)-(-)-3-methylmercaptomalate (cf. below) for both the *Tt* and *Mtb* IPMDHs (not shown).

Enzyme Kinetic Parameters of *Mtb* IPMDH in Comparison with *Tt* and *Ec* IPMDHs

Activity of *Mtb* IPMDH was tested at varying concentrations of each substrate. The kinetic constants derived from the substrate saturation curves by fitting according to the Michaelis-Menten equation, are summarized in Table **1**, in

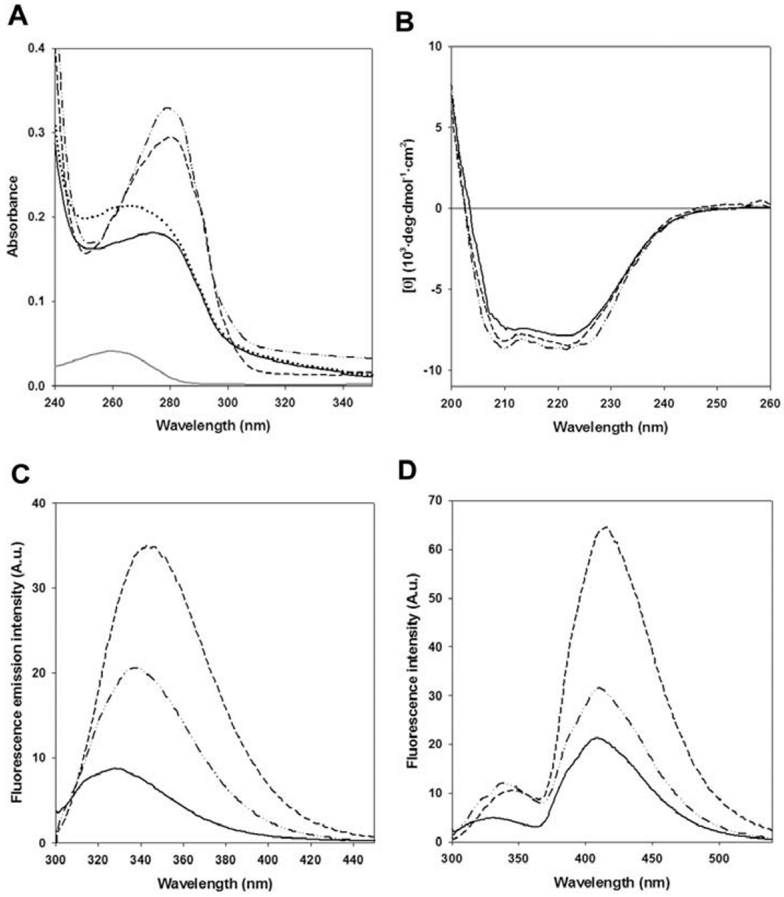

Figure 3. Spectral properties of *Mtb* IPMDH as compared to *Tt* and *Ec* IPMDHs. The spectra of *Mtb* (dotted), *Tt* (dashed) and *Ec* (dashed-dot-dot) IPMDHs were recorded by UV absorbance (**A**). The spectrum of the bound nucleotide (grey solid) was subtracted from the original spectrum of the native *Mtb* IPMDH (dotted), the spectrum of the nucleotide-free *Mtb* IPMDH (black solid line) can be obtained. The spectra of *Mtb* (black solid), *Tt* (dashed) and *Ec* (dashed-dot-dot) IPMDHs were also recorded by far-UV CD (**B**), protein fluorescence emission (**C**) and fluorescence resonance energy transfer (**D**). Protein concentrations of 0.6 mg/mL (15 μM monomer) and 12 μg/mL (0.32 μM monomer) were applied in CD (**B**) and fluorescence (**C** and **D**) measurements, respectively. The excitation was carried out at 295 nm and the excitation and emission slits were 2-4 nm in (**C** and **D**).

