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Crystallization and Crystallographic Analyses of Triosephosphate Isomerase from *Naegleria gruberi*

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Cristalização e Análises Cristalográficas da Triose Fosfato Isomerase de *Naegleria gruberi*

Resumo: A enzima Triose fosfato isomerase de *Naegleria gruberi* (*Ng*TIM) participa da via glicolítica, na qual atua na conversão de dihidroxiacetona fosfato em D-gliceraldeído-3-fosfato. Atualmente, a TIM mais semelhante com estrutura 3D relatada é a da planta *Arabidopsis thaliana*, que possui 58% de identidade sequencial com a *Ng*TIM. A proteína recombinante *Ng*TIM foi expressa em *E. coli* BL21(DE3), purificada por cromatografias de afinidade e exclusão de tamanho e cristalizada pelo método de difusão de vapor. Os melhores cristais foram obtidos em 3 diferentes condições, sendo aquele que proveu o melhor conjunto de dados em etanol 15% (*V*/*V*) e propoxilato pentaeritrol 40% (*V*/*V*). Dados de difração de raios X foram coletados para este cristal a 2,64 Å de resolução e processados. Ele pertence ao grupo de espaço $P4_122$ e têm parâmetros de cela unitária *a* = 79,70, *c* = 98,11 Å. A unidade assimétrica contém um monômero, com V_M de 2,68 $Å^3$ Da^{-1} e um conteúdo de solvente de 54,2 %.

Palavras-chave: Triose fosfato isomerase; *Naegleria gruberi*, Meningoencefalite Amebiana Primária.

Abstract

The enzyme Triosephosphate Isomerase (TIM) from *Naegleria gruberi* is involved in the glycolytic pathway, in which it acts upon the conversion of dihydroxyacetone phosphate to D-glyceraldehyde 3-phosphate. Currently, the most similar TIM with 3D structure reported is from the plant *Arabidopsis thaliana*, which has 58% sequence identity with *Ng*TIM. Recombinant *Ng*TIM protein was expressed in *E. coli* BL21(DE3), purified by affinity and size exclusion chromatographies and crystallized by the vapor diffusion method. The best crystals were obtained in 3 different conditions, the one to provide the best dataset in ethanol 15% (*V*/*V*) and pentaerythritol propoxylate 40% (*V*/*V*). X ray diffraction data were collected for this crystal at 2.64 Å resolution and processed. It belongs to the space group $P4_122$ and have unit cell parameters *a* = 79.70, *c* = 98.11 Å. The asymmetric unit contains one monomer, with V_M of 2.68 $Å^3$ Da^{-1} and a solvent content of 54.2 %.

Keywords: Triosephosphate isomerase; *Naegleria gruberi*, primary amoebic meningoencephalitis.

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Crystallization and Crystallographic Analyses of Triosephosphate Isomerase from *Naegleria gruberi*

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1. Introduction

Naegleria gruberi is a free-living heterotrophic protist found in aerobic and microaerobic environments, in fresh water and wet soils around the world. It is found in the flagellated, cystic and, predominantly, amoeboid forms; in the last form it can reproduce each 1.6 h when fed on bacteria.[1,2] It does not represent a risk for human health, nevertheless, its genus includes *Naegleria fowleri*, to which it presents genetic and structural similarities. *N. fowleri* is found in environments alike and in warm waters; it is an opportunistic pathogen to humans, causing an almost always fatal disease, Primary Amoebic Meningoencephalitis (PAM).[3]

N. gruberi complete genome has been made available recently,[4] what fosters the development of the research to find drugs against the cited pathogenic species.[5] Within such process, protein three dimensional structure elucidation is a key component to understand metabolic catalytic processes.

