Structural Insight into the Core of CAD, the Multifunctional Protein Leading De Novo Pyrimidine Biosynthesis

Graphical Abstract

Highlights

- The covalently linked DHO and ATC domains of CAD self-assemble as dimers of trimers
- The crystal structures of the fungal DHO-like dimer and ATC trimer were determined
- The inactive DHO-like domain has a conserved structural role in CAD-like from fungi
- A DHO-ATC hexamer is proposed as the central structural element of CAD particles

Authors

María Moreno-Morcillo, Araceli Grande-García, Alba Ruiz-Ramos, Francisco del Caño-Ochoa, Jasminka Boskovic, Santiago Ramón-Maiques

Correspondence

santiago.ramon@cbm.csic.es

In Brief

Moreno-Morcillo et al. provide insights into human CAD, the multifunctional protein initiating and controlling de novo biosynthesis of pyrimidines, by characterizing a construct covering DHO and ATC domains. The authors show that this construct forms hexamers and that this assembly does not depend on whether DHO domain is active. They propose a model with the DHO and ATC domains as the central supporting framework of CAD particles.
SUMMARY

CAD, the multifunctional protein initiating and controlling de novo biosynthesis of pyrimidines in animals, self-assembles into ~1.5 MDa hexamers. The structures of the dihydroorotase (DHO) and aspartate transcarbamoylase (ATC) domains of human CAD have been previously determined, but we lack information on how these domains associate and interact with the rest of CAD forming a multienzymatic unit. Here, we prove that a construct covering human DHO and ATC oligomerizes as a dimer of trimers and that this arrangement is conserved in CAD-like from fungi, which holds an inactive DHO-like domain. The crystal structures of the ATC trimer and DHO-like dimer from the fungus Chaetomium thermophilum confirm the similarity with the human CAD homologs. These results demonstrate that, despite being inactive, the fungal DHO-like domain has a conserved structural function. We propose a model that sets the DHO and ATC complex as the central element in the architecture of CAD.

INTRODUCTION

CAD is a 243 kDa polypeptide formed by the fusion of four enzymatic domains that initiate the de novo biosynthesis of pyrimidines (Figure 1A) (Coleman et al., 1977; Evans and Guy, 2004; Jones, 1980). The first two domains, glutaminase (GLN) and carbamoyl phosphate synthetase (CPS-II), initiate the pathway, catalyzing the formation of carbamoyl phosphate (CP) from bicarbonate, glutamine, and two ATP molecules. Next, the labile CP is partially channeled to the C-terminal aspartate transcarbamoylase (ATC) domain (Christopherson and Jones, 1980; Irvine et al., 1997; Mally et al., 1980), where it reacts with aspartate to form carbamoyl aspartate. Then, carbamoyl aspartate is condensed to dihydroorotate, the cyclic precursor of the pyrimidine ring, by the dihydroorotase (DHO), a Zn metalloenzyme fused between CPS and ATC domains.

The upregulation of CAD activity is essential to fuel the high demand of pyrimidines during cell growth and proliferation, and, thus, this multifunctional unit is under precise metabolic control (reviewed in Evans and Guy, 2004). CPS-II catalyzes the rate-limiting step of the pathway and its activity is allosterically inhibited by the end-product uridine-5’-triphosphate (UTP) and activated by PRPP (5-phosphoribosyl-1-H-pyrophosphate), a substrate for the synthesis of both purines and pyrimidines (Jones, 1980). The affinity for the allosteric effectors is modulated by phosphorylation of CPS-II through the MAP kinase and PKA cascades (Carrey et al., 1985; Graves et al., 2000). CAD is also activated by phosphorylation at the linker connecting the DHO and ATC domains through S6 kinase in the downstream of mTORC1 pathway (Figure 1A) (Ben-Sahra et al., 2013; Robitaille et al., 2013).

The initial steps of the de novo pyrimidine pathway are conserved in all organisms (Jones, 1980). However, the fusion of the first enzymatic activities into a single multifunctional unit is unique to animals. In fungi, GLN, CPS-II, and ATC are fused in a CAD-like protein (named URA2 in Saccharomyces cerevisiae) that contains an inactive DHO-like domain (Denis-Duphil, 1989). In these organisms, the DHO activity is encoded in a separate gene as a monofunctional enzyme (Denis-Duphil, 1989). In prokaryotes and plants on the other hand, GLN, CPS, ATC, and DHO are synthesized as distinct proteins that function separately or forming non-covalent complexes (Jones, 1980). The crystal structures of Escherichia coli CPS and of DHO and ATC from different bacteria and archaea have guided in the detailed description of their catalytic and regulatory properties (Lipscomb and Kantrowitz, 2011; Thoden et al., 1997, 2001; Zhang et al., 2009). In contrast, we lack structural information about CAD that allow us to understand the evolutionary advantage of the association of the distinct activities into a single multienzymatic protein (Davidson et al., 1993).