Table 1. Comparison of the kinetic parameters and substrate binding constants of the three different IPMDHs at pH=7.6. The kinetic and binding experiments are described in the Methods section.

IPMDH	K_m (μM)			k_{cat} (min^{-1})	$\frac{k_{cat}}{K_{m,IPM}}$ ($M^{-1}min^{-1}$)	$K_{d,IPM}$ (μM)
	IPM	NAD^+	Mn^{2+}			
Mtb	5.8±2	134±30	15±5	200±20	$(3.45±1.3)\cdot10^7$	5.3±2
Tt	15.8±5	328±50	10.2±3	238±30	$(1.51±0.6)\cdot10^7$	17.0±4
Ec	4.9±2	159±25	17.6±4	700±80	$(1.43±0.5)\cdot10^8$	5.5±2

comparison with those of *Tt* and *Ec* IPMDHs. The catalytic efficiencies of IPMDHs of the different origins are of similar magnitude. As for the k_{cat} values, *Mtb* IPMDH exhibited a value comparable with the one characteristic of *Tt* IPMDH. On the other hand, the K_m values of all three substrates were about 2-3 fold smaller for *Mtb* IPMDH, indicating its somewhat tighter interactions with the substrates, more similar to those of *Ec* IPMDH.

Inhibitory Effects of O-methyl Oxalohydroxamate and (2*S*,3*S*)-(-)-2-hydroxy-3-Sulfanylbutanedioic Acid

In order to find inhibitors against *Mtb* IPMDH, we have synthesized two different previously described compounds that were reported to be good inhibitors against *Tt* IPMDH. The compound O-methyl oxalohydroxamate [22] however, in our experiments (not shown) exhibited significantly weaker inhibitory properties towards *Tt* IPMDH (K_I=1.45±0.03 mM) compared to its previously reported value (K_I=1.2 μM). This is surprising because both data sets have been determined under closely similar experimental conditions. Thus, we decided to synthesize a recently published thia-analogue of IPM, 3-methylmercaptomalate or, according to the IUPAC nomenclature, (2*S*,3*S*)-(-)-2-hydroxy-3-sulfanylbutanedioic acid [23]. The intersecting double reciprocal Lineweaver-Burk plots of the kinetic data of the IPM saturation curves (Fig. **4A**) in the absence and presence of the inhibitor clearly demonstrated the purely competitive character of the inhibition for both *Tt* and *Mtb* IPMDHs. The competitive inhibitory constants (K_I) were found to be closely similar, 349±50 nM and 427±50 nM, respectively, for these two different IPMDHs. These values are not very different from K_I=62 nM, determined earlier by Nango and coworkers with *Tt* IPMDH under somewhat different conditions but at much higher temperature, 60 °C [23].

Further, Nango and coworkers reported that this substrate analogue inhibitor also exhibits weak substrate activity, but the extent has not been quantified. In agreement with this finding, under our experimental conditions, we found k_{cat} values as low as 1.2±0.2 min^{-1} and 1.3±0.2 min^{-1} for *Tt* and *Mtb* IPMDHs, respectively. The K_m value of the analogue could not be quantified but was estimated to be in the nanomolar range.

K_d Value of MnIPM Binding to *Mtb* IPMDH in Comparison with *Tt* and *Ec* IPMDHs

As shown above (Fig. **3D**), the FRET signal is formed only when IPM is bound to the enzyme. Thus the FRET signal is an indicator of IPM binding. Therefore, the enzyme could be titrated with IPM by detecting the increase of the FRET emission. The K_d value of MnIPM binding to *Mtb* IPMDH in comparison with *Tt* and *Ec* IPMDHs has been obtained by fitting these titration curves to simple binding hyperbolas (cf. Methods) and the values are listed in Table **1**. It is seen that the K_d values were closely similar to the respective K_m values of the substrate, in agreement with a rapid equilibrium enzyme kinetic mechanism.

