Triose-phosphate Isomerase (TIM) of the Psychrophilic Bacterium *Vibrio marinus*

**KINETIC AND STRUCTURAL PROPERTIES**

Marco Alvarez‡, Johan Ph. Zeelen‡, Véronique Mainfroid‡, Françoise Rentier-Delrue‡, Joseph A. Martial‡, Lode Wyns§, Rik K. Wierenga§, and Dominique Maes†**

From the ‡Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, B6, Sart Tilman, B4000 Liège, Belgium, €European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, D69012 Heidelberg, Germany, and §Ultrastructure Unit, Vlaams Interuniversitair Instituut voor Biotechnologie, Vrije Universiteit Brussel, B1640 Sint-Genesius-Rode, Belgium

The purification and characterization of triose-phosphate isomerase from the psychrophilic bacterium *Vibrio marinus* (vTIM) is described. Crystal structures of the vTIM-sulfate complex and the vTIM-2-phosphoglycerolate complex (at a 2.7-Å resolution) are also presented. The optimal growth temperature of *Vibrio marinus* is 15 °C. Stability studies show that vTIM is an unstable protein with a half-life of only 10 min at 25 °C. The vTIM sequence is most closely related to the sequence of *Escherichia coli* TIM (eTIM) (66% identity), and several unique structural features described for eTIM are also seen in vTIM, but eTIM is considerably more stable. The *T*ₚ values of vTIM and eTIM, determined by calorimetric studies, are 41 and 54 °C, respectively. Amino acid sequence comparison reveals that vTIM has an alanine in loop 8 (at position 238), whereas all other TIM sequences known to date have a serine. The vTIM mutant A238S was produced and characterized. Compared with wild type, the catalytic efficiency of the A238S mutant is somewhat reduced, and its stability is considerably increased.

Triose-phosphate isomerase (TIM; EC 5.3.1.1) is a dimeric glycolytic enzyme formed by two identical subunits consisting of about 250 residues each. It catalyzes the interconversion of dihydroxyacetone phosphate and d-glyceraldehyde-3-phosphate (see Fig. 1). TIM has been the subject of extensive biochemical, enzymological, and computational studies. Its catalytic properties and mechanism have been studied in detail (1, 2). It has been established that TIM is a very efficient catalyst, since the reaction rates are diffusion-controlled. Neither cofactors nor metal ions are required in this reaction, and there is no evidence of allostery or cooperativity among the subunits.

Structurally TIMs do form a well characterized family. To date, x-ray structures of TIM from seven different sources have been solved (wild type or/and in complex with substrate analogues): chicken (3), yeast (4), *Trypanosoma brucei* (5), *Escherichia coli* (6), human (7), *Bacillus stearothermophilus* (8), and *Plasmodium falciparum* (9). In addition, the structures have been determined of *Leishmania mexicana* TIM (10) and of *Thermotoga maritima* TIM. (11) Also, 45 amino acid TIM sequences from a wide variety of organisms have been determined and are available in the data bases.

Each TIM monomer has a globular shape with two protruding loops. The globular part is formed by an 8-fold repeat of a β-strand, loop, α-helix, loop motif. The βα units fold up in a regular way, such that the β-strands (numbered β₁ to β₈) form an eight-stranded β-barrel surrounded by the eight α-helices (numbered α₁ to α₈) on the outside. This folding motif is also referred to as the TIM barrel motif. In TIM, the two protruding loops are after β-strand three and after β-strand six.

The TIM barrel structural motif is found for a large number of other enzymes with widely different functions (10) that have little or no sequence homology. As such this stable framework with tolerant sequence variations has already been used as an interesting scaffold to design loops (11, 12) and even artificial proteins (13, 14). The active sites of all these TIM barrel enzymes are located at the C-terminal end of the β-barrel, with catalytic residues contributed by the β-strands and the loops connecting the β-strands to subsequent α-helices numbered loop 1 to loop 8 in correspondence with the numbering of the preceding β-strands. The loops preceding the β-strands are located on the other side of the barrel and are believed to be important for the stability of the TIM barrel (15).