Triosephosphate isomerase (E.C. 5.3.1.1; TIM) is an enzyme that partakes the glycolytic pathway and is responsible for the stereospecific conversion of dihydroxyacetone phosphate (DHAP) to D-

glyceraldehyde 3-phosphate (D-GAP). This reaction does not require either ions or cofactors. Its activity is of critical importance for the adequate operation of, for example, human cells; when the activity is decreased, a severe disorder is established as observed by Orosz *et al.*.[6] TIM has also been related to be a possible target in order to cause harmful effects on some pathogens, e. g., in the recent report for the protozoan *Giardia lamblia*.[7] The comprehension of its role in *N. gruberi* can be advanced now by the knowledge of the its genome, what provides insights concerning primitive eukaryote metabolic pathways. Its structure determination must supplement valuable additional information for this intent. In this paper, we report the crystallization, X ray diffraction data collection and crystallographic analyses of Triosephosphate isomerase from *Naegleria gruberi*.

2. Materials and Methods

2.1. Protein expression and purification

The recombinant plasmid pET-15b containing the gene encoding the enzyme *Ng*TIM, prepared by synthesis (GenScript), was inserted into *E. coli* strain BL21 (DE3) by electroporation. Cells were grown in 2xYT medium with ampicilin (5 μg mL^{-1}) until an optical density of 0.6-0.8 (at 600 nm) was reached, then induced with IPTG (0.1 mmol L^{-1}) and left at 37 °C and 185 rpm for protein expression during 16 h. Due to the 6×His-tag added to the N-terminal region by the recombinant process, cell lysates were initially subjected to a Ni^{2+} affinity chromatography (HisTrap™ FF crude, GE LifeSciences) in an automated chromatographer (Äkta Purifier, GE LifeSciences). Suitable protein purity was achieved with a subsequent size exclusion chromatography (Superdex™ 75 10/300 GL, GE LifeSciences). The protein was then concentrated to 7.0 mg mL^{-1} using a Vivaspin device 6 (GE LifeSciences). Protein concentration was determined by the Bradford assay.[8]

2.2. Crystallization

Initial crystallization trials involved the hanging drop vapor diffusion method,[9] manually prepared in the crystallization room in the State University of Ponta Grossa (UEPG), with kits Morpheus, Midas, JCSG Screen, Clear Strategy Screen and PGA Screen (Molecular Dimensions). Each drop comprehended a mixture of 2 μL of reservoir solution and 2 μL of protein solution at a concentration of 7.0 mg mL^{-1} in 30 mmol L^{-1} Tris-HCl pH 7.9 and 500 mmol L^{-1} NaCl. The high salt content was necessary to bring the protein at an appropriate concentration for the assays. The drops were then equilibrated against 500 μl of reservoir solution in 24-well plates at 291 K.

2.3. X ray data collection and processing

The crystals were either transferred to a cryoprotectant solution when necessary, which consisted of the original precipitant containing 30% (*V*/*V*) ethylene glycol, or taken directly to a cold nitrogen stream at 100 K. X ray diffraction experiments were performed in the W01B-MX2 station of the Brazilian Synchrotron Light Laboratory (LNLS - Campinas/ Brazil) equipped with a Pilatus 2M detector (Rigaku). Indexing, integration and scaling were performed with the XDS package.[10]

3. Results and Discussion

3.1. Protein expression, purification and crystallization

In the mentioned expression conditions, *Ng*TIM was produced in high

yields (3.8 mg per liter of culture medium) and then purified for structural analysis in an automated chromatographer (Äkta Purifier, GE LifeSciences). Crystals were formed in a number of different conditions of the Midas kit, the best ones in (a) NaCl 0.2 mol L^{-1}, MES-NaOH 0.1 mol L^{-1} buffer pH 6.0, Jeffamine® ED2003 30% (*m*/*V*); (b) ethanol 15% (*V*/*V*) and pentaerythritol propoxylate 40% (*V*/*V*) and (c) KCl 0.2 mol L^{-1}, glycine 0.1 mol L^{-1} buffer pH 9.5, pentaerythritol ethoxylate 20% (*V*/*V*), such that these were to provide a complete dataset collection. In almost all cases, crystals grew about 7 days. Among them, the one to provide the best dataset was in condition (b), shown in Figure 1B.

