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X-ray Structure and Kinetic Properties of Ornithine Transcarbamoylase from the Human Parasite *Giardia lamblia*

Andrey Galkin¹, Liudmila Kulakova¹, Rui Wu², Maozhen Gong², Debra Dunaway-Mariano², and Osnat Herzberg¹

¹ W.M. Keck Laboratory for Structural Biology, Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, Maryland ²Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, New Mexico.

Introduction

Ornithine transcarbamoylase (OTC; EC 2.1.3.3, also known as ornithine carbamoyltransferase) catalyzes the reversible conversion of citrulline to carbamoyl phosphate (CP) and L-ornithine in the arginine dihydrolase pathway. This pathway is used by a number of microorganisms, including *Giardia lamblia* to generate ATP fermentatively from arginine 1,2. *G. lamblia* OCT (*gl*OTC) was shown to be one of the 16 immunodominant proteins, underscoring the importance of the arginine catabolism pathway in the characterization of the parasite³. Moreover, recently *gl*OTC was identified as one of the major proteins released into the medium after brief interaction of *Giardia* with human intestinal epithelial cells. Therefore it was suggested that targeting *gl*OTC for immunoneutralization may represent a useful therapeutic strategy for treating *giardiasis*⁴. Multiple crystal structures of OTCs were determined from prokaryotic and eukaryotic organisms. Currently 26 structures of OTCs from 10 organisms have been deposited in the PDB⁵ under the OTC or ornithine carbamoyltransferase names. In this work we have undertaken *gl*OTC purification and crystal structure determination in order to assist with the characterization of this attractive therapeutic drug target for the potential treatment of the parasitic infection. We have compared the active sites of *gl*OTC and human OTC (*h*OTC)⁶ to identify potential structural features, which may be exploited to develop selective inhibitors of the parasitic enzyme.

Experimental

Gene cloning, protein expression and purification

The trophozoites of *G. lamblia* isolate WB, were grown as described previously⁷. The genomic DNA was isolated by using the DNA STAT kit (Stratagene). The gene encoding *gl*OTC was amplified using PfuTurbo DNA polymerase (Stratagene), genomic DNA, and 5'- and 3'-end primers. The PCR product was inserted into the pDEST-HisMBP expression vector as described by Waugh et al.⁸ For protein production, the *gl*OTC was expressed in *E. coli* strain BL21(DE3)Star as a maltose binding protein (MBP) fusion product. Cells were grown in Overnight Express Instant TB autoinduction medium (Novagen) for 20 hrs at 30°C. The cells were broken by sonication and the soluble fraction was applied on a Ni-NTA affinity column. After elution and concentration the *gl*OTC MBP-fusion protein was

Address correspondence to: Osnat Herzberg, Center for Advanced Research in Biotechnology, 9600 Gudelsky Drive, Rockville MD 20850, Tel: 240-314-6245; Fax: 240-314-6255; E-mail: herzberg@umbi.umd.edu.

applied onto a preparative size exclusion column, Sephacryl 100. MBP was removed by treatment of the fusion protein with tobacco etch virus (TEV) protease⁹, at a molar ratio of 60:1, followed by chromatography on a Ni-NTA affinity column. The protein was further purified by ion-exchange chromatography on a SOURCE 15 Q column (Amersham Biosciences). Fractions containing protein were collected, dialyzed against 50 mM Tris-HCl (pH 7.5), and 50 mM NaCl, and concentrated up to 35 mg/mL. Protein integrity and purity was assessed by polyacrylamide gel electrophoresis in the presence of SDS. The native mass was measured by size exclusion chromatography using a Superdex-200 HR 10/30 column (Amersham Bioscience) in conjunction with an ÄKTA purifier 10.

Steady-state kinetic analysis: conversion of L-citrulline + phosphate to L-ornithine + carbamoyl phosphate

Reaction solutions (1 mL) initially contained L-citrulline at varied concentration (0.5–5 fold K_m) and 20 mM sodium phosphate (or sodium phosphate at varied concentration (0.5–5 fold K_m) and 60 mM L-citrulline), *g*/OTC, 2 mM glucose, 0.9 mM ADP, 30 mM $MgCl_2$, 0.2 mM NADP, 0.03 μM *G. lamblia* carbamate kinase, 10 u hexokinase, 5 u glucose 6-phosphate dehydrogenase in 50 mM Tris-HCl (pH 8.0, 37°C). The reaction progress was monitored at 340 nm ($\Delta\epsilon = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). The initial velocities (V_o) measured as a function of the concentration of the varied substrate, [S], were fitted with KinetAsyst to the equation $V_o = V_{max}[S]/(K_m + [S])$ to obtain the maximum velocity (V_{max}) and the Michaelis constant (K_m). The k_{cat} was calculated from the ratio of the V_{max} and the enzyme concentration.