Early studies reported that purified CAD was a mixture of different oligomers (Coleman et al., 1977), and that the protein predominantly self-assembles forming hexamers of ~1.5 MDa,
Figure 1. DHO-ATC Forms Hexamers in Solution
(A) Graphical representation of CAD and SDS-PAGE of purified human DHO-ATC. Dashed lines represent proteolytic cleavage. The contaminant endogenous CAD is indicated by an arrowhead.
(B) SEC-MALS analysis of huDHO-ATC wild-type (black) and S1859E mutant (red). The molecular weight of the complexes is indicated.
(C) DHO and ATC activities of the proteins represented as mean ± SEM.
(D) SEC-MALS analysis of huDHO-ATC<sup>D2009A</sup> (green) and huDHO<sup>M1601E</sup>-ATC (purple) mutants.
(E) Schematic model of huDHO-ATC oligomers. The scissors represent nicking at the linker and the stars indicate site-specific mutations.
(F) SEC-MALS analysis of ctDHOlike-ATC. Sample homogeneity is shown on SDS-PAGE. See also Figures S1–S3.
nearly half the size of a ribosome (Lee et al., 1985). The high sensitivity of the linkers to proteolytic cleavage allowed the isolation and characterization of discrete functional domains. Different groups demonstrated that the isolated DHO and ATC domains form dimers and trimers, respectively, in solution (Davidson et al., 1981; Hemmens and Carrey, 1995; Kelly et al., 1986). These observations led to the general assumption that CAD hexamers could result from the association of three polypeptide chains through their respective ATC domains, followed by dimerization of two of these trimers through DHO-mediated interactions (Carrey, 1995). A similar organization was proposed for the CAD-like protein in the fungus Neurospora crassa, although in this model, the association of the ATC-mediated trimers would be facilitated by the formation of CPS-II dimers (Makoff et al., 1978). Confirmation of the central role of the ATC domain in the molecular architecture of mammalian CAD was provided by Qiu and Davidson (1998, 2000), who showed that mutations at the ATC trimer interface caused the dissociation of CAD hexamers into monomers. However, this study suggested that neither DHO nor CPS-II domains appear to participate directly in the oligomerization of CAD. Thus, the attractive idea that CAD might assemble as a “dimer of trimers” remains experimentally unchallenged.

In an attempt to get insight into the molecular architecture of CAD, we previously determined the crystal structures of the isolated DHO and ATC domains of human CAD (Grande-Garcia et al., 2014; Ruiz-Ramos et al., 2016). Whereas the human ATC structure corroborated the predicted similarity with bacterial ATC trimers, the quaternary structure of the human DHO dimer differed from the bacterial homologs, suggesting that the interactions with ATC, if any, in the CAD multimer would be different from the bacterial DHO/ATC complexes (Grande-Garcia et al., 2014).

In this study, we made a construct covering the human DHO and ATC domains, including the long inter-domain linker, and demonstrated that it forms hexamers in solution. Site-directed mutagenesis provided experimental evidence that the hexamer, indeed, assembles as a dimer of trimers. Furthermore, we proved that, despite having an inactive DHO domain, the CAD-like protein from fungus presents the same molecular organization. Building on conserved structural features observed in the DHO-like and ATC crystal structures of the fungus Chaetomium thermophilum, we propose a model that sets the DHO and ATC domains as the central supporting framework of CAD particles.

RESULTS

Human DHO-ATC Self-Assembles into Dimers of Trimers

We cloned a region of human CAD spanning the DHO and ATC domains, including the 91 amino acids linking both domains (Figures 1A and S1). The linker has a high content of prolines (22%) and harbors the phosphorylation site (S1859) for S6 kinase and PKA (Ben-Sahra et al., 2013; Carrey et al., 1985; Robitaille et al., 2013). The bifunctional construct (hereafter named huDHO-ATC; residues 1,456–2,225) was produced in HEK293 cells and migrated in SDS-PAGE at the expected position for a molecular weight (MW) of 84.7 kDa (Figure 1A). Proteolytic rupture of the linker resulted in a discrete number of 40–60 kDa truncated forms that accumulated quickly after overnight storage at 4°C. huDHO-ATC also co-purified with small amounts of a ~250 kDa protein, identified by mass spectrometry as endogenous human full-length CAD (Figures 1A and S1).

Size-exclusion chromatography coupled to multi-angle light scattering (SEC-MALS) analysis showed that huDHO-ATC elutes as a single peak with an average MW of 495 kDa (±0.13%) (Figure 1B), in good agreement with the self-assembly into hexamers. The polydispersity in the MW measurement is likely caused by internal oligomer flexibility, nicking of the linker, or incorporation of endogenous CAD.

The activities of huDHO-ATC were comparable with those of the isolated domains (Figure 1C). To measure the ATC activity, we introduced the inactivating mutation D1686N in the DHO domain to prevent the conversion of carbamoyl aspartate to dihydroorotate (Grande-Garcia et al., 2014). huDHO<sub>D1686N</sub>-ATC showed a k<sub>cat</sub><sub>ATC</sub> = 1,427 ± 71 min<sup>−1</sup>, 1.3-fold higher than huATC, whereas wild-type huDHO-ATC exhibited a k<sub>cat</sub><sub>DHO</sub> = 110 ± 10 min<sup>−1</sup>, similar to huDHO.

Next, we made the phospho-mimicking mutation S1859E to test if, as reported, the phosphorylation at the linker promotes the formation of larger CAD oligomers (Robitaille et al., 2013). The S1859E mutant was shown by SEC-MALS to form hexamers (MW of 470 kDa [±0.12%]), but we did not observe association into bigger multimers (Figures 1B and S2).

