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Expression, purification, crystallization and preliminary X-ray diffraction analysis of the aspartate transcarbamoylase domain of human CAD

Aspartate transcarbamoylase (ATCase) catalyzes the synthesis of *N*-carbamoyl-L-aspartate from carbamoyl phosphate and aspartate in the second step of the *de novo* biosynthesis of pyrimidines. In prokaryotes, the first three activities of the pathway, namely carbamoyl phosphate synthetase (CPSase), ATCase and dihydroorotase (DHOase), are encoded as distinct proteins that function independently or in noncovalent association. In animals, CPSase, ATCase and DHOase are part of a 243 kDa multifunctional polypeptide named CAD. Up-regulation of CAD is essential for normal and tumour cell proliferation. Although the structures of numerous prokaryotic ATCases have been determined, there is no structural information about any eukaryotic ATCase. In fact, the only detailed structural information about CAD is that it self-assembles into hexamers and trimers through interactions of the ATCase domains. Here, the expression, purification and crystallization of the ATCase domain of human CAD is reported. The recombinant protein, which was expressed in bacteria and purified with good yield, formed homotrimers in solution. Crystallization experiments both in the absence and in the presence of the inhibitor PALA yielded small crystals that diffracted X-rays to 2.1 Å resolution using synchrotron radiation. The crystals appeared to belong to the hexagonal space group $P6_322$, and Matthews coefficient calculation indicated the presence of one ATCase subunit per asymmetric unit, with a solvent content of 48%. However, analysis of the intensity statistics suggests a special case of the $P2_1$ lattice with pseudo-symmetry and possibly twinning.

1. Introduction

Aspartate transcarbamoylase (ATCase; EC 2.1.3.2) catalyzes the condensation of carbamoyl phosphate and aspartate to produce *N*-carbamoyl-L-aspartate and inorganic phosphate in the second step of the *de novo* biosynthesis of pyrimidines (Jones *et al.*, 1955; Fig. 1*a*). In prokaryotes and plants the first three enzymes of this pathway, namely carbamoyl phosphate synthetase (CPSase), ATCase and dihydroorotase (DHOase), are encoded as separate proteins that either act independently or associated into complexes. In contrast, in animals the CPSase, ATCase and DHOase activities are gathered as different domains within a single multifunctional polypeptide of 243 kDa named CAD (an acronym for the three catalytic activities), in which the ATCase occupies the most C-terminal position (reviewed in Evans & Guy, 2004; Fig. 1*a*).

The catalytic unit of ATCase is composed of a homotrimer with threefold rotational symmetry (Fig. 1*b*). In prokaryotes, there are three classes of ATCases depending on whether the catalytic trimer functions independently (type C) or in association with another catalytic trimer through interaction with DHOase (type A) or with a regulatory subunit (type B) (Bethell & Jones, 1969). The structure and the catalytic and regulatory mechanisms of the ATCase from *Escherichia coli* have been the most extensively studied (reviewed in Kantrowitz, 2012; Allewell, 1989). Indeed, this enzyme has become a textbook protein, since it provided the first example of a feedback-inhibition mechanism (Yates & Pardee, 1956; Gerhart & Pardee, 1962), and was used as a model to explain protein cooperativity and allosteric regulation (Monod *et al.*, 1965). *E. coli* ATCase is a 310 kDa heterododecamer formed by two catalytic trimers of a 34 kDa catalytic subunit bridged by three dimers of a 17 kDa regulatory chain

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(Wiley & Lipscomb, 1968; Fig. 1*b*). Binding of the allosteric effectors (CTP and ATP are negative and positive regulators, respectively) to the regulatory dimers induces large quaternary conformational changes between a low-activity T state and a high-activity R state (reviewed in Kantrowitz, 2012). However, when separated from the regulatory dimers the catalytic trimers are stable and still active on their own. In other prokaryotes [*e.g. Aquifex aeolicus* (Zhang *et al.*, 2009) and *Thermus aquaticus* (Van de Castele *et al.*, 1997)] ATCase also forms heterododecamers, but the association between the two catalytic trimers occurs through interaction with three DHOase dimers that replace the regulatory subunits. Although these ATCase trimers are active on their own, the association with DHOase is reported to be required for the activity of the latter (Ahuja *et al.*, 2004). Puzzlingly, other bacteria, such as *Pseudomonas aeruginosa* (Vickrey *et al.*, 2002), present a similar ATCase arrangement but the DHOase-like subunits are inactive and must fulfil an as yet unknown structural role. Lastly, other prokaryotes (*e.g. Bacillus subtilis*) have the simplest form of ATCase consisting of unregulated free catalytic trimers (Harris *et al.*, 2011; Brabson *et al.*, 1985).