In the TIM dimer, the dimer interface is formed mainly by loops 1, 2, 3, and 4. The protruding loop 3 of one subunit docks into a deep pocket between loop 1 and loop 4 of the other subunit (16). The catalytic residues, a lysine, a histidine, and a glutamate are located in loop 1, loop 4, and loop 6, respectively. Binding of substrate or substrate analogues is accompanied by

1 The abbreviations used are: TIM, triose-phosphate isomerase; vTIM, eTIM, tTIM, TIM of *V. marinus*, *E. coli*, *T. brucei*, respectively; 2PG, 2-phosphoglycerolate; bp, base pair(s).

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The atomic coordinates and structure factors (1AW1 and 1AW2) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

To whom correspondence may be addressed: European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 102209, D69012 Heidelberg, Germany. Tel.: 49 6221 3870256; Fax: 49 6221 387306; E-mail: Rik.wierenga@embli-heidelberg.de.

A research associate of the Belgian National Science Foundation (FWO) and to whom correspondence may be addressed: ULTR, IMOL, Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium. Tel.: 32 2 359 0263; Fax: 32 2 359 0289; E-mail: dommaes@vub.ac.be.

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changes in conformation of loops 5, 6, and 7. The most remarkable change occurs in loop 6, "the flexible loop," the tip of which undergoes a Cα movement of some 7 Å in response to ligand binding (17, 18). As such, the active site is closed off from solvent to prevent an unwanted phosphate elimination reaction that may otherwise occur (1). This state is called "the closed state," in contrast to the unliganded "open state." In the liganded form the oxygen atoms of the phosphate interact with main chain atoms of loops 6, 7, and 8. Loop 8 is called the phosphate binding loop, because it comprises a 3/10 helix (the phosphate chain atoms of loops 6, 7, and 8). Loop 8 is called the phosphate binding helix whose N terminus points directly to the phosphate moiety, allowing for hydrogen bonds between main chain NH groups and phosphate oxygen atoms. These interactions keep the phosphate moiety rigidly in place, whereas in the triose part of the ligand molecule, the catalytic conversion takes place.

The glutamate at the beginning of loop 6 is implicated in the transfer of a proton between the two substrate C atoms. The side chain of this glutamate can occupy two different positions, depending on the contents of the active site. In ligand-free TIM, it is found in a "swung out" conformation, whereas in liganded TIM, it occupies a "swung in" conformation, ideal for carrying the phosphate moiety rigidly in place, whereas in the triose part of the ligand molecule, the catalytic conversion takes place.

In this paper, we focus on the TIM enzyme from the psychrophilic bacterium Vibrio marinus. We will refer to the sequence numbering of V. marinus TIM (vTIM); according to this numbering, the catalytic residues are Lys-11, His-92, and Glu-169 (see Fig. 2). V. marinus is a member of the Vibrionaceae family, able to grow at 4 °C and having an optimal growth temperature of about 15 °C (20). We cloned the gene encoding vTIM. Recombinant vTIM was overproduced in Escherichia coli and purified. The kinetic properties as well as the stability of the enzyme were determined.

Two structures of this enzyme, one complexed with sulfate and one with the substrate analogue 2-phosphoglycolate (2PG) (Fig. 1), were solved by x-ray crystallography at a resolution of 2.7 Å. Because the sequence of vTIM is most closely related to that of E. coli TIM (eTIM) (66% identity) (see Fig. 2), the latter enzyme will be used as a reference for comparison throughout this paper.

Furthermore, sequence comparison of the vTIM sequence with all other known TIM sequences reveals that vTIM has a unique residue in the phosphate binding helix, where the otherwise completely conserved Ser-238 is replaced by an alanine. The implications of this unique sequence feature are discussed by comparing the stability and the kinetic parameters from the native vTIM with its A238S mutant. It turns out that this point mutation decreases the physiological efficiency at 10 °C and it increases the thermal stability, as evidenced by calorimetric studies.
The crystallization conditions were found using the “Fast crystallization protocol” (28) and are summarized in Table I.