Formation of FRET Spectrum in the Presence of (2*S*,3*S*)-(-)-2-Hydroxy-3-Sulfanylbutanedioic Acid and its Binding Parameters

As noted above, FRET spectra of both *Tt* and *Mtb* IPMDHs are also formed with 3-methylmercaptomalate. This is not surprising because this compound is a weak substrate, thus, it may be able to induce conformational changes similar to those caused by the real substrate, IPM. Using this FRET signal, we have titrated both *Tt* and *Mtb* IPMDHs with this analogue in order to determine its binding constants

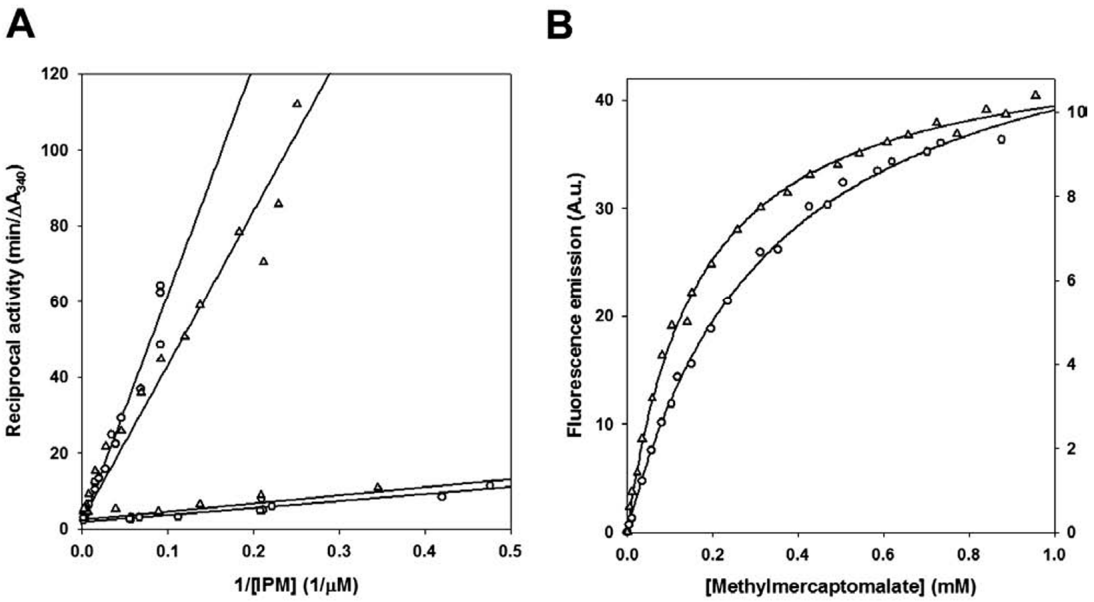

Figure 4. Characterization of the inhibitor (2*S*,3*S*)-(-)-2-hydroxy-3-sulfanylbutanedioic acid. The inhibitory effect of (2*S*,3*S*)-(-)-2-hydroxy-3-sulfanylbutanedioic acid (or 3-methylmercaptomalate) on *Mtb* (△) and *Tt* (○) IPMDHs is illustrated by a Lineweaver-Burk plot (**A**). The activities were recorded in the absence and in the presence of 37 μM inhibitor. The competitive inhibitory constant (K_I) values are obtained to be 427±50 nM and 349±50 nM, respectively, for *Mtb* and *Tt* IPMDHs. Binding of 3-methylmercaptomalate to the complex of native IPMDH-NADH binary complex was monitored by recording the appearance of the FRET signal (**B**) upon addition of increasing concentration of 3-methylmercaptomalate to *Mtb* (△, right axis) and *Tt* (○, left axis) IPMDHs. The K_d values of 87±15 μM and 178±20 μM were derived for *Mtb* and *Tt* IPMDHs, respectively, by fitting the experimental data to Eq. 2 (cf. Experimental part).

