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Differential effects on enzyme stability and kinetic parameters of mutants related to human triosephosphate isomerase deficiency

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ABSTRACT

Human triosephosphate isomerase (TIM) deficiency is a very rare disease, but there are several mutations reported to be causing the illness. In this work, we produced nine recombinant human triosephosphate isomerases which have the mutations reported to produce TIM deficiency. These enzymes were characterized biophysically and biochemically to determine their kinetic and stability parameters, and also to substitute TIM activity in supporting the growth of an *Escherichia coli* strain lacking the tim gene. Our results allowed us to rate the deleteriousness of the human TIM mutants based on the type and severity of the alterations observed, to classify four "unknown severity mutants" with altered residues in positions 62, 72, 122 and 154 and to explain in structural terms the mutation V231M, the most affected mutant from the kinetic point of view and the only homozygous mutation reported besides E104D.

1. Introduction

The fifth enzyme of the glycolytic pathway, triosephosphate isomerase (TIM, E.C. 5.3.1.1), catalyzes the reversible conversion between dihydroxyactetone 3 phosphate (DHAP) and glyceraldehyde 3 phosphate (GAP). After this enzymatic reaction, GAP can be further metabolized by subsequent enzymes in the pathway, continuing the breakdown of glucose, which produces two ATPs per molecule of the carbohydrate [1,2].

Almost all organisms have this enzyme, which, in most cases, is a homodimer with two identical subunits with a molecular mass of 27 kDa composed of approximately 250 amino acids. Both monomers have all catalytic residues but TIM is only active as a dimer. In some archaea TIM has an active and stable homotetrameric quaternary structure, but, in all cases, monomers of this enzyme are inactive and unstable, emphasizing that the enzyme acts as either a dimer or a tetramer [3].

Human triosephosphate isomerase (HsTIM) is formed by two subunits, which have 248 amino acids, that associate to form a 54 kDa homodimer. The enzyme with the wild type (WT) sequence of HsTIM has kinetic parameters that make it a nearly perfect enzyme, because the reaction is a diffusion-limited process. It also has a high thermal resistance and its monomers interact with high affinity making it a very stable dimer [4,5].

The sequence of TIM has high homology for many species. A few key residues are completely conserved among all known sequences, and in some other positions have relatively little variation. In the sequence of HsTIM, very few mutations occur naturally that allow an individual to survive. The extremely small number of persons who have their HsTIM with an altered sequence suffer from HsTIM deficiency. This condition represents the most severe glycolytic enzyme defect in humans which is almost always lethal in early childhood. Although this disease, and the mutations in HsTIM that produce it, have been extensively reviewed [6–8], biochemical characterization using purified recombinant mutant enzymes has not been investigated. There have been several attempts to predict in silico the effect of mutations in HsTIM as they might reflect in the patients with TIM deficiency. Schneider [6] and Oliver and Timson [9] proposed that mutant proteins associated with pathological phenotypes are less stable than those associated with a less severe disease. Schneider [6], based on structural information, also predicted that catalytic abnormalities might be associated with the severity of the disease. Oliver and Timson [9] could not include kinetic parameters of enzyme function into their bioinformatics investigations. So, both these analyses lack the direct study of the

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function of the mutant HsTIMs having the mutated amino acids described in patients with HsTIM deficiency and the characterization of these enzymes *in vitro*. In this study, we offer such a comparison of several mutants related to HsTIM deficiency. We rate these mutants in terms of their deleterious effects according to kinetic, thermal and dimerization stability assays, and also bacterial growth inhibition. In addition, using X-ray crystallography, we analyze the molecular basis of the deficiency produced by mutant HsTIMV231M, the only other known homozygous mutant reported besides HsTIME104D.

2. Materials and methods

2.1. Cloning and purification

The DNA sequence X69723.1 (NCBI database) for HsTIM was used to make the single mutants. All mutants were constructed on a modified plasmid pET-3a [5] using the QuikChange protocol (Agilent Technologies, CA). The plasmids containing the different mutants were transformed into the *Escherichia coli* BL21 Codon Plus (DE3)-RIL strain (Agilent Technologies, CA). Cells were grown at 37 °C in Luria-Bertani medium containing ampicillin and chloramphenicol until an A_{600} of 0.8 was obtained. At that time, they were induced with 1 mM isopropyl-D-1-thiogalactopyranoside following the conditions described in Supplementary Table 1, which were obtained after testing two periods of time for induction (3 and 18 h), and three different temperatures (18, 30 and 37 °C).

The cell pellet from 1 L of culture was suspended in 20 mL of buffer A containing 50 mM sodium phosphate pH 8.0, 10 mM imidazole and different concentrations of NaCl (Supplementary Table 1). Cells were lysed by sonication and centrifuged at 20,000 ×*g* for 20 min. The supernatant was loaded on a 5 mL HisTrap (GE Healthcare) column. A 1 mL column was used, for the HsTIMA62D mutant. The proteins were eluted with a linear gradient of buffer A containing 500 mM imidazole and dialyzed for 2 h against a buffer containing 50 mM Tris pH 8.0, 0.5 mM EDTA and 1 mM dithiothreitol (DTT). The proteins were then cleaved using purified recombinant His tagged tobacco etch virus (TEV) protease expressed using vector pRK508 [10]. The protease was added in a proportion of 1:20 (w/w) and incubated at 30 °C for 18 h. Subsequently, the His-tagged TEV protease was removed using a HisTrap column equilibrated with buffer A. The enzymes were precipitated with ammonium sulfate at 75% saturation and maintained at 4 °C until their use.