For the vTIM(sulfate) crystallization, 100 mM ammonium sulfate was added, and for the vTIM(2PG) crystallization, 20 mM 2PG was added. The crystallization conditions were found using the hanging drop vapor diffusion technique. The protein solutions from 15 to 60 °C, and the scan rate was 1 °C/min.

The rotation function showed eight unique solutions, as expected for noncrystallographic symmetry in dimer in four dimers per asymmetric unit. A translation function was calculated for each of the eight rotation peaks. The best translation function showed that the protein solution was used to find the position of the second dimer. This process was repeated with the available solutions to find the correct orientation of the two TIM dimers in the unit cell, molecular replacement was performed with the program AMORE (30) using the crystal structure of the eTIM dimer (1TRE; Ref. 6) as the search model. The flexible loop (residues 169–180) known to be variable in conformation was deleted in both monomers.

Hydro-3-phosphate (Sigma) (0.13–9.75 mM) to the enzyme system (glycerol-3-phosphate dehydrogenase/NADH) and consecutively measuring the residual activity. Kinetic parameters were determined with the Enzfitter program (26). $K_i$ was measured with 2-phosphoglycerate (2PG) as the inhibitor (27).

Stability Assays—For the stability assays, samples containing 100 mM triethanolamine-HCl (pH 7.6) and TIM were incubated at various temperatures (5–25 °C) in a thermocycler (Programmable TRIO-thermoblock TB-1, Biometra BAU) for 10–60 min. The remaining activity was measured as a function of incubation time. From these measurements an inactivation rate constant, $k_{inact}$ (sec$^{-1}$), was calculated according to Rentier-Delrue et al. (22).

Differential Scanning Calorimetry—Thermal unfolding of TIM was studied using a microcal scanning calorimeter differential scanning calorimetric unit (MicroCal, Inc.). Protein samples of 1.3 mg/ml in triethanolamine-HCl buffer (pH 7.6) were used. Samples were heated from 15 to 60 °C, and the scan rate was 1 °C/min.

Crystals of a size up to 0.3 × 0.3 × 0.5 mm$^3$ grew within a week, and one of each was used for data collection. Data were collected at 25 °C on a BigMar image plate detector mounted on an Enraf Nonius rotating anode FR571. Data collection was set up to provide a 2.7 Å data set of the vTIM(2PG) crystal and a 2.65 Å data set of the vTIM(sulfate) crystal. The data were processed with DENZO (29). The crystal lattice for both growth conditions is primitive monoclinic. The β-angle for both data sets is about 91° and the a and c cell dimensions are very similar (89.7 and 89.5 Å), agreeing closely with tetragonal symmetry. Indeed at low resolution the data could be merged in space group P4, but for the higher resolution shells this results in a R-merge above 40%. The systematic absences of the 0k0 reflections indicated space group P2$_1$. This symmetry and the cell volume (1.11 $\times$ 10$^6$ Å$^3$) suggest that the asymmetric unit contained four TIM dimers, giving a $V_m$ of 2.5 $\times$ 10$^5$ Å$^3$. Data collection statistics for both data sets are shown in Table I.