Regardless of the quaternary organization, the numerous crystal structures of ATCase from a variety of bacteria and archaea have

proven that the catalytic chains exhibit a conserved fold, with two α/β -domains each containing the binding site for carbamoyl phosphate or aspartate. Residues from the adjacent subunit complete the active site, thus explaining why the enzyme is only active as a trimer. It has been clearly demonstrated for *E. coli* ATCase that the reaction mechanism is ordered, with carbamoyl phosphate binding first and inducing the formation of the binding site for aspartate (Hsuanyu & Wedler, 1987). The binding of aspartate triggers the closure of the active site that is required for catalysis to occur.

The ATCase domain of mammalian CAD is predicted to be very similar to the catalytic chain of *E. coli* ATCase, since the two proteins share 44% sequence identity and the residues involved in substrate binding and catalysis are fully conserved (Grayson & Evans, 1983; Maley & Davidson, 1988; Simmer *et al.*, 1989; Scully & Evans, 1991). However, no crystal structure of a eukaryotic ATCase has been reported to date. In fact, no detailed structural information about CAD exists other than that it self-associates, forming hexamers and trimers (Coleman *et al.*, 1977; Lee *et al.*, 1985). Importantly, interactions through the ATCase domains play a central role in CAD oligomer formation (Qiu & Davidson, 2000). It has been reported that CAD activity is modulated by phosphorylation through different

Escherichia coli ATCase

Figure 1

ATCase and *de novo* biosynthesis of pyrimidines. (a) Scheme of CAD organization and of the reactions catalyzed in *de novo* pyrimidine synthesis. (b) Diagram of the quaternary structure of *E. coli* ATCase and a cartoon representation of the catalytic trimer of *E. coli* ATCase with each chain in a different colour and a molecule of PALA bound per active site.

signalling cascades (Graves *et al.*, 2000; Carrey *et al.*, 1985; Ben-Sahra *et al.*, 2013; Robitaille *et al.*, 2013) and that the up-regulation of CAD is essential for normal and tumour cell proliferation (Aoki & Weber, 1981; Fairbanks *et al.*, 1995). This has increased the interest in this central metabolic pathway as a target for new therapeutical treatments. Thus, deciphering the architecture of CAD is important not only to understand the synthesis of pyrimidines but also to provide valuable information for the potential design of new antitumour drugs. Recently, we obtained the first high-resolution information about CAD by solving the crystal structure of the DHOase domain of human CAD (Grande-García *et al.*, 2013). Here, we report the cloning, expression, purification and crystallization of the ATCase domain of human CAD (huATCase). We also report the crystallization of huATCase in the presence of *N*-phosphonoacetyl-L-aspartate (PALA; Collins & Stark, 1971), a potent inhibitor of CAD activity (Swyryd *et al.*, 1974) that has been shown to stop the proliferation of normal and tumour cells in culture (Leyva *et al.*, 1981; Tsuboi *et al.*, 1977). huATCase crystals grown with or without PALA diffracted X-rays to 2.1 Å resolution.

2. Materials and methods

2.1. Cloning of the human ATCase domain

The cDNA of human CAD (UniProt P27708) was purchased from Open Biosystems (clone ID 5551082). A gene fragment covering the ATCase domain of CAD (residues 1915–2225) was amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs). The forward (5'-AAGTTCTGTTTCAGGGCCCCGatgtcacccctgctgactca) and reverse (5'-ATGGTCTAGAAAGCTTTAgaaacggccagcagcgt) primers contained specific regions (shown in capitals) for cloning into the pOPIN-M expression vector (Oxford Protein Production Facility) using In-Fusion technology (Clontech). The resulting plasmids were verified by sequencing and transformed into *E. coli* BL21 (DE3) pLysS cells (Novagen).