We also mutated the interfaces of the DHO dimers and ATC trimers to test the effect on the DHO-ATC hexamer. Previously, we proved that mutation M1601E hampers the dimerization of huDHO (Grande-Garcia et al., 2014), whereas Qiu and Davidson (1998, 2000) demonstrated that mutation D2009A dissociates the ATC trimers. SEC-MALS analysis showed that mutant huDHO<sub>M1601E</sub>-ATC eluted in a single peak with an estimated MW of 271 kDa (±0.33%), in good agreement with the formation of a trimer (Figures 1D, 1E, and S2). In turn, mutant huDHO-ATC<sub>D2009A</sub> eluted in a major peak of 182 kDa (±0.50%), indicating the association as dimers.

These results demonstrated that the covalently linked DHO and ATC domains self-assemble as dimers of trimers, although the association does not influence significantly on their kinetic properties.

C. thermophillum DHOlike-ATC Forms Hexamers

The susceptibility to proteolytic cleavage turned huDHO-ATC into a challenging target for structural studies. To explore other sources of CAD that could be more stable, we purchased a synthetic gene encoding the CAD-like protein from the fungus C. thermophillum (Bock et al., 2014) (Figure S3A), expected to have GLN-CPS and ATC activities bridged by an inactive DHO-like domain. The C. thermophillum DHO-like and ATC domains (hereafter ctDHOlike and ctATC) share 35% and 53% sequence identity with human. The sequence connecting both domains is 12 residues shorter and shares low homology (21%) with the human linker, although the high proline content (17%) suggested a conserved structural function (Figure S3B).

We produced an 80.8 kDa construct covering the ctDHOlike and ctATC domains including the linker region (hereafter named ctDHOlike-ATC; residues 1,519–2,253). The construct exhibits an ATC activity 2.5-fold higher than huDHO<sub>D1686N</sub>-ATC, and as
expected, it lacks DHO activity (Figure 1C). SEC-MALS analysis showed that ctDHOlike-ATC behaves as a single species with an estimated MW of 432 kDa (±0.19%) (Figures 1F and S2), indicating that, as observed in huDHO-ATC, the construct from fungus forms hexamers in solution.

We did not notice significant degradation after several days at 4°C (data not shown), and, thus, ctDHOlike-ATC was used in crystallization trials. We obtained two different crystal forms that turned out to contain exclusively either ctDHOlike or ctATC (Figure S4A). Although more stable than the human homolog, cleavage at the linker during the crystallization process (2–3 weeks at 18°C) led to the fortuitous determination of the first structures of the DHO-like and ATC domains from a fungus (Figure S4B).

Structure of the Inactive DHO-like Domain
Wing-shaped crystals diffracted X-rays to 2 Å resolution and belong to orthorhombic space group P2₁2₁2 with unit cell parameters a = 101 Å, b = 109 Å, and c = 58 Å (Table 1). Phases were obtained by molecular replacement using the structure of huDHO as search model (PDB: 4C6M; Grande-Garcia et al., 2014). The crystals had 37% solvent content and two ctDHOlike domains per asymmetric unit. The electron density was unambiguous for residues 1,519–1,855, with no extra density attributable to ctATC, and a model was refined to R and Rfree values of 19.89% and 23.20%, respectively (Table 1).

The two ctDHOlike proteins in the asymmetric unit are similar, with a root-mean-square deviation (RMSD) of 0.6 Å for 336 Cα atoms. The domain folds in an (α/β)₈ barrel (residues 1,526–1,772) with eight parallel β strands (named β₁ to β₈) connected to eight external α helices (α₁ to α₈) by loops of different length (loops 1 to 8) (Figures 2A and S4C). The barrel connects through two helices (α₆ and α₇) with an adjacent β stranded domain (residues 1,519–1,525 and 1,793–1,840) that clamps one side of the barrel. The overall fold is similar to huDHO, with both proteins superimposing with an RMSD of 1.8 Å for 311 Cα atoms (Figure S4D).

cTDHOlike, however, lacks the metal-coordinating and substrate-binding residues that, in human and bacterial homologs, shape the active site (Ruiz-Ramos et al., 2015; Thoden et al., 2001) (Figure 2B). The position of the Zn²⁺ atoms is partially taken by α helix a₅ (in loop 8) that protrudes from the center of the barrel and forms, together with loop 7 and the adjacent domain β hairpin, the top surface of the protein (Figures 2A–2C). The extensive hydrophobic interactions and the presence of a disulfide bridge between cysteines C1709 and C1712 at the tip of loop 7 suggest that these elements form a rigid structure (Figures 2B–2D).

