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Artigo

Crystallization and X Ray Diffraction Data Analyses of the Enzyme Urocanate Hydratase from *Trypanosoma cruzi*

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Cristalização e Análises dos Dados de Difração de Raios X da Enzima Urocanato Hidratase de *Trypanosoma cruzi*

Resumo: *Trypanosoma cruzi* é o único tripanossomatídeo patogênico de humanos no qual as quatro enzimas da via de degradação de histidina foram encontradas até agora. A enzima urocanato hidratase de *Trypanosoma cruzi* catalisa o segundo passo da degradação da histidina e é essencial para a conversão catabólica de urocanato a 4-imidazolona-5-propionato, o qual finalmente produz glutamato e depois glutamina e outros intermediários do ciclo dos ácidos tricarboxílicos, tais como o α-cetoglutarato. A proteína recombinante foi expressa com cauda His6 na região N-terminal em *Escherichia coli*, purificada de forma homogênea e cristalizada em várias condições, tal que os melhores cristais foram obtidos com 0,04 M de dihidrogenofosfato de potássio, 16,00 % (w/v) de polietileno glicol (PEG) 8000 e 22,00 % (v/v) de glicerol. Dados de difração de raios X foram coletados a 2,61 Å de resolução. Os cristais pertencem ao grupo de espaço $P2_1$, com parâmetros de célula unitária *a* = 85,12, *b* = 137,63, *c* = 117,69 Å, *β* = 94,69 ° e são adequados para faseamento por substituição molecular. A unidade assimétrica contém quatro monômeros, com V_M de 2,3 $Å^3$/ Da e um conteúdo de solvente de 45,9 %.

Palavras-chave: Doença de Chagas; *Trypanosoma cruzi*; metabolismo da histidina urocanato hidratase.

Abstract

Trypanosoma cruzi is the only trypanosomatid pathogenic to humans in which the four enzymes of the histidine degradation pathway were found up to now. The enzyme urocanate hydratase from *Trypanosoma cruzi* catalyzes the second step of histidine degradation and is essential for the catabolic conversion of urocanate to 4-imidazolone-5-propionate, which finally yields glutamate and further glutamine and tricarboxylic acid cycle intermediates such as α-ketoglutarate. The recombinant C-terminally His6-tagged protein was expressed in *Escherichia coli*, purified in a homogenous form and crystallized in several conditions, with the best crystals obtained with 0.04 M dipotassium hydrogen phosphate, 16.00 % (*w*/*v*) polyethylene glycol 8,000 and 22.00 % (*v*/*v*) glycerol. X ray diffraction data were collected to 2.61 Å resolution. The crystals belong to the monoclinic space group $P2_1$, with unit cell parameters *a* = 85.12, *b* = 137.63, *c* = 117.69 Å, *β* = 94.69 ° and are suitable for molecular replacement phasing. The asymmetric unit contains four monomers, with a V_M of 2.3 $Å^3$/Da and a solvent content of 45.9 %.

Keywords: Chagas disease; *Trypanosoma cruzi*; histidine metabolism; urocanate hydratase.

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Crystallization and X Ray Diffraction Data Analyses of the Enzyme Urocanate Hydratase from *Trypanosoma cruzi*

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1. Introduction

Trypanosoma cruzi is the protozoan parasite that causes Chagas disease, a neglected disease affecting about 10 million people in the Americas.[1] Over the past two decades, as a result of the genome sequencing of the protozoan *Trypanosoma cruzi*, specific metabolic pathways have been and are presently being studied to better understand their relationship with different aspects of its biology.[2] In this point, the uptake and metabolism of several amino acids have been approached with increasing detail, revealing that beyond their obvious participation as raw materials for protein synthesis, amino acids, and their metabolites, have relevant roles in different aspects of of *T. cruzi* biology.[3-16] As a consequence, amino acid-related enzymes and transporters started to be evaluated as potential therapeutic targets, opening up the prospect for the development of more specific and less toxic drugs for the treatment of Chagas disease.[17] In this regard, some of the main amino acid-metabolism therapeutic targets considered up to now are arginine kinase,[18] proline racemase,[19] as well as the proline uptake system[5] and glutamate uptake/receptor system.[20]

More recently, other possibilities that have been considered in the search for therapeutic targets are enzymes of amino acid metabolism pathways, which contribute

as energy sources to *T. cruzi*.[7,9] Among amino acids, L-histidine degradation leads to glutamate and other final products. Through database searches in the *T. cruzi* genome and proteome, it was possible to identify the enzymes that are part of this pathway: histidine ammonia-lyase (EC 4.3.1.3), urocanate hydratase (UH) (EC 4.2.1.49), 4-imidazolona-5-propionase (EC 3.5.2.7) and formimine glutamase (EC 3.5.3.8). This complete pathway drives the conversion of histidine into glutamate, which in turn can be deaminated into α-ketoglutarate, allowing its complete oxidation.