2.2. Protein expression and purification

Transformed bacterial cells were grown in a shaking incubator in autoinduction medium (Studier, 2005) supplemented with 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol for 6 h at 310 K followed by 21 h at 293 K. The cells were harvested by centrifugation, washed with phosphate-buffered saline and stored at 193 K. The bacterial pellet was thawed and resuspended in buffer A (20 mM Tris-HCl pH 8, 0.5 M NaCl, 10 mM imidazole, 5% glycerol, 2 mM β-mercaptoethanol) with 2 mM phenylmethanesulfonyl-fluoride (PMSF) and the cells were disrupted by sonication. The lysate was clarified by centrifugation in a Beckman 45 Ti rotor at 40 000 rev min⁻¹ for 40 min. The supernatant was filtered through a 0.45 µm pore filter and applied onto a 5 ml Ni²⁺-loaded HisTrap Chelating FF column (GE Healthcare, USA). Following extensive washing of the column with buffer A containing 39 mM imidazole, the protein, which has an N-terminal His₆-tagged maltose-binding protein (MBP), was eluted by increasing the imidazole concentration to 300 mM. Excess imidazole was removed and the tag was cleaved off by overnight dialysis against buffer S (20 mM Tris-HCl pH 7, 75 mM NaCl, 5% glycerol, 2 mM β-mercaptoethanol), with inclusion of GST-tagged PreScission protease (1/20th of the protein weight) within the dialysis bag. Three extra residues were left at the N-terminus of huATCase (GPM¹⁹¹⁵SP) after removal of the tag. The dialyzed and cleaved sample was passed through a 5 ml HiTrap SP HP column (GE Healthcare, USA) equilibrated in buffer S. The noncleaved protein, the His₆-MBP tag and the protease pass through

the column, whereas the cleaved huATCase elutes early (~90 mM) in a linear gradient of salt. The fractions containing the protein were pooled and concentrated to ~3 mg ml⁻¹ in an Amicon Ultra system with a 10 kDa cutoff membrane. The sample was then further purified by size-exclusion chromatography on a Superdex 75 10/300 column (GE Healthcare, USA) equilibrated in buffer GF [20 mM Tris pH 7, 100 mM NaCl, 2% glycerol, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP)]. huATCase eluted as a single peak that was pooled and concentrated as before to 5 mg ml⁻¹. The sample was directly used for crystallization studies. In some instances, the excess protein was supplemented with 40% glycerol, flash-frozen in liquid nitrogen before the gel-filtration step and stored at 193 K. However, freezing of the protein had a noticeable effect on the crystallization trials and diffraction-quality crystals were only obtained with unfrozen sample. All purification steps were carried out at 277 K. The final sample purity was evaluated by SDS-PAGE and Coomassie staining. huATCase contains no tryptophans and the protein concentration was therefore determined using the Bradford method (Bradford, 1976).

2.3. Gel-filtration and multi-angle light-scattering (MALS) measurements

For molar-mass determination, 400 µl purified huATCase at 0.8 mg ml⁻¹ was fractionated by gel filtration on a Superdex 200 10/300 column equilibrated in buffer GF using an ÄKTA purifier at a flow rate of 0.5 ml min⁻¹. The eluted sample was characterized by in-line measurement of the refractive index and multi-angle light scattering using Optilab T-rEX and DAWN 8⁺ instruments, respectively (Wyatt). The data were analyzed using the ASTRA 6 software (Wyatt) to obtain the molar mass (Wyatt, 1993).

2.4. Crystallization

Crystallization screenings were performed at 291 K using a Cartesian MicroSys robot (Genomic Solutions) and the sitting-drop vapour-diffusion method in 96-well MRC plates. Nanodrops consisting of 0.2 µl protein solution plus 0.2 µl reservoir solution were equilibrated against 60 µl reservoir solution. Initial screening involved different protein concentrations and the commercial crystallization screens The JCSG+, PACT, Protein Complex, pHClear and pHClear II Suites (all from Qiagen). Small crystals with similar thin-plate morphology were obtained in multiple conditions containing polyethylene glycol (PEG; average molecular weights in the range 1500–8000 and concentrations in the range 8–25%) as precipitant, 0.1–0.2 M of different salts and pH values of between 7.5 and 9. The initial hits were reproduced in 15-well plates with drops consisting of 1 µl protein solution and 1 µl reservoir solution and were further optimized by varying the protein and precipitant concentrations as well as the pH. Optimal crystallization conditions for huATCase free of ligands consisted of protein at 1.5 mg ml⁻¹ and 0.1 M Tris pH 8.5, 6% PEG 8000, 9% ethylene glycol (EG) as the crystallization solution. To crystallize the protein with the inhibitor PALA, the protein in buffer GF at 3 mg ml⁻¹ was diluted with an equal volume of 0.1 M CAPS pH 10, 150 mM NaCl following addition of PALA to a final concentration of 0.5 mM. PALA (CAS 51321-79-0) was obtained from the Developmental Therapeutics Program (NCI, National Institutes of Health) Open Chemical Repository. Screening of crystallization conditions for the protein with PALA were performed as described above. Optimal conditions for the co-crystallization with PALA were obtained using protein at 1.5 mg ml⁻¹ and 10 mM ZnCl₂, 15% PEG 6000, 50 mM sodium acetate pH 4.8 as the crystallization solution. Prior to cooling in liquid nitrogen, the crystals were

Table 1

Data-collection statistics for huATCase.