(Fig. **4B**). While for IPM, the binding constant (K_d) was in close agreement with its K_m value (cf. Table **1**), these two constants greatly differ for 3-methylmercaptomalate. While the K_m values are in nanomolar range (cf. above), the K_d-values are in micromolar range (178±20 μM and 87±15 μM for *Tt* and *Mtb* IPMDHs, respectively). It is possible that the binding modes of this weak substrate are different in the presence of simultaneously bound NAD^+ (functioning complex) and NADH (non-functioning complex). These different binding modes might be reflected in these greatly different constants. Thus, the different binding modes of inhibitors in the functioning and non-functioning complexes are possibly a general characteristic of all IPMDHs.

pH-Dependence of Activity of the Investigated IPMDHs

As IPMDH, similar to other oxidative decarboxylases [34], probably functions by the aid of acid-base catalysis, the pH dependence of the enzyme activity is an informative characteristic of the enzyme. We investigated this property of the *Mtb* IPMDH, and compared it to the other two better-characterised *Tt* and *Ec* IPMDHs. As shown in (Fig. **5A**), the k_{cat} of *Mtb* IPMDH exhibits a maximum at pH≈9, similar to *Tt* and *Ec* IPMDHs. From analysing the normalised pH vs. activity profiles (Fig. **5B**) according to Eq. 1, it became clear that the pH dependence of the activity of *Mtb* IPMDH can be attributed to the dissociation of an ionisable group with p*K*=7.5, similar to *Tt* enzyme (p*K*=7.4), but somewhat different from the pH dependence of *Ec* IPMDH (p*K*=6.7). The larger specific activity of *Ec* IPMDH compared to that of the *Tt* enzyme at pH=7.6 (Table **1**) can most probably be attributed to the different pH dependences of their activities (Fig. **5**). Since the CD spectra of the enzymes (recorded in the pH range of 6 and 9, not shown) do not indicate the occurrence of any significant structural changes, the observed pH dependences of the activities can most probably be attributed to the ionisation curves of at least one (or more) specific active site residue(s). Site-directed mutagenesis studies with *Tt* IPMDH are underway in our laboratory and are expected to identify the catalytic residue(s) responsible for the observed pH-dependence.

Denaturation and Renaturation Experiments with *Mtb* IPMDH

The spectral changes observed in the protein fluorescence of *Mtb* IPMDH upon ~1 h incubation in the presence of 8 M urea are shown in (Fig. **6A**). During denaturation, it was a considerable decrease in the emitted fluorescence intensity with a redshift of the λ_{max} from 330 nm to 353 nm. These changes are very similar to those observed previously for the *Tt* and *Ec* IPMDHs [9]. The time dependences of these spectral changes upon denaturation of the *Mtb* IPMDH have been registered and shown in (Figs. **6B** and **6C**), respectively. The half-times of denaturation as derived from these curves are compared to those for *Tt* and *Ec* IPMDHs in Table **2**. It is clear that the stability properties of *Mtb* IPMDH resembled more the behaviour of the similarly mesophilic *Ec* IPMDH.

Table 2. Protective effect of the substrate MnIPM against denaturation of the investigated IPMDHs. The half time of urea-denaturation ($t_{1/2}$, min) was determined in the absence and presence of MnIPM.

IPMDH	No IPM ($t_{1/2}$, min)	With IPM ($t_{1/2}$, min)
Mtb	8.6±3	29.9±5
Tt	46.2±8	53.3±8
Ec	5.0±2	30.1±7