### Table 1. Data Collection and Refinement Statistics

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>ctDHOlike</th>
<th>ctATC apo</th>
<th>ctATC CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beamline</td>
<td>ID30B, ESRF</td>
<td>XALOC, ALBA</td>
<td>XALOC, ALBA</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.93927 Å</td>
<td>0.919760 Å</td>
<td>0.919760 Å</td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁2₁2</td>
<td>P4₁32</td>
<td>P4₁32</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>100.8, 109.2, 58.3</td>
<td>138.6, 138.6, 138.6</td>
<td>139.4, 139.4, 139.4</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>48.00–2.13 Å (2.25–2.13)</td>
<td>49.00–2.26 Å (2.38–2.26)</td>
<td>49.30–1.96 Å (2.07–1.96)</td>
</tr>
<tr>
<td>Mosaicity</td>
<td>0.17</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>11.0 (98.4)</td>
<td>13.0 (273.9)</td>
<td>14.1 (341.4)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.8 (97.9)</td>
<td>100.0 (99.7)</td>
<td>99.8 (100.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>98.8 (97.9)</td>
<td>100.0 (99.7)</td>
<td>99.8 (100.0)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>6.5 (6.6)</td>
<td>38.5 (38.3)</td>
<td>39.0 (39.7)</td>
</tr>
<tr>
<td>CC₁/₂ (%)</td>
<td>99.8 (70.2)</td>
<td>100.0 (70.4)</td>
<td>100.0 (72.1)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>45.81–2.13 Å</td>
<td>49.02–2.26 Å</td>
<td>49.28–1.96 Å</td>
</tr>
<tr>
<td>No. of reflections</td>
<td>36,184</td>
<td>21,926</td>
<td>33,774</td>
</tr>
<tr>
<td>R factor/Rfree (%)</td>
<td>19.89/23.20</td>
<td>19.73/24.35</td>
<td>17.82/20.84</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.003</td>
<td>0.014</td>
<td>0.011</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>0.606</td>
<td>1.291</td>
<td>1.093</td>
</tr>
<tr>
<td>No. of atoms protein/solvent</td>
<td>5,240/199</td>
<td>2,535/54</td>
<td>2,537/176</td>
</tr>
<tr>
<td>Ramachandran plot (%)</td>
<td>96.59</td>
<td>94.44</td>
<td>97.12</td>
</tr>
<tr>
<td>Favored</td>
<td>3.11</td>
<td>5.56</td>
<td>2.88</td>
</tr>
<tr>
<td>Allowed</td>
<td>0.30</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

RMSD, root-mean-square deviation. Values in parentheses correspond to the highest-resolution shell.
Figure 2. Crystal Structure of the Inactive Fungal DHO-like Domain

(A) Cartoon representation of ctDHOlike structure in two perpendicular views. α Helices and β strands from the central barrel are colored green and red, respectively, whereas the adjacent domain is shown in orange. The dimer interface is indicated with a gray shadow.

(B) Detailed view showing the side chains of residues replacing Zn-coordinating and substrate-binding residues in active DHOs.

(C) Zoom-in view of the hydrophobic interactions between loop 7 (pink), loop 8 (blue), and the adjacent domain.

(D) Structure-guided sequence alignment of ctDHOlike and huDHO. Sequences are colored according to secondary structure. Zn-coordinating and substrate-interacting residues in huDHO are highlighted with yellow and brown backgrounds, respectively. Residues depicted in bold are highly conserved (90% identity) among animals (for huDHO) or fungi (for ctDHOlike). Residues buried in the dimerization surface are shown with gray background. The disulfide bridge at the tip of loop 7 is indicated in the alignment and shown in ball-and-sticks in the other panels. See also Figure S4.
It was uncertain whether or not the inactive DHO-like from fungi forms dimers in solution. Interestingly, the two ctDHO-like proteins in the asymmetric unit interact through a hydrophobic interface of 900 Å² formed by helices $\alpha_4$, $\alpha_5$, and $\alpha_6$, the same structural elements involved in the dimerization of huDHO (Figures 3 and 4A). To test if the crystal dimers also formed in solution, we produced the isolated ctDHO-like domain (residues 1,519–1,855, MW = 36.8 kDa) and a variant with the mutation L1660C to disrupt the interaction between the crossing $\alpha_5$ helices at the center of the putative dimer interface (Figures 4A and S2). SEC-MALS analysis proved that ctDHO-like forms homodimers in solution (MW = 69.3 kDa), whereas mutant L1660C is mostly monomeric (MW = 39.9 kDa) (Figure 4B). Together, these results demonstrate that the inactive DHO-like domain from fungi forms elongated (100–200 Å) dimers virtually identical to huDHO, with a flat bottom and an upper surface with rigid loops protruding as a two-pin plug.

To further test the role of the ctDHO-like dimers, we introduced the mutation L1660C in the ctDHO-like-ATC construct. SEC-MALS analysis showed that the mutant ctDHO-like$^{L1660C}$-ATC did not form hexamers but eluted in a single peak with an MW of 255 kDa, fitting the theoretical mass of a trimer (Figures 4C and S2). These results strongly suggest that, despite being inactive, the ctDHO-like domain plays a conserved architectural role in the assembly of CAD-like complex.