Values in parentheses are for the outermost resolution shell.

	Apo form	With PALA
Processing software	<i>XDS/XSCALE</i>	<i>XDS/XSCALE</i>
Beamline	PX-1, SLS	XALOC, ALBA
Wavelength (Å)	1.0	1.148
Space group	<i>P2₁</i>	<i>P2₁</i>
Unit-cell parameters (Å, °)	<i>a</i> = 82.8, <i>b</i> = 157.9, <i>c</i> = 82.7, α = 90, β = 120, γ = 90	<i>a</i> = 83.0, <i>b</i> = 145.1, <i>c</i> = 83.2, α = 90, β = 120, γ = 90
Resolution range (Å)	42.4–2.1 (2.15–2.10)	50–2.1 (2.15–2.10)
Reflections (observed/unique)	344600/101090 (15125/5053)	332141/97555 (24831/7218)
Multiplicity	3.4 (3.0)	3.4 (3.4)
Completeness (%)	94.3 (64.4)	98.0 (97.9)
<i>R</i> _{meas}	0.12 (0.51)	0.07 (0.60)
$\langle I/\sigma(I) \rangle$	8.9 (2.8)	13.9 (2.5)
CC _{1/2} †	98.9 (78.4)	99.8 (77.5)
Wilson <i>B</i> factor (Å ²)	38.9	42.6

† Percentage of correlation between intensities from random half data sets.

transferred to cryoprotectant solution consisting of the mother liquor plus 20% glycerol.

2.5. Data collection and reduction

The huATCase crystals diffracted X-rays to 2.0 Å resolution using synchrotron radiation. Complete data sets for huATCase crystals grown in the absence or presence of PALA were collected at 100 K on beamlines PX-I (SLS synchrotron, Villigen) and XALOC (ALBA synchrotron, Barcelona), respectively, using a Pilatus 6M detector in both cases. Data processing and scaling was performed using *XDS* and *XSCALE* (Kabsch, 2010). The statistics of the crystallographic data are summarized in Table 1. Analysis of the intensity distribution and statistics were performed with *phenix.xtriage* from the *PHENIX* package (Adams *et al.*, 2010).

3. Results and discussion

The ATCase domain of human CAD (huATCase; residues 1915–2225) was cloned, expressed, purified and crystallized for structural characterization. The recombinant protein was produced with an N-terminal His₆-MBP tag that was subsequently removed by digestion with PreScission protease. After Ni²⁺-chelating chromatography and a tag-cleavage step, followed by ion-exchange and size-exclusion chromatography steps, huATCase was obtained without tags and at high purity as judged by SDS-PAGE analysis (Fig. 2*a*). The purified sample had a minor contamination with an ~20 kDa protein (Fig. 2*a*, marked with an asterisk), which was identified as *E. coli* cAMP receptor protein (UniProt P0ACJ8) by mass-spectrometric analysis. The approximate yield was of ~2 mg pure protein per litre of culture. The mobility of the purified protein on SDS-PAGE showed that the protein has the expected molecular weight of 34.8 kDa. However, the molecular mass of the protein in solution, as measured by gel filtration coupled to multi-angle light scattering (Fig. 2*b*), demonstrates the formation of a single species of average molecular mass 102 ± 10 kDa, which corresponds to the formation of a homotrimer.

Initial crystallization screening yielded multiple hits in PEG-containing conditions. The crystals were small thin plates that most often clustered together. Varying the crystallization conditions did not significantly improve the size or morphology of the crystals in most of the cases. Optimization of condition No. 16 of The JCSG+ Suite [0.1 M HEPES pH 7.5, 10% (w/v) PEG 8000, 8% (w/v) EG] resulted in thin trapezoidal crystals with a maximum dimension of

0.2 mm that grew without clustering at 291 K after 1–2 d (Fig. 3*a*). The final crystallization condition was 0.1 M Tris pH 8.5, 6% PEG 8000, 9% EG and the protein concentration was increased to 1.5 mg ml⁻¹. Similar thin but hexagonal-shaped crystals appeared in the same drops (Fig. 3*a*, inset). These crystals exhibited concave faces, which has been suggested to be a possible indicator of crystal twinning (Yeates, 1997). To crystallize the protein in complex with the inhibitor PALA, huATCase at 1.5 mg ml⁻¹ in buffer GF was mixed with PALA at concentrations ranging from 0.1 to 1 mM. However, the complex with the inhibitor has very low solubility and most of the protein precipitated, and crystal screening with the soluble fraction at a concentration of 0.5 mg ml⁻¹ did not yield crystals. Since the reported crystals of bacterial ATCases in complex with PALA or other substrate analogues were mostly obtained in conditions with extreme acidic or basic pH, we exchanged the protein to a new buffer at pH 10 or 5.5 and then added PALA to a final concentration of 0.5 mM. In this way, the addition of PALA did not cause protein precipitation and the sample could be used for crystallization. Initial crystals (30 × 30 µm) of huATCase with PALA were obtained in condition No. 12 of The PACT Suite [0.1 M sodium acetate pH 5, 10 mM zinc chloride, 20% (w/v) PEG 6000] at 291 K. Optimization of the conditions allowed the growth of rugby-ball-shaped crystals with a maximal dimension of 80 µm (Fig. 3*b*), using as final conditions 0.05 M sodium acetate pH 4.8, 10 mM zinc chloride, 15% (w/v) PEG

