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Structural studies of the *Trypanosoma cruzi* Old Yellow Enzyme: Insights into enzyme dynamics and specificity

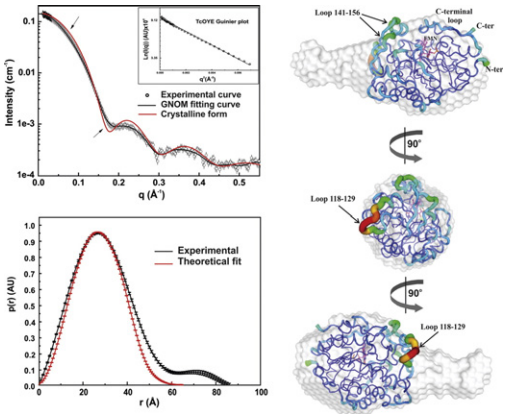
Mário T. Murakami [a,1], Nathalia C. Rodrigues [b,1], Lisandra M. Gava [c], Rodrigo V. Honorato [a], Fernanda Canduri [d], Leandro R.S. Barbosa [e], Glaucius Oliva [b], Júlio C. Borges [d,*]

[a] *Laboratório Nacional de Biociências - LNBio/CNPEM-ABTLuS, Campinas, SP 13083-970, Brazil*
[b] *Instituto de Física de São Carlos, USP, São Carlos, SP 13566-590, Brazil*
[c] *Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, SP 13565-905, Brazil*
[d] *Instituto de Química de São Carlos, USP, São Carlos, SP 13560-970, Brazil*
[e] *Instituto de Física, USP, São Paulo, SP 05508-090, Brazil*

HIGHLIGHTS

- The high-resolution crystallographic structure of TcOYE was solved at 1.27 Å resolution.
- It has a classical $(\alpha/\beta)_8$ fold with the FMN prosthetic group bound to a deep cleft.
- In solution, TcOYE is a monomer and displays a distinct conformational state compared to the crystal structure due to long and flexible loops.
- Molecular dynamics shed light in the interaction mechanism with three naphthoquinones.

GRAPHICAL ABSTRACT



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ABSTRACT

The flavoprotein old yellow enzyme of *Trypanosoma cruzi* (TcOYE) is an oxidoreductase that uses NAD(P)H as co-factor. This enzyme is clinically relevant due to its role in the action mechanism of some trypanocidal drugs used in the treatment of Chagas' disease by producing reactive oxygen species. In this work, the recombinant enzyme TcOYE was produced and collectively, X-ray crystallography, small angle X-ray scattering, analytical ultracentrifugation and molecular dynamics provided a detailed description of its structure, specificity and hydrodynamic behavior. The crystallographic structure at 1.27 Å showed a classical $(\alpha/\beta)_8$ fold with the FMN prosthetic group buried at the positively-charged active-site cleft. In solution, TcOYE behaved as a globular monomer, but it exhibited a molecular envelope larger than that observed in the crystal structure, suggesting intrinsic protein flexibility. Moreover, the binding mode of β-lapachone, a trypanocidal agent, and other naphthoquinones was investigated by molecular docking and dynamics suggesting that their binding to TcOYE are stabilized mainly by interactions with the isoalloxazine ring from FMN and residues from the active-site pocket.

Abbreviations: AUC, analytical ultracentrifugation; CD, circular dichroism; D_{max}, maximum distance; f/f_0, frictional ratio; MM, molecular mass; R_s, Stokes radius; R_g, radius of gyration; $s_{20,w}$, sedimentation coefficient at standard conditions; $s^0_{20,w}$, standard sedimentation coefficient at 0 mg mL^{-1} of protein; [Θ], mean residue ellipticity.

* Corresponding author at: Instituto de Química de São Carlos, Universidade de São Paulo, USP, P.O. Box 780, Zip Code 13560-970, São Carlos, SP, Brazil. Tel.: +55 16 3373 8637; fax: +55 16 3373 9982.
E-mail address: borgesjc@iqsc.usp.br (J.C. Borges).