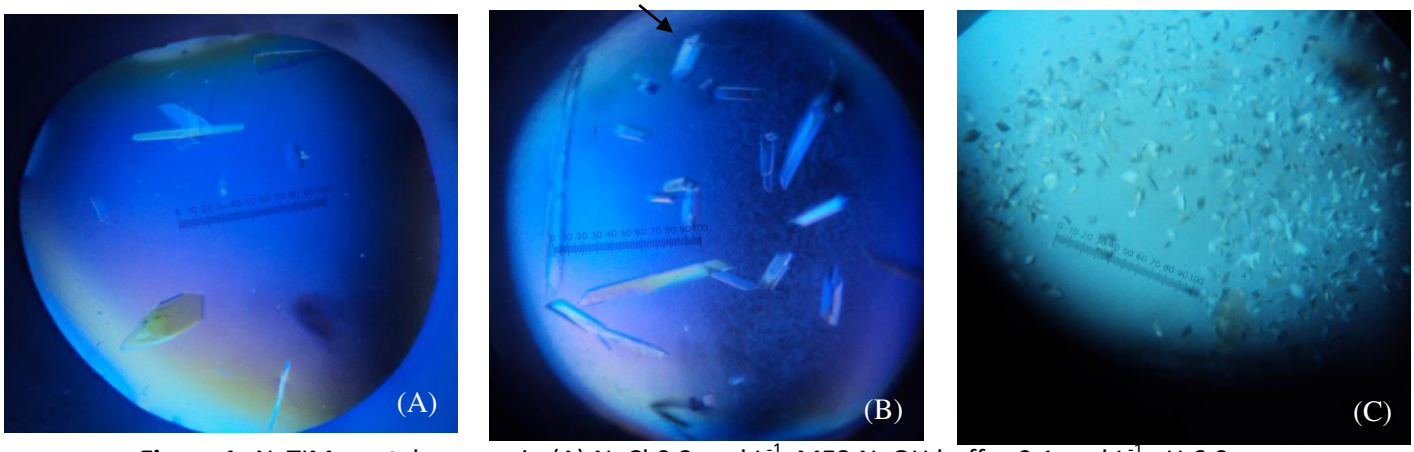

Figure 1. *Ng*TIM crystals grown in (A) NaCl 0.2 mol L^{-1}, MES-NaOH buffer 0.1 mol L^{-1} pH 6.0, Jeffamine® ED2003 30% (*V*/*V*) (B) ethanol 15% (*V*/*V*) and pentaerythritol propoxylate 40% (*V*/*V*) and (C) KCl 0.2 mol L^{-1}, glycine 0.1 mol L^{-1} buffer pH 9.5, pentaerythritol ethoxylate 20% (*V*/*V*). The crystal used for the data collection presented in this text is indicated by an arrow. Ten units of the ruler correspond to 0.143 µm

3.2. X ray data collection and processing

The best crystal allowed the collection of a complete data set to 2.64 Å resolution, a conservative cutoff value used after a number of data processing tests, such that in the last resolution shell R_{merge} = 73.3%, notwithstanding, due to the high multiplicity, ‹(*I*/σ(*I*)› = 6.93 and $CC_{1/2}$ = 97.8%.[11] Figure 2 presents an image of the X ray diffraction pattern. The 2.64 Å resolution circumference indicates the limit for diffraction spots that were used in data processing.

The crystal belongs to the tetragonal space group $P4_122$, with unit cell parameters *a* = *b* = 79.70 and *c* = 98.11 Å. Other crystal information and data collection statistics are shown in Table 1.