**Table I**

| Crystallization buffer | vTIM(sulfate) | vTIM(2PG) |
|------------------------|--------------|-----------|
| pH                     | 7.0          | 7.5       |
| Precipitants           | 1.26 M sodium citrate | 2.0 M ammonium sulfate |
| Additions              | 1 mM diethiothreitol, EDTA, NaN$_3$ | 1 mM diethiothreitol, EDTA, NaN$_3$ |
| 100 mM ammonium sulfate | 20 mM 2PG |
| Space group            | P2$_1$       | P2$_1$    |
| Cell dimensions (Å)    | 89.66 137.82 89.54 | 89.51 138.08 89.52 |
| Cell dimensions (%)    | 90.00 90.94 90.00 | 90.00 91.00 90.00 |
| Subunits per asymmetric unit | 8     | 8         |
| $V_m$ (Å$^3$/Dalton)   | 2.5          | 2.5       |
| Data collection statistics |             |           |
| Observed reflections   | 166,818      | 332,463   |
| Unique reflections     | 60,048       | 59,692    |
| Overall range (Å)      | 25–2.65      | 25–2.70   |
| Overall R-merge (%)    | 10.1         | 8.4       |
| Overall completeness (%)| 95.7       | 99.9      |
| Last shell range (Å)   | 2.70–2.65    | 2.75–2.70 |
| Last shell R-merge (%) | 29.8         | 19.8      |
| Last shell completeness (%)| 95.8       | 98.2      |
| Refinement             |              |           |
| Protein atoms          | 3,733        | 3,733     |
| Ligand atoms           | 5            | 18        |
| Solvent atoms          | 44           | 77        |
| Resolution range (Å)   | 8.0–2.65     | 8.0–2.70  |
| R-factor (%)           | 20.0         | 19.2      |
| R-free (%)             | 21.9         | 21.5      |
| r.m.s. bond-length deviations (Å) | 0.015 | 0.014 |
| r.m.s. bond angle deviations (%) | 1.81 | 1.70 |
| r.m.s. dihedral angles (%) | 24.2 | 24.5 |
| r.m.s. impropers (%)   | 1.64         | 1.50      |
| r.m.s. $\Delta B$ for covalently bonded atoms (Å$^2$) | 3.60 | 3.65 |
| $x_i$ imperfection (%) | 29.1         | 30.2      |
| Average B-factor, all protein atoms (Å$^2$) | 18.5 | 12.6 |
| Average B-factor, backbone atoms (Å$^2$) | 17.7 | 11.7 |
| Average B-factor, side chain atoms (Å$^2$) | 19.5 | 13.7 |
| Average B-factor, ligand atoms (Å$^2$) | 70.9 | 27.2 |
| Average B-factor, solvent atoms (Å$^2$) | 28.9 | 20.2 |
| Ramachandran plot$^b$ |              |           |
| Most favored regions (%) | 90.3       | 92.8      |
| Additional allowed regions (%) | 8.8    | 7.0       |
| Generously allowed regions (%) | 0.7    | 0.2       |
| Disallowed regions (%)  | 0.2          | 0.0       |
| Noncrystallographic symmetry in dimer |           |           |
| r.m.s. $\Delta C_n$ (Å) | 0.45 | 0.28 |
| r.m.s. $\Delta g$ (%)  | 22.0         | 15.9      |
| r.m.s. $\Delta d$ (%)  | 22.2         | 15.4      |
| r.m.s. $\Delta B$ (Å$^2$) | 13.3 | 8.8      |

$^a$ The $x_i$$\Delta B$ imperfection value (6) is the r.m.s. difference between observed $x_i$$\Delta B$ value and the nearest preferred cluster values, as observed in a data base of well refined structures.

$^b$ As defined by PROCHECK (34).
dure was repeated until the positions of all four dimers were found. The R-factor for data between 15 and 3.5 Å for this complete solution was 49.1%, which dropped to 45.7% after rigid body refinement. The correctness of the solution was confirmed by the good packing of the four dimers and the quality of the fit of the omitted loop in the electron density map.

**Structure Refinement**—First the structure of the vTIM(sulfate) complex was refined. The sequence of the molecular replacement model was mutated from that of eTIM to that of vTIM in both monomers. The model was optimized to improve its fit in a 2Fo-Fc SIGMA A weighted map (CCP4; Ref. 31) using O (32) running on an Evans and Sutherland workstation. For refinement, a subset of 5% of the data (the test set) was used for R-free calculations. Refinement was pursued with a mixture of simulated annealing x-ray refinement and conventional positional and thermal factor refinement using the X-PLOR package (33) for all refinement calculations. The positions and the thermal B-factors of the atoms of both monomers in the dimer were refined independently. The atom positions of the three other dimers were generated by strict noncrystallographic symmetry operators, recalculated at several stages of the refinement by rigid body refinement.

Electron density maps indicated an open conformation for loop 6, which was manually rebuilt into the density. In addition, a sulfate ion was found in the active site of the first subunit. Its coordinates were included in the model. In the final stages of the refinement, water molecules were added at sites displaying a peak larger than 3 S.D. above the mean in an Fo-Fc map and having a potential hydrogen bonding partner. In total, 44 water molecules were added per dimer. The final R-factor was 20.3% and R-free was 22.0% to a resolution of 2.65 Å (Table I).