[1] Both authors have equally contributed to this work.

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1. Introduction

Chagas' disease is caused by the protozoan *Trypanosoma cruzi* and represents a still serious public health problem in developing countries throughout South and Central America, with nearly 8 million people infected while 28 million are at risk of infection [1]. This disease leads to severe acute and chronic complications, which may compromise the economic life of the patient [1]. Nifurtimox and benznidazole are the current drug treatments for this disease, which act by forming free radical anions and reactive oxygen species. However, these drugs have limited efficacy and often provoke harmful side effects [1]. In addition, several cases have been reported for the resistance of *T. cruzi* to these compounds [1–3]. The current scenario highlights the importance of studies on the development of new and more effective drugs against *T. cruzi*. Consequently, efforts to elucidate the action mechanism of new potential molecular targets for rational drug design strategies have been conducted by several groups (reviewed in [2,4]).

It was recently shown that the enzyme prostaglandin F2α synthase, which reduces the 9,11-endoperoxide PGH2 to PGF2α, is responsible for the drug-metabolizing activity of trypanocidal drugs, such as nifurtimox, benznidazole and the diterpene komaroviquinone in *T. cruzi* [5,6]. This enzyme is also called *T. cruzi* Old Yellow Enzyme (TcOYE) due to the presence of its flavin mononucleotide prosthetic group [6], which gives its characteristic coloring. The oxidoreductase activity of TcOYE is dependent of NAD(P)H that acts as cofactor. Its drug-metabolizing activity has been suggested by the TcOYE over-expression in *T. cruzi* strains susceptible to benznidazole [7] and by the *T. cruzi* benznidazole resistance when TcOYE gene copies were deleted [8]. Moreover, TcOYE immunoprecipitation from *T. cruzi* cell lysates abolished, under anaerobic conditions, the reductase activity for peroxides, naphthoquinones and nitroheterocyclic drugs supporting the role of TcOYE in drug metabolism [6].

Despite the importance of TcOYE in trypanocidal drugs and prostaglandin metabolism [6], few studies detailing its structure in solution have been reported [9,10]. In this work, we have produced the recombinant TcOYE isoform from *T. cruzi* 17WTS strain and investigated its structural and dynamics properties by X-ray crystallography, small angle X-ray scattering, analytical ultracentrifugation and molecular dynamics. The holoenzyme structure was solved in two crystalline forms at 1.27 and 2.00 Å resolution and SAXS data pointed out that TcOYE displayed some intrinsic flexibility related to interfacial loops with high B-factors. TcOYE molecular docking studies with naphthoquinone compounds, like β-lapachone, a natural compound [11] that is metabolized by TcOYE into a semiquinone radical anion, suggested the TcOYE binding mode. All tested ligands seem to make hydrophobic interactions with the FMN isoalloxazine ring and Phe^{71} and Met^{290} residues as well as hydrogen bonds with Thr^{28}, His^{195} and Tyr^{200}.