**Structure of Fungal ATC Free and Bound to CP**

A second ctDHO-like-ATC crystal type with a diamond shape grew in the absence and presence of CP, and diffracted X-rays to ~2 Å resolution (Table 1; Figure S4A). The crystals belong to cubic space group P4$_3$32 with unit cell dimensions $a = b = c = 139$ Å. Based on solvent content prediction, we estimated that the asymmetric unit was too small to fit one ctDHO-like-ATC subunit. Crystallographic phases were obtained by molecular replacement using huATC structure (PDB: 5G1N; Ruiz-Ramos et al., 2016) as search model. The electron density was unambiguously assigned to one ctATC subunit (residues 1,939–2,253) forming a trimer across the crystal 3-fold axis, with no additional density for ctDHO-like.

cTATC exhibits the conserved architecture of the transcarbamoylase superfamily, a dome-shaped trimer with three active sites at the interface between subunits at the concave face (Lipscomb and Kantrowitz, 2011; Shi et al., 2015) (Figure 5A). Each subunit in the trimer is divided into two domains of similar size with a central $\beta$ sheet of five parallel strands flanked by $\alpha$ helices (Figures 5A, 5B, and S5A). The N-terminal domain (N-domain, residues 1,939–2,088 and 2,237–2,253) provides most of the inter-subunit contacts and binds CP. The C-terminal domain (C-domain; residues 2,089–2,236) occupies an external position and holds the binding site for aspartate.

The crystal grown in presence of CP showed additional density at the active site that was unambiguously assigned to the bound substrate (Figure 5C). CP binds at the N-end of helix $\alpha_2$, interacting with the main chain N atoms of T2003 and T2005 and with the side chains of S2002, R2004, and T2005 (Figure 5C). The O and N atoms of the carbamoyl group make additional H-bonds with the side chains of T2005, H2081, and Q2084, and with the carbonyl oxygen of P2214 (the only residue from...
the C-domain). Binding of CP induces a hinge-closure between the N- and C-domains and the arrangement of the CP-loop (residues 2,022–2,035) from the adjacent subunit, which is partially unfolded in the apo structure (Figure 5B). The CP-loop interacts through the side chains of S2029 and K2032 with the phosphate group of CP (Figure 5C). In other ATCs, including huATC, the occupation of the aspartate site is reported to trigger a further hinge-bending of the two domains with closure of the Asp-loop (Ruiz-Ramos et al., 2016). In both ctATC structures, the two subunits, whereas V1985 establishes hydrophobic contacts with G1991 in the adjacent subunit. Also, the nearby R2019 (A1990 in huATC) makes two additional hydrogen bonds with Q2014 (A1985) and G2018 (G1989) from the adjacent subunit (Figure 5C). Altogether, these contacts that are not present in huATC might reinforce the association between ctATC subunits across the central channel.

**Analysis of DHO-ATC Particles by Electron Microscopy**

To get an insight into the molecular architecture of DHO-ATC hexamers we carried out negative-staining electron microscopy (EM). Initially, freshly purified huDHO-ATC and ctDHOlike-ATC samples were directly applied onto glow-discharged carbon-coated grids and negatively stained for EM analysis. The size and internal symmetry should make these complexes suitable targets for the EM approach. However, close examination of the EM micrographs showed particles with very different shapes and sizes that disallowed further structural analysis (Figure S6A). Likely, the preparation of diluted samples for EM promotes partial dissociation of the complexes resulting in a mixture of various sub-complexes. Indeed, we previously reported that huATC trimer undergoes partial dissociation at low protein concentrations (Ruiz-Ramos et al., 2016). The presence of truncated forms could also contribute to the heterogeneity of the sample, a limitation that appears more severe for the human protein (Figure 1A).

Since chemical crosslinking was successfully used to demonstrate the formation of CAD hexamers (Coleman et al., 1977; Lee et al., 1985), we next attempted to stabilize the ctDHOlike-ATC oligomers using mild chemical crosslinking with diluted solutions of glutaraldehyde. The crosslinked sample was purified by SEC and the elution fractions were used for negative staining. Visual inspection of EM fields from glutaraldehyde-stabilized protein showed a more homogeneous population of larger globular particles compared with a non-crosslinked sample (Figure S6B). We selected ~29,000 particles that were classified and processed to obtain reference-free averages for preferred views of the molecule. Characteristic 2D averages appeared as rectangular particles of approximately 190 × 100 Å with compact globular regions and fuzzy densities likely corresponding to flexible parts of the complex (Figure 6A). Despite crosslinking, the flexibility of the particles introduced sufficient conformational heterogeneity to hamper the reconstruction of a 3D model.
DISCUSSION

Evolutionary Advantages of a Multifunctional Protein

The characterization of a construct covering the DHO and ATC domains of human CAD combined with site-directed mutagenesis have allowed us to experimentally confirm that CAD self-assembles as a dimer of trimers. We also provided the first detailed structural information on the CAD-like protein in fungi, revealing the structural similarities between the inactive DHO-like dimers and the ATC trimers with the human homologs, and demonstrating that the fused domains also self-assemble into hexamers.

Figure 5. Crystal Structure of *C. thermophilum* ATC

(A) Cartoon representation of ctATC trimer bound to CP. One subunit is highlighted in purple background and the structural elements are labeled in another subunit. α Helices in the N- and C-domains are colored blue and green, respectively. CP and Asp loops are depicted in sand and orange, respectively.

(B) Ribbon superposition of apo- (gray) and CP-bound ctATC structures. CP-induced hinge movement is indicated with an arrow.

(C) Detailed view of CP binding. The electron density map for CP is shown as a red mesh. Dashed lines indicate relevant interactions.