Urocanate hydratase, the second enzyme of the histidine-glutamate pathway, acts on the conversion of urocanate to 4-imidazolone-5-propionate. It is found in different organisms, prokaryotes and eukaryotes, including humans; it was shown that the variety from *Pseudomonas putida* is a homodimer in physiological conditions[21] and that each monomer contains one Nicotinamide Adenine Dinucleotide (NAD^+) electrophile coenzyme, essential for its correct folding and catalysis.[21-23] Currently, there are only three UH structures determined crystallographically [Protein Data Bank, PDB, codes 1uwk, 2fkn and 1x87[24]], however, it is noteworthy that the highest identity [to the one from *Pseudomonas putida*[21]] is 36 % and none of them are from a protozoan organism. The elucidation of *Trypanosoma cruzi* Urocanate Hydratase (*Tc*UH) three-dimensional structure by X ray crystallography will aggregate valuable information *per se*, such as the better understanding of the enzyme at molecular level and its catalytic mechanism, which should also be compared to homologous proteins. This information, added to further molecular biology studies, will clarify its role in *T. cruzi* metabolism. Provided that the enzyme is evinced as a therapeutic molecular target for Chagas disease, its 3D structure can be used for *in silico* and guide *in vitro* searches for inhibitors, that in the long term may indicate lead compounds for the development of new antichagasic drugs.

In the present paper we describe for the first time the crystallization, X-ray diffraction data collection and analyses of a protozoan UH.

2. Materials and methods

2.1. Production and purification

By means of a collaboration with Professor Dr. Mariano Ariel Silber from University of São Paulo (USP), Department of Parasitology, *Escherichia coli* BL21-DE3 clones carrying the gene that codes for *Tc*UH inserted into the plasmid pET-24a (+) (Novagen), which introduces a C-terminal His6 tag and contains a kanamicin resistance gene, were obtained and then used to express the protein.

Three liters of Luria-Bertani medium containing the antibiotics kanamycin and tetracycline at final concentrations of 0.03 and 0.005 mg/ml, respectively, were used during protein production. Induction was performed by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 298 K for 16 hours of incubation. Purification was performed by affinity chromatography on an agarose nickel column (HisTrap™ FF crude, GE Life Sciences). *Tc*UH was eluted with five column volumes (CV) of the buffer 40 mM Tris-HCl pH 7.9, 500 mM NaCl and 500 mM imidazole. SDS-PAGE (Laemmli 1970) confirmed purity suitable for crystallization trials.

2.2. Crystallization

For initial crystallization trials, *Tc*UH was concentrated to 5.0, 7.5 and 10.0 mg/ml, as quantified by Bradford method,[25] in 20 mM Tris-HCl pH 7.9 and 500 mM NaCl. The crystallization method used was sitting drop vapor diffusion.[26] Initial trials were performed with the commercial kits Joint Center for Structural Genomics (JCSG, Nextal/Qiagen), polyethylene glycol (PEG),

anion and cation test (PACT Suite, Nextal/Qiagen), Wizard Screens I and II (Emerald BioSystems), Precipitant Synergy (Emerald BioSystems) and Crystal Screen (Hampton Research) in a robot (HoneyBee 963, Digilab Global) at the Brazilian Biosciences National Laboratory (LNBio-Campinas, Brazil). Each drop was prepared by mixing 0.7 µl reservoir solution to 0.7 µl protein solution in buffer 20 mM, Tris-HCl pH 7.9 and 500 mM NaCl. The drops were then equilibrated against 100 µl reservoir solution, in 96-well plates, at 291 K. Initial hits were obtained with the JCSG kit, conditions number 2, 37 and 48. Then, for condition number 48, crystallization condition refinement was performed at State University of Ponta Grossa (UEPG) with manually set plates and the hanging drop vapor diffusion method[26] in which the protein solution at the same initial conditions was mixed with the reservoir solution with variable PEG 8,000 and glycerol concentrations (both from 14 to 24 % - *w*/*v* and *v*/*v*, respectively – 2 % step). In this manual set up, the drops were equilibrated against 500 µl reservoir solution in 24-well plates at 291 K and were prepared by mixing 2 µl reservoir solution to 2 µl protein solution.