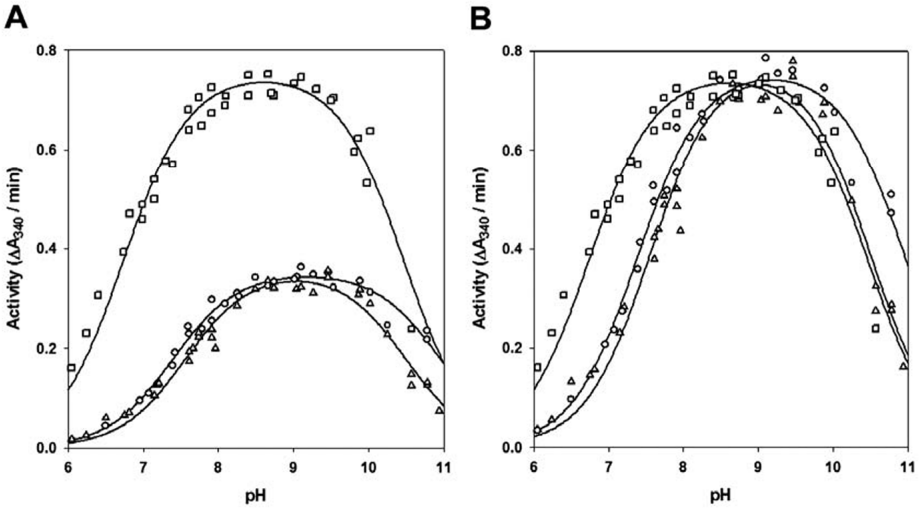

Figure 5. The pH dependence of the activity (k_{cat}) of IPMDHs. The enzyme activities of *Mtb* (△), *Tt* (○) and *Ec* (□) IPMDHs were determined at the protein concentration 6 μg/mL (0.16 μM monomer) at different pH values (**A**). On panel (**B**) the pH dependence curves are normalised to the *Tt* enzyme. The data were fitted by a bell-shaped curve in accordance with the Eq. 1. The pH dependence of activity of *Mtb* IPMDH can be attributed to dissociation of an ionisable group with p*K*=7.5, similar to *Tt* enzyme (p*K*=7.4), but somewhat different from the pH-dependence of *Ec* IPMDH (p*K*=6.7).

We have also monitored urea-denaturation of *Mtb* IPMDH in the presence of the substrate, IPM. Substrate binding, in addition to the effect of stabilizing the catalytically competent protein conformation (e.g. the domain-closed form of IPMDH), in general, can also stabilize the protein structure against the damage caused by denaturing agents. We have reported that binding of MnIPM markedly reduces the rates of unfolding of IPMDHs and this effect is more prominent for the less stable enzyme variants. It is only marginal in case of the thermostable *Tt* IPMDH but pronounced in the cases of the mesophilic *Ec* and the psychrotrophic *Vibrio* sp. I5 IPMDHs [9]. Here, the denaturation experiment with *Mtb* IPMDH in the presence of MnIPM yielded a coherent result (Figs. **6B** and **6C**). It is clear from Table **2** that the rate of denaturation was slowed down significantly in the presence of MnIPM, similarly to the case of the other mesophilic form, *Ec* IPMDH. Thus, the protecting effect of the substrate on the protein structure against denaturation correlates well with the structural flexibilities of the various IPMDHs [8, 35].

Refolding kinetics of the denatured *Mtb* IPMDH was recorded by measuring the change of the fluorescence intensity of the protein-bound hydrophobic probe ANS upon decreasing the urea concentration by dilution. An immediate increase of the ANS fluorescence emission within the mixing time was observed (the fluorescence of ANS bound either to the denatured or the native protein are negligible). This increase is generally characteristic of the formation of a molten globule state, i.e., an intermediate with relatively loosely packed globular structure with the majority of the secondary structural elements formed, but without the rigid tertiary structure [36, 37]. This immediate increase of fluorescence intensity is followed by a slower decrease reflecting the formation of the protein native structure having low affinity for ANS (Fig. **7A**). This decrease can be approximated by a single exponential with a rate constant of 0.29±0.07 min^{-1}. However, the total change was much smaller (around 10-15%) for *Mtb* IPMDH as compared to the other two (*Tt* and *Ec*) IPMDHs (about 60-70% [9]). Consistent with this finding, the reactivation studies upon renaturation of *Mtb* IPMDH also led to only a very low yield of the active enzyme with a rate constant of 0.18±0.04 min^{-1} (Fig. **7B**).