**Subsequently this structure was used for the rigid body refinement against the vTIM(2PG) data set. Electron density maps showed clear density for the closed loop 6 and the 2PG bound in the active site of both subunits. A similar protocol as for the native structure refinement was used, loop 6 was rebuilt, the inhibitor 2PG was built into the active site of both subunits, and 77 waters per dimer were added. It resulted in a final R-factor of 19.2% (R-free is 21.5%) for this vTIM(2PG) structure.**

The refinement statistics are given in Table I.

**Structure and Sequence Analysis**—The quality of the structures was analyzed using the programs PROCHECK (34) and WHAT IF (35). For the purpose of analysis, TIM structures were superimposed on the basis of the 129 Cα positions forming the eight α-helices and eight β-strands using the lsq option in O. Cavities were calculated with the program MSP (36), intermolecular contacts were calculated with WHAT IF, and molecular graphs were drawn with the programs MOLSCRIPT (37) (Fig. 6) and ICM (38) (Figs. 7 and 8). The consensus sequence shown in Fig. 2 was obtained from a sequence alignment of 45 sequences currently available in the databases using PILEUP (39) as well as a superposition analysis of known structures.

**RESULTS AND DISCUSSION**

**Kinetic and Stability Properties of vTIM**

In the present work we have cloned the gene encoding TIM of *V. marinus*. The corresponding protein was expressed in *E. coli*. The thermostability of this recombinant TIM was assessed by measuring the inactivation rate constant (k_inact) at various temperatures (Fig. 3). At 25 °C the value recorded was 1 × 10^{-3} sec^{-1}. The calculated half-life (t_{1/2}) was 10 min at this temperature, showing the intrinsic psychrophilic character of this TIM. Because of its psychrophilic properties, we choose to measure its kinetic parameters at 10 °C, at this temperature t_{1/2} is 68 min. The kinetic properties are summarized in Table II.

It is interesting to point out that the k_inact (vTIM, 10 °C) is very similar to the k_inact (eTIM, 25 °C), as shown in Table II. An important difference between vTIM and eTIM is the K_i (2PG), which for vTIM is approximately 10 times higher, showing that
the affinity of the vTIM active site for 2PG is much weaker. It is not clear from the structure how to explain this difference. For example, an analysis of the presence of the charged side chains within a sphere of radius 8 Å from the phosphate position showed no difference between vTIM and eTIM, except for the presence of an asparagine at position 216 in eTIM, where in vTIM a lysine is found (the NZ atom is at a distance of 7.1 Å from the phosphate). The Asn-216 in eTIM is responsible for the N-capping of helix 7, whereas in vTIM Pro-217 has the same role.

Characterization of the A238S Mutant

To assess the role of the unique Ala-238 in loop 8 of vTIM, we decided to study the point mutant A238S. The mutant was produced and purified in the same manner as the native TIM. Its catalytic efficiency and its stability were analyzed (Table II). Activity measurements at different temperatures revealed that the mutant has a higher thermal stability than the corresponding wild type; for example, the half-lives of wild type and A238S at 25 °C are 10 and 27 min, respectively (Table II). This was confirmed by microcalorimetric studies (Fig. 4), showing that thermal unfolding of the mutant occurs at higher temperatures. In both cases, denaturation was found to be irreversible under the conditions tested. The values of the temperature of half-denaturation $T_{1/2}$, calculated for a protein concentration of 1.3 mg/ml, were 40.8 °C for the wild type enzyme and 45.8 °C for the mutant.