Conversion of L-ornithine + carbamoyl phosphate to L-citrulline + phosphate

Reaction solutions (1 mL) initially contained L-ornithine at varied concentration (0.5–5 fold K_m), 10 mM carbamoyl phosphate (or carbamoyl phosphate at varied concentration (0.5–5 fold K_m) and 10 mM L-ornithine) and *g*/OTC in 50 mM Tris-HCl (pH 8.0) at 37°C. The reaction progress was determined using a fixed-time colorimetric assay for L-citrulline¹⁰. The k_{cat} and K_m values were calculated from the initial velocity data as described above.

Determination of the inhibition constant for N-phosphonoacetyl-L-ornithine (PALO)

PALO was synthesized as described previously by Morizono *et al.*¹¹ The competitive inhibition constant for PALO was determined by fitting the initial velocities measured for reactions initially containing (0.0, 0.05, 0.09 or 0.13 μM PALO), L-citrulline (5–100 mM), 20 mM sodium phosphate, 2 mM glucose, 0.9 mM ADP, 30 mM $MgCl_2$, 0.2 mM NADP, 0.03 μM carbamate kinase, 10 u hexokinase, 5 u glucose 6-phosphate dehydrogenase and *g*/OTC in 50 mM Tris-HCl (pH 8.0, 37°C) with KinetAsyst. The initial velocity data were analyzed using the equation $V_o = V_{max}[S]/(K_m(1 + [I]/K_i) + [S])$, where K_i is the inhibition constant and [I] is the PALO concentration.

Crystallization, data collection and structure determination

Crystals were obtained at 20°C by the vapor diffusion method in hanging drops. The protein solution was mixed with an equal volume of mother liquor containing 30% Polyethylene Glycol Monomethyl Ether 550, 0.1 M NaCl and 0.1 M Bicine buffer (pH 9.0), and equilibrated against the mother liquor reservoir. The crystals diffracted X-rays to a resolution of 2.0 Å, and required a period of 3–5 days to appear. Diffraction data were acquired using an RAXIS IV++ image plate detector mounted on a Rigaku rotating anode MicroMax 007 X-ray generator (Rigaku MSC Inc.). Data processing was carried out using the CrystalClear program, version 1.3.6 (Rigaku MSC Inc.). The statistics of data collection are provided in Table I.

The structure was determined by the Molecular Replacement method using the computer program Phaser 12 and the *Pyrococcus furiosus* OTC structure¹³ as the search model. The difference Fourier maps indicated that some *glOTC* segments had to be modified compared with the *P. furiosus* OTC (residues 3–4, 34–37, 149–156, 210–216, and 323–325). Structure refinement was carried out using the CNS program 14. The resulting models were inspected and modified on a graphics workstation using program ‘O’ 15. Water molecules were added to the model based on the $F_o - F_c$ Difference Fourier electron density map (where F_o and F_c are the observed and calculated structure factors, respectively), using peaks with density $\geq 3\sigma$ as the acceptance criteria. Refinement statistics are provided in Table I.

Results and Discussion

Expression, purification and kinetic properties of *glOTC*

The N-terminally His-tagged MBP fusion *glOTC* was expressed in soluble form in *E. coli* and remained soluble after digestion with TEV protease and removal of the MBP. The mass of the purified *glOTC* was determined to be approximately 90 kDa (using the molecular marker from Amersham), which based on a theoretical subunit mass of 36494.2 Da defines the quaternary structure as homotrimeric. The steady-state catalytic rate constants measured at pH 8.0 and 37°C are reported in Table II. The bisubstrate analog PALO is a competitive inhibitor *vs.* L-citrulline at saturating phosphate (10-fold K_m). Because the phosphate is expected to compete with the PALO for binding to the free enzyme, the experimental K_i value of $0.025 \pm 0.009 \mu\text{M}$ is expected to be somewhat larger than the actual K_i value. The kinetic and inhibition constants of *glOTC* are in the range observed for other OTCs and similar to that of the human¹¹ and porcine¹⁶ enzymes.

Overall structure of *glOTC*

The *glOTC* crystal contains one protein molecule in the asymmetric unit and the crystallographic 3-fold symmetry generates a trimeric structure (Fig 1A), consistent with the association state measured in solution (*vide supra*). Human OTC also associates into trimers. Crystal contacts are mediated by a Ni^{2+} ion that presumably originated from the Ni-NTA column. The Ni^{2+} occupies a special position on the 3-fold symmetry axis and it coordinates three His71 residues and three water molecules. The model of the *glOTC* molecule includes 304 of the 326 amino acid residues. Missing from the model are residues that were not associated with interpretable electron density: one N-terminal residue remaining after TEV cleavage (Gly), three OTC N-terminal residues, and 17 residues of two flexible loops associated with the active site (loop1 - residues 78–87, and loop2 – residues 244–251). Both of these loops play an important role in sequestering the substrates from bulk solvent.