The rate constants of renaturation of *Mtb* IPMDH are about the same magnitude as those of *Tt* and *Ec* IPMDHs (0.12±0.01 min^{-1} and 0.064±0.006 min^{-1} [9]). However, the extent of renaturation of *Mtb* IPMDH was found to be much smaller compared to the other two investigated IPMDHs. We have checked that this different behaviour of *Mtb* IPMDH cannot be due to its somewhat larger hydrophobic molecular surface compared to *Tt* and *Ec* IPMDHs. The hydrophobic fraction of the accessible surface is in the 56-58% range for each one of the three IPMDHs as determined by using the program ALPHASURF [29]. It is possible, therefore, that the greatly different amino acid sequence of *Mtb* IPMDH (cf. below) is associated with an altered folding pathway of this protein and thereby led to off-pathway irreversible intermediates.

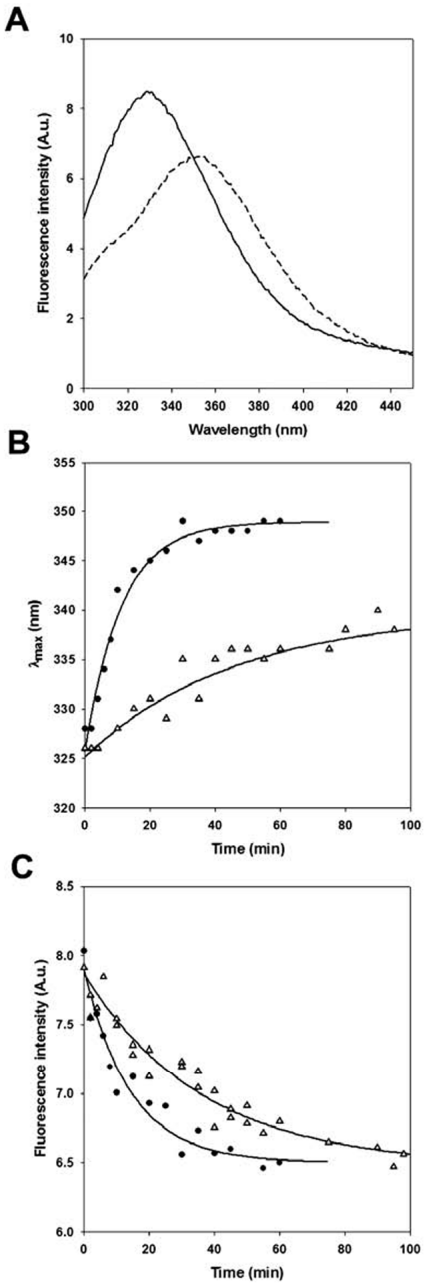

Figure 6. Denaturation of *Mtb* IPMDH as followed by protein fluorescence. The spectral changes accompanying the denaturation of *Mtb* IPMDH are represented by the spectra of the native (solid line) and denatured (dashed line) enzymes (**A**). During denaturation the emission spectra were recorded in the absence (●) and in the presence (△) of IPM in every 5 minutes. The values of λ_{max} (**B**) and the fluorescence intensities at 335 nm (**C**) were plotted at different time intervals during denaturation. Half-times of 8.6±1 and 29.9±5 min were obtained by fitting the curves to a single exponential.