Figure 2
The recombinant ATCase domain of human CAD is a trimer in solution. (*a*) SDS-PAGE of huATCase purification. Mass standards are indicated in kDa; lane Ni, elution from Ni-affinity chromatography; lane DD, dialyzed and digested with PreScission protease; lane S, sample after ion exchange; lane GF, final sample after gel filtration. The presence of contaminating *E. coli* cAMP receptor protein is indicated with an asterisk. (*b*) The molar mass of huATCase measured using gel filtration coupled with light scattering indicates the formation of homotrimers in solution.

Figure 3
Crystallization of huATCase in the absence or presence of PALA. (a) Crystals of recombinant huATCase grown by the hanging-drop method in the absence of PALA. Inset: hexagonal-shaped crystals with an apparent concave face appeared together with the more common trapezoidal-shaped crystals. The dotted line indicates the relation between the crystal types. (b) Crystals of huATCase grown in the presence of PALA. (c) Diffraction pattern from an huATCase crystal grown in the presence of PALA obtained on the XALOC beamline (ALBA). Circles are labelled with the resolution limits in Å. (d) Twinning analysis of the X-ray diffraction data of both crystal types processed in space group $P2_1$. The L -test for acentric data indicates a deviation from the expected distribution for a normal untwinned crystal (Padilla & Yeates, 2003).

6000, mixing huATCase at 3 mg ml^{-1} in buffer GF with an equal volume of 0.1 M CAPS pH 10, 150 mM NaCl and adding PALA to a final concentration of 0.5 mM .

A crystal grown in the absence or the presence of PALA diffracted X-rays to a maximum resolution of $\sim 2.0 \text{ Å}$ using synchrotron radiation (Fig. 3c). The data sets were processed and scaled to 2.1 Å resolution and the statistics are presented in Table 1. Both crystal types appeared to belong to the hexagonal space group $P6_322$, with unit-cell parameters $a = b = 83$, $c = 158 \text{ Å}$ in the absence of PALA and $a = b = 83$, $c = 145 \text{ Å}$ in the presence of PALA. Matthews coefficient calculation predicted the presence of one huATCase protomer per asymmetric unit, with a V_M of $2.35 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 48%. Thus, the threefold axis of the huATCase trimer is coincident with the crystallographic symmetry axis. However, analysis of the diffraction data with *phenix.xtriage* from the *PHENIX* package (Adams *et al.*, 2010) indicated a significant deviation of the intensity statistics from the expected distribution for a normal crystal and suggested the over-merging of pseudo-symmetric intensities. Reprocessing the diffraction data in the lower symmetry space group $P2_1$ yielded significantly better statistics. The monoclinic unit-cell parameters of the crystals without PALA were $a = 82.8$, $b = 157.9$, $c = 82.7 \text{ Å}$, $\alpha = 90$, $\beta = 120$, $\gamma = 90^\circ$, whereas the unit-cell parameters of the crystals grown in the presence of PALA were $a = 83.0$, $b = 145.1$, $c = 83.2 \text{ Å}$, $\alpha = 90$, $\beta = 120$, $\gamma = 90^\circ$. The predicted content of the asymmetric unit is six huATCase subunits that would form two trimers. This unusual $P2_1$ unit-cell geometry allows pseudo-merohedral twinning. Indeed, an L -test analysis (Padilla & Yeates, 2003) of the X-ray diffraction intensity statistics of both crystal types diagnoses the existence of twinning (Fig. 3d), which will have to be taken into account during structural determination and model building.

The crystal structure of huATCase will provide the first view of a eukaryotic ATCase, and the differences between the free structure and that bound to PALA should provide information about the differences between the open and closed forms of the enzyme. The ATCase domain plays a central role in the oligomerization of CAD and therefore the structure of huATCase should reveal important information towards deciphering the architecture of the complex.

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