As with all OTCs, the molecule consists of N-terminal and C-terminal domains of similar size, known as the CP and amino-acid binding domains. A DALI¹⁷ search identified the *Mycobacterium tuberculosis* OTC as the *glOTC* closest structure (pdb code 2i6u, with Z-score of 42.8 and a root-mean-squares deviation (RMSD) of 1.2 Å for 289 aligned α -carbon atoms that share 37% sequence identity).

Each domain exhibits an α/β topology with a central β -pleated sheet flanked by α -helices. The domains are connected by two inter-domain helices (residues 133–149 and 301–332), and the cleft between two domains comprises the active site. The CP binding domains are located in the interior of the trimer, and the amino-acid binding domains are located further from the trimer center. By analogy to the many known OTC structures, residues from both domains are involved in catalysis.

Active site comparison with human OTC (hOTC)

The *g*lOTC is a potential anti *giardiasis* drug target, and therefore features that distinguish it from the human counterpart are of special interest for the development of selective inhibitors. The two enzymes share only 30% sequence identity, suggesting an opportunity to exploit their differences in drug development. In contrast to the catabolic *g*lOTC, human OTC is a ureotelic enzyme that catalyzes the formation of citrulline from CP and L-ornithine in the urea cycle¹⁸. Superposition of the *g*lOTC and *h*OTC structures using the *h*OTC/PALO complex structure (pdb entry code 1oth)6 yielded a RMSD of 1.6 Å for 288 aligned α -carbon atoms.

The superposition and the sequence alignment show that the active sites of both enzymes are very similar and most of the residues around the bound PALO are identical (Fig.1B&C). However, there are two notable exceptions related to residues located on the disordered loops of the *g*lOTC structure. His117 in *h*OTC is located on a loop analogous to the disordered loop 1 of *g*lOTC. The His117 from an adjacent *h*OTC subunit interacts with the phosphonate group of PALO (Fig.1B). The *g*lOTC loop1 is 1 amino acid residue longer than that of *h*OTC and its sequence, GANSNVGGKE (residues 78–87), does not contain a His residue. Modeling indicates that the most probable equivalent *g*lOTC residue to the *h*OTC His117 is Ser81. This is an important difference because all ureotelic OTCs have His at this position linking the phosphate oxygen of CP to Asn121 (*h*OTC numbering) in a potential pathway for transferring a proton between the solvent and CP6. The second significant sequence difference between the *h*OTC and *g*lOTC active sites occurs in the disordered loop2 (*g*lOTC residues 244–251). In *h*OTC Met286 is stationed close to the carboxyl end of the bound PALO (Fig.1B). In contrast to *h*OTC the equivalent residue in *g*lOTC sequence is Tyr245, which introduces different space and electrostatic constraints. Other residues of both loops may play an important role in defining the active site cleft, in affecting ligand interactions and in sequestering the catalytic site from solvent. Yet, these loops exhibit no sequence homology between *h*OTC and *g*lOTC, thus providing opportunities for designing selective inhibitors. Structures of liganded *g*lOTC will provide better insight into strategies of inhibitor design.

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Overall fold, active site architecture of *g/OTC*, and sequence alignment of *g/OTC* and *h/OTC*. (A) Ribbon diagram representation of the protein trimer. The Ni²⁺ ion at the center of the trimer is shown as magenta sphere. (B) Stereoscopic view of the *g/OTC* active site superposed with the *h/OTC/PALO* active site. The carbon atoms are colored green (*g/OTC*) and magenta (*h/OTC*). Other atomic colors are as follows: oxygen, red; nitrogen, blue; phosphor, orange; and sulfur, yellow. Black residue labels correspond to *g/OTC*, except that the two residues labeled in magenta color (His117 of a neighboring subunit and Met268) are *h/OTC* residues that are disordered in the *g/OTC* structure (Ser82 and Tyr245, respectively). (C) Sequence alignment of *g/OTC* and *h/OTC*. Identical residues are blocked in blue and residues surrounding the active site are indicated by red triangles.

TABLE I

X-Ray Data Collection and Refinement Statistics

Space group	P6 ₃
% solvent	42
No. molecule in asymmetric unit	1
<u>Data collection</u>	
Cell dimension: a, b, c (Å)	a= b=95.49 c= 56.96
Resolution range (Å)	20–2.0
No. observations	146433
No. unique reflections	19980
Completeness (%) ^a	99.3(99.9)
R_{merge}^b	0.069(0.306)
<u>Refinement statistics</u>	
No. reflections	19952
No. residues	304
No. water molecules	267
R_{cryst}^c	0.197
R_{free}^d	0.255
RMS deviation	
Bonds (Å)	0.017
Angles (°)	2.0

^aThe values in parentheses are for the highest resolution shell

^b $R_{merge} = \sum_{hkl} [(\sum_j |I_j - \langle I \rangle|) / \sum_j |I_j|]$, for equivalent reflections

^c $R_{cryst} = \sum_{hkl} ||F_o| - |F_c|| / \sum_{hkl} |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively

^d R_{free} is computed for 5% of reflections that were randomly selected and omitted from the refinement