(D) Structure-guided sequence alignment of *C. thermophilum* and human ATCs. Sequences are colored according to their secondary structure. Residues interacting with the substrates or with the inhibitor phosphonacetyl-L-aspartate (PALA) in huATC and equivalent residues in ctATC are shown on yellow background. See also Figures S4A, S4B, and S5.
prokaryotic counterparts (Davidson et al., 1993). In addition to facilitating the simultaneous production of the different proteins in stoichiometric amounts (Stark, 1977), the linkage ensures the co-localization and association between enzymes that would otherwise depend on strong and highly specific interactions to form a complex. Indeed, whereas in some bacteria ATC and DHO form stable non-covalent complexes (Zhang et al., 2009), we did not detect direct interactions between the isolated huATC and huDHO (data not shown) and, to the best of our knowledge, there is no experimental evidence on the formation of stable complexes between isolated CAD domains. Thus, we conclude that the covalent linkage of the DHO/DHO-like and ATC domains is required for the assembly of the conserved hexameric sub-complex, and, by extension, of the CAD and CAD-like particles. The fusion of the domains might also favor the folding or increase the integrity of individual components that would otherwise be unstable on their own. Indeed, in agreement with previous work, we noticed that the mammalian ATC domain can only be produced independently if it is N-terminal fused to the maltose binding protein or, as shown here, to the DHO domain (Figure 1A) (Qiu and Davidson, 1998; Ruiz-Ramos et al., 2013). On the other hand, we failed so far in producing the human or C. thermophilum CPS-II as an independent protein, regardless of the addition or not of the GLN domain (data not shown). GLN-CPS-II appears to be less stable on its own, as also suggested by limited proteolysis and complementation studies (Mally et al., 1981; Musmanno et al., 1992; Souciet et al., 1982). Thus, it is possible that the interactions within the multienzymatic particle stabilize the tertiary structure of GLN-CPS-II, and that this domain needs to be engrafted into the DHO-ATC framework to be functional.

Another advantage for the fusion into a multifunctional unit is that it may improve the metabolic efficiency of the pathway. The comparison of the overall CAD reaction with the partial steps suggested that the linked system is more efficient and responds faster to regulatory signals (Christopherson and Jones, 1980; Mally et al., 1980). Different studies in mammalian CAD and in yeast URA2 indicated that the CP released by CPS-II is partially channeled to the ATC active site without substantial exposure to the bulk solvent (Christopherson and Jones, 1980; Denis-Duphil, 1989; Mally et al., 1980; Otsuki et al., 1982; Penverne et al., 1994). The passage of CP requires a correct assembly of the domains and the integrity of the DHO-ATC linker (Guy and Evans, 1994), and is facilitated by reciprocal communication of conformational changes between the CPS-II and ATC domains (Irvine et al., 1997). Similarly, reciprocal conformational changes

Figure 6. EM Analysis and DHO-ATC Model
(A) Representative class averages of crosslinked and negatively stained cTDHOlike-ATC particles. (B and C) Selected 2D projections (B) filtered to 25 Å of (C), a DHO-ATC model formed by association of two ATC trimers (purple) and three DHO dimers (green). The fuzzy appearance and thick arrows illustrate the flexibility between the ATC and DHO domains. Movements around the 3- and 2-fold axes are indicated. The N- and C-ends of the domains are labeled. (D) Cartoon representation of human CPS-I dimer (PDB: 5DOU; de Cima et al., 2015). In one subunit, the GLN domain is colored yellow and the CPS regulatory subdomain is shown with salmon background. The position of the two phosphorylation sites is indicated with ADP molecules shown as spheres. The internal tunnel connecting the active sites is represented with a blue line. See also Figure S6.
between the CPS-II and ATC domains of URA2 have been proposed to explain the mechanism of allosteric feedback inhibition by UTP (Serre et al., 2004). We asked how the DHO-ATC complex reported in this study contributes to the CP channeling and to the communication of conformational changes between ATC and CPS-II domains.

**DHO-ATC as the Framework of CAD Particles**

Building on the schematic drawings of D. Evans (Evans, 1986) and E. Carrey (Carrey, 1995), we propose a model for the architecture of CAD that sets the DHO-ATC hexamer as the central element of the particle (Figures 6B and 6C). The model places two ATC trimers at the apical positions, with their concave faces facing each other. Three DHO dimers are interposed between the ATC trimers, with their long axes in parallel to the 3-fold axis and with the top of the ATC barrel oriented inwards. Thus, the linked domains associate in a closed architecture of the particle (Figures 6B and 6C). The model places the catalytic cycle (Lipscomb and Kantrowitz, 2011; Ruiz-Ramos et al., 2016) could be transmitted to the outer GLN-CPS-II dimers, explaining the reciprocal communication between both domains (Irvine et al., 1997). The concerted movements in the particle would modulate the rate and coupling of the different reactions, the channeling of intermediates, and the flux of substrates and products in and out the particle.