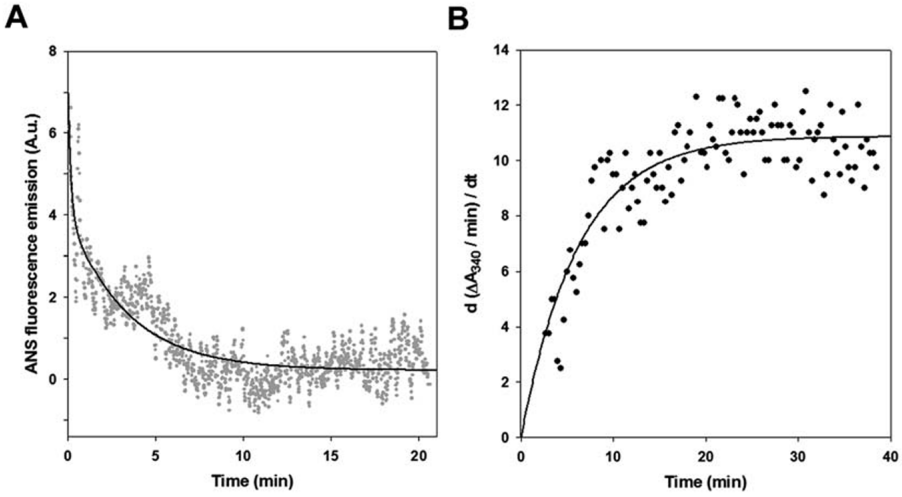

Figure 7. Renaturation time courses of *Mtb* IPMDH. Refolding kinetic curves of *Mtb* IPMDH were determined by recording the changes of the bound ANS fluorescence (**A**) and enzyme activity (**B**). The protein concentrations were 6 μg/mL (0.16 μM monomer) (**A**) and 60 μg/mL (1.6 μM monomer) (**B**). The corresponding rate constants are 0.29±0.07 and 0.18±0.04 min^{-1}.

Table 3. Sequential comparison of various IPMDHs. Percentage of the identical amino acid residues are indicated in pairwise comparisons of the alighned sequences.

	Mtb	*Thermotoga maritima*	*Thiobacillus ferrooxidans*	*Ec*	*Salmonella typhimurium*	*Tt*
Tt	40.9 %	53.5 %	52.8 %	48.8 %	47.9 %	100 %
Salmonella typhimurium	37.2 %	55.2 %	48.9 %	94.5 %	100 %	
Ec	37.2 %	55.5 %	49.5 %	100 %		
Thiobacillus ferrooxidans	38.9 %	50.8 %	100 %			
Thermotoga maritima	37.5 %	100 %				
Mtb	100 %					

Structural Comparison of *Mtb* IPMDH with IPMDHs of Different Origins

Pairwise comparison of the various IPMDH sequences has revealed that the sequence of *Mtb* enzyme is rather different from that of any other IPMDHs while the other IPMDHs have smaller differences among them. Sequence comparison of *Mtb* IPMDH with five other bacterial IPMDHs with known crystal structures has revealed that it is only identical in 37-41% with them, while the identities among these other sequences are more extensive, at least 48-55% (Table **3**). The highest agreement (41%) of *Mtb* IPMDH sequence is found with the *Tt* IPMDH. This observation might be correlated with the closer similarities in the kinetic properties (k_{cat} and the substrate K_m) of *Mtb* and *Tt* IPMDHs. Indeed, the sequence alignment of *Mtb* IPMDH with *Tt* and *Ec* IPMDHs has revealed the identity of 40 and 29 non-conserved amino acid residues, respectively (Fig. **S1**).

In order to look for the possible differences in the three-dimensional structure of *Mtb* IPMDH relative to other IPMDHs that might be due to the observed sequential differences, we have analyzed the molecular contacts of the conserved side-chains in the crystal structure of *Mtb* IPMDH, similar to the previous analysis carried out with *Tt* IPMDH [19]. A disturbance of the important contacts of conserved side-chains by the non-conserved side-chains in the structure of *Mtb* IPMDH might be expected. However, no meaningful structural effects could be observed. This can probably be attributed to the fact that in most cases, chemically similar non-conserved side-chains (e.g. Leu/Ile, Phe/His/Pro, Ser/Thr/Asn) replace each other in the structures of various IPMDHs.