2.2. Growth curves of E. coli Δtim- BL21-gold (DE3) cells complemented with WT HsTIM and different mutants

We used a genetically manipulated strain BL21-Gold (DE3) without the tim gene (*E. coli* Δtim-BL21-Gold (DE3)) [Saab et al., unpublished data]. Cells were grown at 37 °C in M9 minimal medium supplemented with glucose (0.2%), casamino acids (0.006%) and ampicillin (100 μg/mL). The assay was performed in triplicate in 96-well, clear, flat-bottom, plates (Corning Costar 3697), in a total volume of 80 μl inoculated either with a single colony of WT bacteria, mutants or cells containing the plasmid without the gene, using a Synergy MX equipment (BioTek, VT). The growth was followed for 16 h and the results were monitored with the software of the equipment (Gen 1.11).

2.3. Activity assay

Enzyme activity was measured at 25 °C following the conversion of glyceraldehyde 3 phosphate (GAP) to dihydroxyacetone phosphate using α-glycerolphosphate dehydrogenase (α-GDH) as a coupling enzyme, as described by [11]. NADH oxidation was monitored at 340 nm. The assay system (1 mL) had 100 mM triethanolamine (TEA) pH 7.4, 10 mM EDTA,1 mM GAP, 0.2 mM NADH and 20 μg/mL of α-GDH. The reaction was initiated by the addition of different quantities of the corresponding mutant enzymes (Table 1). To calculate the kinetic parameters, GAP concentration was varied between 0.05 and 3 mM. The data were adjusted to the Michaelis-Menten model and the values of *K*m and Vmax were calculated using non-linear regression. Activity was measured in a Cary 60 spectrophotometer (Agilent Technology, CA) with a multi-cell attachment. All assays were performed in triplicate.

Table 1
Enzymatic kinetic parameters for WT HsTIM and the mutant enzymes. The mean of three independent experiments is shown. For simplicity, a common color code is followed in all tables and figures to describe the behavior of the enzymes.

Enzyme	*K*m (mM)	*k*cat (10^5 min^{-1})	*k*cat/ *K*m (10^6 M^{-1} s^{-1})	*k*cat/ *K*m relative
WT	0.46 ± 0.07	2.73	9.8	1
C41Y	0.62 ± 0.03	2.16	5.8	0.59
A62D	nd	nd	nd	nd
G72A	3.0 ± 0.36	4.11	2.3	0.2
E104D	0.57 ± 0.08	3.31	9.6	0.97
G122R	0.57 ± 0.08	3.57	10.4	1.06
V154M	0.49 ± 0.04	2.7	9.0	0.91
I170V	0.03 ± 0.004	0.11	5.8	0.59
V231M	4.02 ± 0.99	2.67	1.1	0.1
F240L	0.83 ± 0.06	3.32	6.6	0.6

2.4. Thermal shift assay (differential scanning fluorimetry)

We followed the protocol described by Niesen and collaborators [12]. Briefly, the assay system had 0.2 μg of each mutant protein, 8 μl of 100 mM TEA pH 7.4,10 mM EDTA and a 1:100 dilution of SYPRO Orange dye (Invitrogen, CA), in a final volume of 10 μl. The dye was excited at 490 nm and the emitted light intensity was recorded at 575 nm. Data were collected at 1 °C intervals from 25 to 99 °C on a StepOnePlus real time PCR system using a 96-well reaction plate (Applied Biosystems 4346907, MA) and analyzed with the Protein Thermal Shift Software v1.3 from Applied Biosystems to define the thermal melting temperature (Tm). All assays were performed in triplicate.

2.5. Dimer stability

All enzymes we analyzed were incubated at the following concentrations: 0.01, 0.05, 0.1, 0.5, 1, 2 and 5 μg of protein/mL for 2 h at 36 °C. At that time, the specific activities of the samples were determined. The amount of enzyme used to measure the specific activity was different for each mutant (Supplementary Table 1). All assays were performed in triplicate. The percentage of residual catalytic activity was plotted against the logarithm of protein concentration and the data were adjusted to a non-linear-fit-three-parameter equation, included in the GraphPad Prism 7.0 software.

2.6. Crystallization and data collection of HsTIMV231M

HsTIMV231M was crystallized via vapor diffusion using the sitting drop method. One microliter of a solution at 35 mg/ of protein mL was mixed with 1 μL of reservoir solution. Crystals were obtained after three weeks of incubation at 20 °C in condition A6 of the Crystal Screen HT kit from Hampton Research (200 mM $MgCl_2$, 100 mM Tris pH 8.5, 30% polyethylene glycol 4000). The crystals were cryoprotected in a solution prepared with the mother liquor supplemented with 20% glycerol; they were immediately frozen in liquid nitrogen. Diffraction data were collected at 100 K using a wavelength of 0.9785 Å at the Life Sciences Collaborative Access Team (LS-CAT) 21-ID-G beamline at the Advanced Photon Source (Argonne National Laboratory, IL). The data were processed with iMOSFLM [13] and reduced with Aimless [14].