The kinetic parameters of the A238S mutant were determined at 10 °C, whereas those of eTIM were measured at 25 °C. $t_{1/2}$ values were calculated with data as shown in Fig. 3; $T_d$, the temperature of half-denaturation, was determined from Fig. 4.

| Table II | Kinetic parameters (for the conversion of α-glyceraldehyde-3-phosphate into dihydroxyacetone phosphate) and stability data of vTIM, its A238S variant, and eTIM |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| Unit | vTIM | A238S | eTIM |
| $K_m$ (mM) | $1.9 \pm 0.2$ | $4.8 \pm 0.6$ | $1.03 \pm 0.1$ |
| $k_{cat}$ (min$^{-1}$) | $4.2 \pm 0.1 \times 10^7$ | $2.5 \pm 0.1 \times 10^5$ | $5.4 \pm 0.1 \times 10^5$ |
| $k_{cat}/K_m$ (min$^{-1}$ mol$^{-1}$) | $2.2 \pm 0.2 \times 10^5$ | $0.6 \pm 0.06 \times 10^5$ | $5.2 \pm 0.6 \times 10^5$ |
| $K_c$ (2PG) (mM) | 80 | 101 | 6 |

$\Delta H$ (cal/mole/K) | 100 | 80 | 60 |

$\Delta T$ (K) | 40 | 30 | 20 |

$\Delta C_p$ (cal/mole/K) | 20 | 10 | 0 |

$\Delta T$ (K) | 15 | 20 | 25 |

$\Delta C_p$ (cal/mole/K) | 0 | -10 | -20 |

In Fig. 5 the real space density correlation coefficient for the main chain atoms is displayed as a function of residue number for both monomers. The lowest values are found in loop 6, which is poorly defined in the electron density map. Loop 6 has the highest B-factors as also shown in Fig. 5. The B-factors of the second subunit are consistently higher than the B-factors of the first subunit. This is due to the different packing interactions; for each of the dimers in the asymmetric unit it is observed that the first subunit has more crystallographic contacts when compared with the second subunit. For the first subunit approximately 45 intermolecular contacts are observed, and for the second subunit only 23 (when using a cutoff of 3.5 Å) are observed.

Both subunits are very similar with a Cα r.m.s. deviation of 0.29 Å for the framework Cα atoms and of 0.45 Å for all main chain Cα atoms. Loop 6 is in the open conformation, and Glu-169 adopts the swung-out conformation (19), contacting Ser-98 of loop 4. A sulfate ion occupies the active-site pocket of subunit A but not of subunit B. The average thermal B-factor of the atoms of this ion is high (70 Å$^2$), suggesting a low occupancy of this site.
The vTIM(2PG) Structure—The structure of vTIM(2PG) is very similar to the structure of vTIM(sulfate), with a Cₐ r.m.s. deviation of 0.18 Å for the framework Cₐ atoms and of 0.95 Å for all main chain Cₐ atoms of the first monomer. The most important differences are the presence of 2PG in both active sites of the dimer and, consequently, the closed conformation of loop 6 in both subunits (Fig. 6). In the vTIM(2PG) structure, loop 6 is well defined by the electron density map, and the loop 6 atoms do have lower B-factors when compared with the vTIM(sulfate) structure (Fig. 5). The catalytic glutamate, Glu-169, at the beginning of loop 6 is now seen in the swung-in conformation, contacting the 2PG molecule, as already seen in other TIM complexes. The 2PG phosphate position does not exactly superimpose on the sulfate of the vTIM(sulfate) structure. The distance between the equivalent S and P atoms is 1.25 Å. As shown in Fig. 6, the phosphate moiety is bound
Comparison of the vTIM with the eTIM Structure—The sequences of vTIM and eTIM have almost the same length (256 for vTIM and 255 for eTIM) with a quite high sequence homology (66% sequence identity), suggesting a close evolutionary relationship. There is only one insertion of two residues at the C-terminal end of helix $\alpha_2$ (Fig. 1), resulting in a longer helix $\alpha_2$ in vTIM (Fig. 6). This difference is located at the N-terminal end of the $\beta$-barrel, and concerns residues not involved in catalysis and dimer formation. The overall fold of both vTIM structures and the unliganded eTIM structure has been compared in Fig. 6. The rms difference after superposition of the 129 framework C$_\alpha$ atoms of the vTIM(sulfate) and the eTIM structures is 0.59 Å for these framework atoms. A detailed structural analysis of the eTIM structure has shown some unique structural features (6), the majority of which are also present in vTIM. For example in vTIM and eTIM a methionine is located at position 7 in strand $\beta_1$, where in other known TIM structures a glycine or an alanine is located. This methionine points into the interior of the barrel. The larger space required is compensated for by smaller residues in the environment. Another unique common feature of vTIM and eTIM is found in the region between loop 1 and loop 8, as shown in Fig. 7, in which the structures of vTIM, eTIM, and tTIM are superimposed. A buried water molecule is observed in this region in vTIM and eTIM, hydrogen-bonded to NE1(Trp-10) and O(Ala-238). The presence of this water molecule does correlate with two sequence changes in this region; in particular it concerns the mutation of an alanine (in tTIM) to a glycine (in vTIM and eTIM) at position 8 and of a phenylalanine (in tTIM) to a leucine (in vTIM and eTIM) at position 26. As a result of these two changes to a smaller side chain, a cavity is formed that is filled up by a water molecule. A small rotation of the Trp side chain is also observed. Consequently, the direct hydrogen bond of NE1(Trp-10) and O(Ala-238) between loop 1 and loop 8 as seen in tTIM is replaced by the above-mentioned water-mediated hydrogen bond (Fig. 7). Another common feature of vTIM and eTIM is an intersubunit salt bridge between residue 79 and His-104 that is not observed in the other structures, where an intersubunit salt bridge exists between Glu-79 and Arg-100. The latter contact is absent in eTIM due to a reorientation of the side chains from the residues involved. In vTIM an aspartate is found at the position of Glu-79 in eTIM, and the previously mentioned salt bridge between residue 79 of one subunit and residue 100 of the other subunit is also present, as well as the Asp-79–His-104 salt bridge.