2. Material and methods

2.1. Cloning, expression and purification

The genomic DNA from *T. cruzi* 17WTS strain was used as template to amplify the DNA sequence of TcOYE (GenBank ID: 61741940) with the following primers 5′-TTTCATATGGCGACGTTCCCTG-3′ and 5′-GAGGTATCAAGCTTATTTGTTGTACG-3′. The amplified DNA was cloned into the pET28a expression vector (Novagen) yielding TcOYE with a His-tag fused to its N-terminal (His-TcOYE). The cloning process was checked by automated DNA sequencing. The recombinant protein was expressed in *E. coli* BL21(DE3) strain, at 30 °C, in LB medium containing 30 μg mL^{-1} of kanamycin. The cells were grown to an optical density at 600 nm of 0.4, with addition of 0.1 mM of isopropyl thio-β-D-galactoside. After 4 h of induction, the cells were harvested by centrifugation at 2600 ×*g* for 10 min. The cells were disrupted by sonication, after incubation for 30 min on ice with 5 U of DNAse (Promega) and 30 μg mL^{-1} of lysozyme (Sigma), and centrifuged 2 times at 20,000 ×*g* for 30 min at 4 °C. The supernatant was submitted to affinity chromatography in a HiTrap Chelating column (GE Healthcare) coupled to an ÄKTA Prime system (GE Healthcare) equilibrated with 20 mM phosphate (pH 7.5) with 500 mM NaCl. The bound fractions were eluted with 500 mM imidazole solved in the aforementioned buffer. The His-tag was released from the His-TcOYE by incubating of 10–15 mg of protein with 10 U of thrombin (Sigma) at 4 °C for 24 h; after the incubation time the noncleaved His-tagged protein was separated by nickel affinity chromatography. The cleaved protein (named TcOYE) was loaded onto a HiLoad Superdex 200 pg 16/60 size exclusion column (GE Healthcare) equilibrated with 25 mM Tris–HCl (pH 8.0) containing 100 mM NaCl and 1 mM β-mercaptoethanol. The efficacy of each purification step was assessed by 12% SDS-PAGE. The protein concentration was determined by UV absorbance at 280 nm, using the calculated extinction coefficient of 64,600 M^{-1} cm^{-1}, calculated at water conditions by the Sednterp program (www.jphilo.mailway.com/download.htm) using the TcOYE amino acid sequence, and summing the FMN extinction coefficient at 280 nm.

2.2. Circular dichroism spectroscopy

Circular dichroism (CD) measurements were performed in a Jasco J-815 spectropolarimeter connected to a Peltier-type temperature control system PFD 425S. The TcOYE was tested in 25 mM Tris–HCl (pH 8.0) containing 100 mM NaCl and 1 mM β-mercaptoethanol, at 4 different molar concentrations ranging from 2 to 25 μM in a 0.2 and 1.0 mm pathlength Hellma cuvette. The CD spectra were normalized to residual molar ellipticity ([Θ]) and averaged.

2.3. Crystallization

Crystallization experiments were performed at 292 K by the hanging-drop vapor diffusion method. Typically, 2 μL of protein solution at 14 mg mL^{-1} in 25 mM Tris–HCl (pH 8.0) containing 100 mM NaCl and 1 mM β-mercaptoethanol was mixed with an equal volume of the reservoir solution and equilibrated against 0.5 mL of reservoir solution. The better-quality crystals grew within 10 days from a condition containing 28% ($w\ v^{-1}$) polyethylene glycol 1500 and 0.3 M ammonium fluoride.

2.4. Data collection and processing

A single crystal was harvested using a nylon loop (Hampton Research) and transferred from the crystallization drop to a fresh 2 μL cryo-solution containing 10% glycerol, 28% PEG 1500 and 0.3 M ammonium fluoride. The crystal was then flash-cooled to 100 K in a nitrogen gas stream in order to prevent radiation damage during data collection. Data sets were collected on the MX2 beamline at the Brazilian Synchrotron Light Laboratory (Campinas, Brazil) using a MARMOSAIC 225 detector to record the intensities. Depending on crystal symmetry, different data collection strategies were adopted in order to optimize oscillation range, oscillation per frame, sample-to-detector distance and exposure time. The data were indexed, integrated and scaled using the HKL2000 package [12].

2.5. Structure determination and refinement

The structure was solved by molecular replacement using the *Molrep* program [13] and the atomic coordinates of TcOYE isoform (PDB code: 3ATY) [9]. The refinement was performed by using the REFMAC5 program as implemented in the *CCP*4 program suite [14]. The electron density maps were examined and model building was carried out using the *COOT* program [15]. The final model was analyzed using the *MOLPROBITY* program [16]. The atomic coordinates and structure factors of the TcOYE structure in $P2_12_12_1$ and $P2_1$ crystalline forms have