2.3. X ray data collection and processing

Crystals from the initial hits were taken to a diffractometer (D8 Venture, Bruker) at Federal University of Paraná, but no diffraction spots were observed, what, nevertheless, indicated them to likely be protein crystals. After the crystallization refinement, with better crystals, X ray diffraction data could be collected at a wavelength of 1.458 Å (at 100 K) using a synchrotron radiation source, at the W01B-MX2 station in the Brazilian Synchrotron Light Laboratory (LNLS), with a PILATUS 2M detector (Dectris). The best protein crystal was harvested with a cryoloop and flash-frozen directly in a nitrogen stream at 100 K since the crystallization condition was already cryoprotectant. Indexing, integration and scaling were performed with the X-ray Detector Software, XDS suite.[27] To obtain phase information, molecular replacement was performed using an edited model from the structure of *Pseudomonas putida* (36 % sequence identity and 81 % coverage), PDB code 1UWK[21] as template, prepared by the program CHAINSAW[28] with the "maximum" mode. Rotation and translation solutions for the four monomers were found by the program Phaser.[29]

3. Results and discussion

*Tc*UH was overexpressed in *E. coli* and purified through the usage of a single nickel-affinity chromatography step. The purification efficiency and quality were followed by electrophoresis (Fig. 1A). The chromatography fractions containing the pure recombinant protein were pooled, dialyzed and concentrated for crystallization trials. Crystals were formed in various conditions and at protein concentrations of 5.0, 7.5 and 10 mg/ml. The best crystal in quality was the largest one (Fig. 1B) and was suitable, after growth for two weeks, to provide a complete dataset. The optimized final crystallization condition consisted of 0.04 M potassium dihydrogen phosphate, 16.00 % (*w*/*v*) polyethylene glycol 8,000 and 24.00 % (*v*/*v*) glycerol, using a protein concentration of 10 mg/ml.

The dataset was collected to 2.61 Å resolution at 100 K from a single crystal (Fig. 1C, D). The crystal belongs to space group $P2_1$, with unit-cell parameters a = 85.12, b = 137.63, c = 117.69 Å, β = 94.69 °. A summary of the data collection statistics is given in Table 1. The calculated Matthews coefficient[30] was 2.3 Å^3/Da, with a solvent content of 45.9 %, which corresponds to the presence of four monomers in the asymmetric unit. The molecular replacement calculations indicated four promising

solutions that at rigid body refinement indicated an overall residual factor (R_{factor}) of 47.6 %. Structure inspection and refinement at the currently available resolution is being evaluated, though trials to eventually get even better crystals will be performed.