There are three unique residues in vTIM when compared with the other nine known structures: Asp-79, Ala-182, and Ala-238. Only the latter is a unique residue when comparing the 45 known sequences of TIM. Position 79 is at the tip of loop 3; in most sequences (41 times) this is an aspartate (three times) or a threonine (one time) is observed. As previously mentioned, at position 238 an
more than 190 lysozyme variants (only 48 stabilizing, from larger than the most stabilizing point mutation observed in of approximately 5 °C is quite significant. For example it is that the kinetic properties of vTIM and A238S are rather variant (Table II). The assumption of only minor structural hydrogen bonds, which rationalizes the extra stability of this serine mutation in vTIM will generate two additional buried NH. These hydrogen bonds are also present in wild type vTIM. (Fig. 8), each of these two buried main chain oxygens is making another hydrogen bond with a neighboring main chain peptide NH. These hydrogen bonds are also present in wild type vTIM. Assuming no major structural rearrangements, the alanine to serine mutation in vTIM will generate two additional buried hydrogen bonds, which rationalizes the extra stability of this variant (Table II). The assumption of only minor structural rearrangements is entirely consistent with the observation that the kinetic properties of vTIM and A238S are rather similar. The increase in stability, as expressed in a $T_d$ increase of approximately 5 °C is quite significant. For example it is larger than the most stabilizing point mutation observed in more than 190 lysozyme variants (only 48 stabilizing, from which only 4 with an increase of $T_d$ higher than 1.5 °C and with a maximum of 2.8 °C) (42). This is partly due to the fact that TIM is a dimer; therefore the more favorable hydrogen bond interactions occur twice. Furthermore it is also due to the very good hydrogen bonding complementarity of the environment for a serine at this site (Fig. 8).

Concluding Remarks—In this paper we reported the purification and the kinetic and stability properties of triose-phosphate isomerase from the psychrophilic bacterium V. marinus and its A238S mutant as well as two x-ray structures of the native enzyme, one complexed with sulfate and one complexed with the substrate analogue 2PG. The vTIM structure is very similar to the structure of eTIM, in agreement with the high sequence identity. The comparison of the structures of vTIM and eTIM does not provide an explanation for the 10-fold lower affinity of 2PG for vTIM, when compared with eTIM. One unique sequence feature of vTIM has been found, which is Ala-238, whereas in all other TIM sequences a serine is observed. The importance of the alanine at position 238 has been investigated further by studying the properties of the A238S mutant. A238S is considerable more stable than wild type, and it catalyzes the reaction less efficiently; therefore, there is good evidence that the alanine at position 238 is a successful adaptation to the psychrophilic character of V. marinus.

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