Our model ultimately suggests that CAD is more than the sum of its parts, and that the detailed study of the pieces will fall short in comparison with the complexity that will be disclosed by the structural determination of the full-length particle.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **CONTACT FOR REAGENT AND RESOURCE SHARING**
- **METHOD DETAILS**
  - Cloning of Constructs and Mutagenesis
  - Protein Expression
  - Protein Purification
  - Gel-Filtration Coupled to Multi-Angle Light-Scattering (SEC-MALS) Measurements
  - Enzymatic Assays
SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.str.2017.04.012.

AUTHOR CONTRIBUTIONS


ACKNOWLEDGMENTS

We thank Luisa Gleich for cloning of C. thermophilum constructs. X-ray diffraction experiments were performed at ALBA and ESRF Synchrotrons with the collaboration of beamline staff. This work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO; BFU2013-48365-P and BFU2016-80570-R) and the CNIO Intramural Program. M.M.-M. is a fellow of the Marie Curie/WHRI-Academy and Juan de la Cierva-Incorporation Program (MINECO). A.R.-R. was the recipient of an FPI PhD fellowship and F.d.C.-O. is a fellow of the Severo Ochoa Excellence Program, both funded by MINECO.


# STAR★METHODS

## KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial and Virus Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21 (DE3) pLysS <em>Escherichia coli</em> cells</td>
<td>Novagen</td>
<td>Cat# 70236</td>
</tr>
<tr>
<td><strong>Chemicals, Peptides, and Recombinant Proteins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Dihydroorotic acid</td>
<td>SIGMA ALDRICH</td>
<td>Cat# D7128-500MG</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>SIGMA ALDRICH</td>
<td>Cat# A7219-100G</td>
</tr>
<tr>
<td>Lithium Carbamoyl Phosphate</td>
<td>SIGMA ALDRICH</td>
<td>Cat# C5625-10MG</td>
</tr>
<tr>
<td>Carbamoyl-L-Aspartate</td>
<td>CYMIT QUIMICA S.L</td>
<td>Cat# 01-M-2240.0001-1G</td>
</tr>
<tr>
<td>Crystal Screen</td>
<td>Hampton</td>
<td>Cat# HR2-110</td>
</tr>
<tr>
<td>Crystal Screen 2</td>
<td>Hampton</td>
<td>Cat# HR2-112</td>
</tr>
<tr>
<td>JCSG+</td>
<td>Qiagen</td>
<td>Cat# 130720</td>
</tr>
<tr>
<td>PACT</td>
<td>Qiagen</td>
<td>Cat# 130718</td>
</tr>
<tr>
<td>Pro-Complex</td>
<td>Qiagen</td>
<td>Cat# 130715</td>
</tr>
<tr>
<td>pHClear Suites</td>
<td>Qiagen</td>
<td>Cat# 130709</td>
</tr>
<tr>
<td>pHClear II Suites</td>
<td>Qiagen</td>
<td>Cat# 130710</td>
</tr>
<tr>
<td>Wizard I/II</td>
<td>Molecular Dimensions</td>
<td>Cat# MD15-W12-T</td>
</tr>
<tr>
<td>MRC 48-well sitting-drop plates</td>
<td>Hampton</td>
<td>Cat# HR3-180</td>
</tr>
<tr>
<td>Polyethylenimine, 25 kDa branched</td>
<td>SIGMA ALDRICH</td>
<td>Cat# 408727-100ML</td>
</tr>
<tr>
<td>FreeStyle™ 293 Expression Medium</td>
<td>Gibco</td>
<td>Cat# BE12-723F</td>
</tr>
<tr>
<td>UltraDOMA serum-free</td>
<td>Lonza</td>
<td>Cat# BE12-723F</td>
</tr>
<tr>
<td><strong>Critical Commercial Assays</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-Fusion® HD Cloning Kit</td>
<td>Takara Bio USA, Inc. (Clontech)</td>
<td>Cat# E39648</td>
</tr>
<tr>
<td>Phusion® High-Fidelity DNA Polymerase</td>
<td>New England Biolabs</td>
<td>Cat# M0530S</td>
</tr>
<tr>
<td><strong>Deposited Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ctATC</td>
<td>This paper</td>
<td>PDB: SNNN</td>
</tr>
<tr>
<td>ctATC (with CP)</td>
<td>This paper</td>
<td>PDB: SNNQ</td>
</tr>
<tr>
<td>ctDHOlike</td>
<td>This paper</td>
<td>PDB: SNNL</td>
</tr>
<tr>
<td><strong>Experimental Models: Cell Lines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEK293S GnTI⁺</td>
<td>ATCC</td>
<td>Cat# CRL-3022</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers for huDHO, see Table S1</td>
<td>Lalrous et al., 2012</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for huATC, see Table S1</td>
<td>Ruiz-Ramos et al., 2013</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for ctDHOlike and ctATC, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for D1668N mutation, see Table S1</td>
<td>Grande-Garcia et al., 2014</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for M1601E mutation, see Table S1</td>
<td>Grande-Garcia et al., 2014</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for D2009A mutation, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for S1859E mutation, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for L1660C mutation, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Recombinant DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cDNA human CAD (clone ID 5551082)</td>
<td>Open Biosystems</td>
<td>Cat# MHS1010-9206052</td>
</tr>
<tr>
<td>Synthetic gene C. thermophilum CAD-like</td>
<td>GenScript</td>
<td>N/A</td>
</tr>
<tr>
<td>Plasmid: pOPIN-M</td>
<td>Oxford Protein Production Facility</td>
<td><a href="https://www.oppf.rc-harwell.ac.uk/OPPF/">https://www.oppf.rc-harwell.ac.uk/OPPF/</a></td>
</tr>
<tr>
<td>Plasmid: pOPIN-F</td>
<td>Oxford Protein Production Facility</td>
<td><a href="https://www.oppf.rc-harwell.ac.uk/OPPF/">https://www.oppf.rc-harwell.ac.uk/OPPF/</a></td>
</tr>
<tr>
<td>huDHO-pOPIN-M</td>
<td>Lalrous et al., 2012</td>
<td>N/A</td>
</tr>
</tbody>
</table>