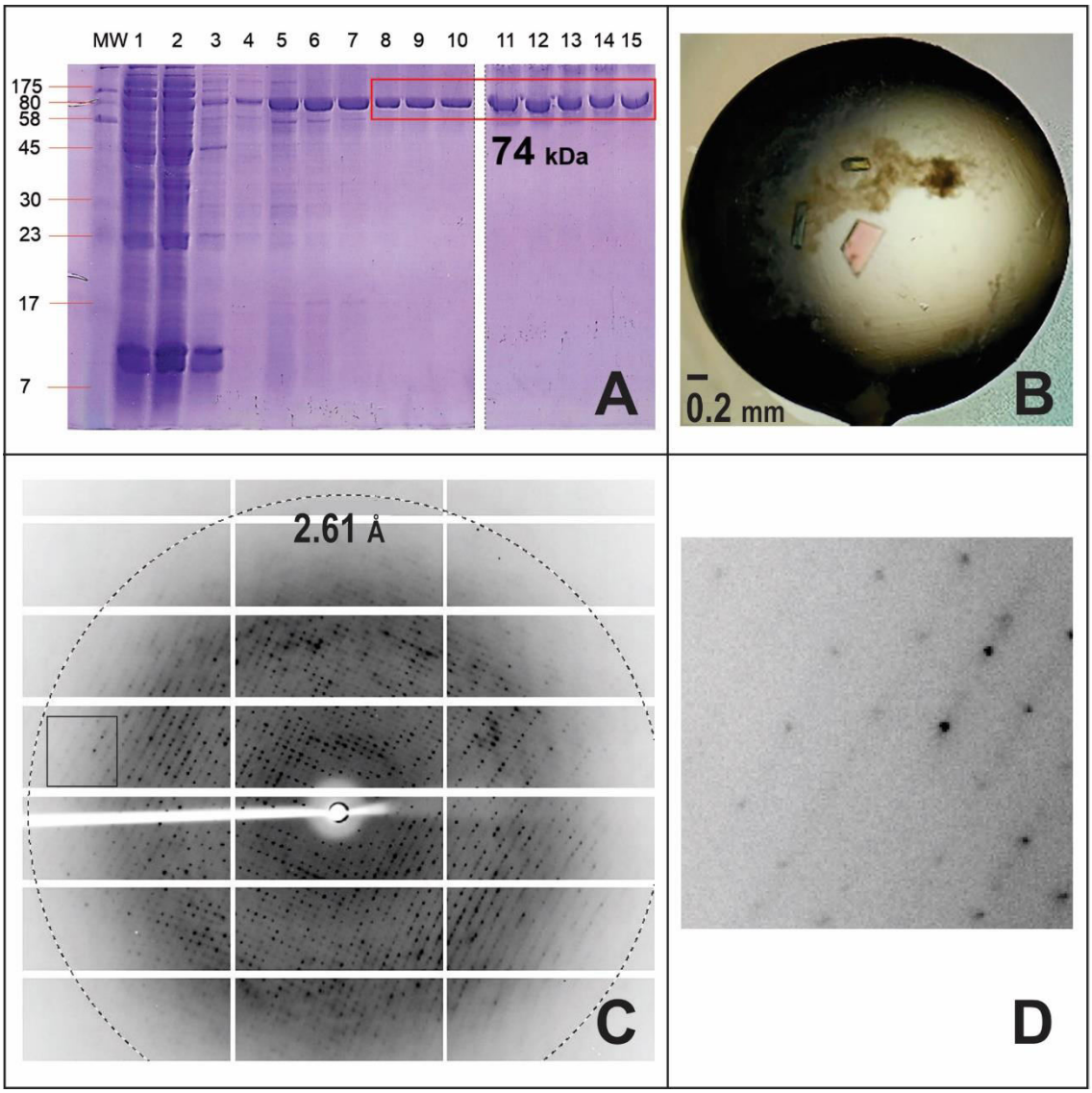

Figure 1. **A**. Picture of the 12 % SDS-PAGE of the affinity chromatography fractions. The ones sampled in lanes 8-15 were pooled for subsequent assays. MW: Molecular Weight Markers. **B**. Crystals of *Tc*UH grown in a solution containing 0.04 M di-potassium hydrogen phosphate, 16.00 % (*w*/*v*) PEG 8,000 and 22.00 % (*v*/*v*) glycerol. The larger crystal in the picture, with approximate dimensions of 0.5 x 0.3 x 0.1 mm, provided the best diffraction dataset. **C.** Crystal diffraction images of *Tc*UH. A. Overall pattern, with diffracted points to 2.61 Å of resolution limit indicated. **D**. Expansion of the diffraction image of the highlighted region in 2A.

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

Diffraction source	W01B-MX2, LNLS
Wavelength (Å)	1.458
Temperature (K)	100
Detector	PILATUS 2M
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	20
Space group	$P2_1$
a, *b*, *c* (Å)	85.12, 137.63, 117.69
β (°)	94.69
Mosaicity (°)	0.428
Resolution range (Å)	117.3 - 2.61 (2.69-2.61)
Total No. of reflections	231844 (15163)
No. of unique reflections	76789 (6279)
Completeness (%)	93.5 (88.3)
Multiplicity	3.02 (2.41)
‹(*I*/σ(*I*)›	11.31 (1.52)
R_{merge} (%)	9.7 (76.6)
R_{meas} (%)	11.8 (98.5)
$CC_{1/2}$ (%)	99.4 (51.4)
Overall *B* factor from Wilson plot (Å^2)	61.6
No. of molecules per asymmetric unit	4
V_M (Å^3 Da^{-1})	2.3
Solvent content (%)	45.9

4. Conclusions

Urocanate Hydratase from *Trypanosoma cruzi* could be crystallized in the presence of glycerol and PEG 8,000. The crystals are monoclinic, space group $P2_1$, and are suitable for molecular replacement phasing. These successful crystallization conditions related for *Tc*UH might guide crystallization assays for other related proteins. Further structure solution must be pursued and reveal structural aspects of the histidine degradation pathway.

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