In agreement with these findings, we could model the binding of both substrates into the known crystal structure [18] of *Mtb* IPMDH (Fig. **8**). As it is seen, almost all conserved substrate binding residues in the crystal structure of substrate-free *Mtb* IPMDH are located in a steric arrangement allowing formation of contacts with both substrates. A few side-chains (e.g. R97 or D275 in *Mtb* enzyme) are exceptions, but their positions are possibly optimized as soon

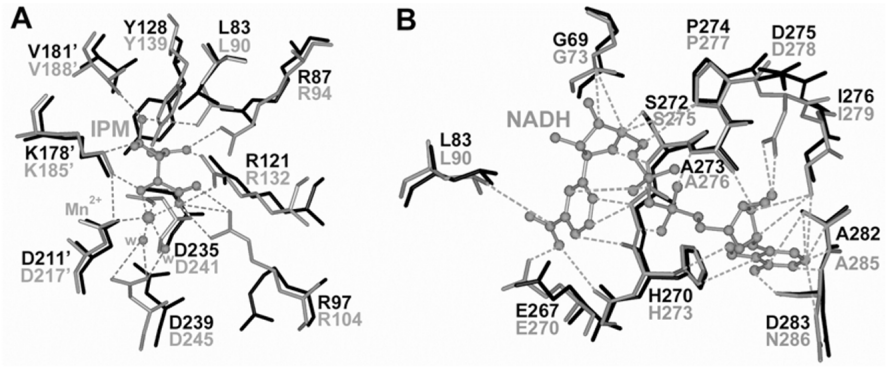

Figure 8. Demonstration of the similar active site architectures of *Mtb* and *Tt* IPMDHs: IPM (A) and NADH (B) binding. The *Tt* (grey, pdb entries 2Y41 and 2Y42 for the IPM and NADH bound complexes, respectively, [19]) and *Mtb* (black, pdb 1W0D, [18]) IPMDH structures were superimposed by the secondary structural elements of the Domain 2 (**A**) or Domain 1 (**B**), respectively. The conserved side-chains involved in the substrate binding are represented by stick models. The substrates MnIPM and NADH are illustrated by ball-and-stick models, respectively. The dashed lines represent atomic interactions of *Tt* IPMDH with the substrates.

as the substrates are bound. This is illustrated by superimposition of the respective crystal structures of complexes of *Tt* IPMDH with IPM (Fig. **8A**) and with NADH (Fig. **8B**) onto the *Mtb* IPMDH structure. Furthermore, the contact lists of all other conserved residues, important for domain-domain communications, are very similar for all three IPMDHs investigated here (Table **S1**).

It is notable, however, that the static pictures derived from the crystal structures are not fully suitable to explain all fine details reflected in the kinetic properties of the three IPMDHs (e.g. quantitative values of the catalytic constants cf. Table **1**) investigated here. Yet, the main features of the catalysis and its structural background are very similar for all IPMDHs. Thus, any designed new inhibitor of *Mtb* IPMDH, as a potential drug against tuberculosis, can be tested with any one of the other IPMDHs. This can reasonably facilitate designing and testing new inhibitors, as unlike the *Mtb* enzyme, the other IPMDHs can generally be produced by simpler expression procedures.

ABBREVIATIONS

IPMDH = 3-isopropylmalate dehydrogenase [EC 1.1.1.85]

IPM = Threo-D-3-isopropylmalate or (2*R*,3*S*), 2-hydroxy-3-(propan-2-yl)butanedioic acid

Tt = *Thermus thermophilus*

Ec = *Escherichia coli*

Mtb = *Mycobacterium tuberculosis*

BD = Blue dextrane (cf. Fig. **1C**)

DLS = Dynamic light scattering

FRET = Förster resonance energy transfer

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY MATERIALS

Figure **S1** provides structure-based sequence alignment of *Mtb*, *Tt and Ec* IPMDHs.

Table **S1** gives information about the main atomic contacts of the conserved residues in comparison of *Mtb* and *Tt* IPMDHs.

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