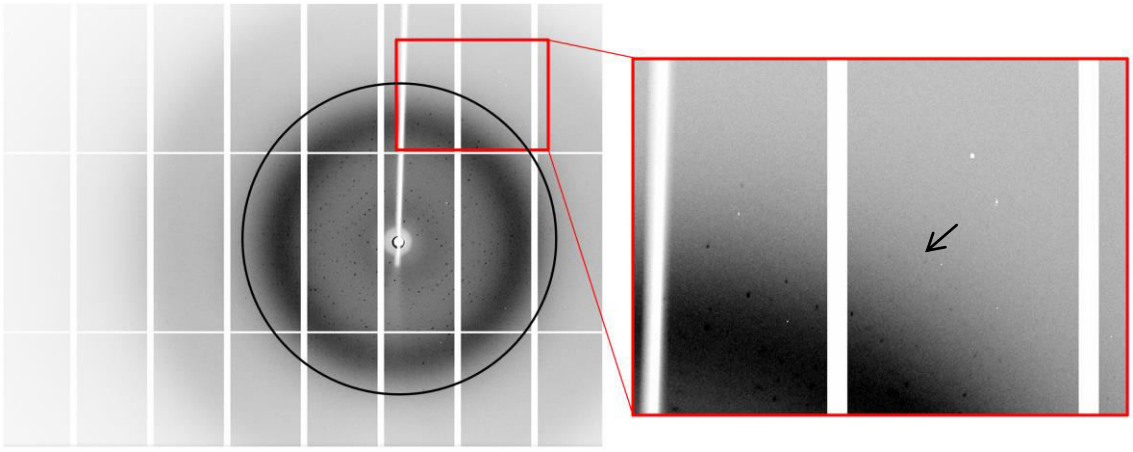

Figure 2. (A) Diffraction image of *Ng*TIM, with diffraction spots up to 2.64 Å resolution limit (indicated by the circumference). (B) Image magnification of the highlighted area with an arrow to indicate a reflection spot collected at 2.64 Å resolution

Table 1. Data collection and processing statistics. The numbers in parentheses correspond to the values in the highest resolution shell

Diffraction source	W01B-MX2
Wavelength (Å)	1.46
Temperature (K)	100
Detector	PILATUS 2M
Crystal-to-detector distance (mm)	130
Rotation range per image (°)	1.0
Total rotation range (°)	534
Exposure time per image (s)	10
Space group	$P4_122$
a, c (Å)	79.70; 98.11
Mosaicity (°)	0.20
Resolution range (Å)	98.0 - 2.64 (2.72-2.64)
Total No. of reflections	367311 (30675)
No. of unique reflections	9781 (804)
Completeness (%)	99.9 (100.0)
Multiplicity	37.55 (38.15)
‹(*I*/σ(*I*)›	35.68 (6.93)
R_{merge} (%)	10.2 (73.3)
R_{meas} (%)	10.4 (74.3)
$CC_{1/2}$ (%)	99.9 (97.8)
Overall *B* factor from Wilson plot (Å^2)	61.92
No. of molecules per asymmetric unit	1
V_M (Å^3 Da^{-1})	2.68
Solvent content (%)	54.2

Based on the monomer molecular mass of 29054.7 Da (which includes the tail with the His residue tag that was added to the native sequence of 252 residues for expression), the crystal packaging parameter (Matthews coefficient) of 2.68 Å^3/Da was calculated,[12] what leads to a volume solvent content of 54.2% for one single monomer in the asymmetric unit. Initial phases were determined by molecular replacement using

as initial model the edited TIM structure from *Arabidopsis thaliana*[13] to which *Ng*TIM share 58% sequence identity. Structure refinement is currently being evaluated concomitantly with trials to get better crystals.

4. Conclusions

Conditions for triosephosphate isomerase from *Naegleria gruberi* expression in high yields in *E. coli* were found. After expression, the protein was purified, concentrated and then crystallized in the presence of ethanol 15% (*V*/*V*) and pentaerythritol propoxylate 40% (*V*/*V*), among other suitable crystallization conditions that were also encountered. The crystals are tetragonal, space group $P4_122$, and are suitable for molecular replacement phasing. These successful crystallization conditions reported here for *Ng*TIM might guide crystallization assays for homologous proteins to the species related genus, especially pathogenic *Naegleria fowleri*. Further structure solution must be pursued and reveal structural aspects of this protein and its action in the amoeba metabolic pathway, specially the glycolytic one. Allied to genome studies, this should shed light on primitive eukaryote metabolism.

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