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Santiago Ramón-Maiques (santiago.ramon@cbm.csic.es).

METHOD DETAILS

Cloning of Constructs and Mutagenesis

cDNA of human full-length CAD (UniProt P27708) was purchased from Open Biosystems (clone ID 5551082). Chaetomium thermophilum CAD-like (ctCAD) sequence was retrieved from the genome resource (http://ct.bork.embl.de/) and the synthetic gene, optimized for human and insect cells expression, was purchased from GenScript. Gene fragments covering the region of interest were amplified by PCR using Phusion High-Fidelity DNA Polymerase (New England Biolabs). Specific pairs of oligonucleotides used for each construct are detailed in Table S1. Both, the forward and the reverse primers contained specific regions (underlined in Table S1) for cloning into expression vectors from the pOPIN series (Oxford Protein Production Facility) using In-Fusion technology (Clontech). huDHO and huATC domains were inserted into pOPIN-M and huDHO-ATC, ctDHOlike and ctDHOlike-ATC into pOPIN-F. The resulting plasmids were verified by sequencing.

Site-directed mutagenesis was carried out by overlap extension PCR (Ho et al., 1989) using mutagenic primers and the corresponding flanking primers. The mutated genes were inserted into a pOPIN vector with In-Fusion cloning kit (Clontech) (Table S1).
Protein Expression

huDHO-ATC was expressed in HEK293S-GnTI- cells adapted to suspension culture in Freestyle medium (Gibco) with 1% fetal bovine serum and grown in an orbital stirrer at 125 rpm under standard humidified conditions (Grande-Garcia et al., 2014). The culture (1.5 million cells/ml) was transfected with a pOPIN-F vector carrying the huDHO-ATC, in a 1:3 ratio mixture of DNA (1 μg/ml) and polyethyleneimine (PEI 25 kDa branched; Sigma). Prior to the transfection, DNA and PEI were diluted to 20 and 60 μg/ml, respectively, in UltraDOMA medium (Lonza) and incubated separately for 5 min at room temperature. Then, the solutions were mixed and incubated 10 min before adding the mixture to the cells. Transfected cultures were harvested by centrifugation after 2–3 days of incubation, washed with PBS and stored at −80°C for further use.

Protein expression of the fungi constructs was performed in BL21 (DE3) pLysS Escherichia coli cells (Novagen) grown at 37°C in LB medium supplemented with 100 μg ml⁻¹ ampicillin and 34 μg ml⁻¹ chloramphenicol to mid-exponential phase (OD₆₀₀ = 0.6–0.8). Protein expression was induced with 0.8 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubated 16 h at 20°C. Cells were harvested by centrifugation and stored at −80°C.

huATC was expressed as detailed in (Ruiz-Ramos et al., 2013). In summary, transformed BL21 (DE3) pLysS E. coli cells grew under shaking conditions in autoinduction media (Studier, 2005) supplemented with 100 μg ml⁻¹ ampicillin and 34 μg ml⁻¹ chloramphenicol for 6 h at 37°C and then transferred to 20°C for 21 h.

In all cases, mutants were expressed as the corresponding wild-type proteins.

Protein Purification

The cell pellet was thawed and resuspended in buffer A [20 mM Tris-HCl pH 8.0, 0.5 M NaCl, 10 mM imidazole, 5% glycerol, 2 mM β-mercaptoethanol and 2 mM phenylmethanesulfonyl fluoride (PMSF)]. The HEK293 cells were mechanically disrupted using a dounce homogenizer Potter-Elvehjem followed by brief sonication, whereas bacterial cells were directly sonicated. The lysate was clarified by centrifugation in a Beckman JA-25.50 rotor at 50000 × g for 45 min at 4°C. The supernatant was filtered through a 0.45 μm pore filter and loaded onto a 5 ml HisTrap FF column (GE Healthcare, USA). After extensively washing with buffer A supplemented with 35 mM imidazole, proteins were eluted in a single step by increasing the imidazole concentration to 250 mM.

huDHO-ATC, huDHO and ctDHOlike were dialyzed overnight against buffer A with 35 mM imidazole, in presence of GST-tagged PreScission protease (Bowman et al., 2004) (in a relation of 1/20th of the protein weight), and passed through two connected 5 ml HisTrap and GSTrap FF columns (GE Healthcare) equilibrated in buffer A. After tag removal, the unbound proteins were concentrated up to 1-1.7 mg ml⁻¹ using the Amicon Ultra system with a 30 kDa cut-off membrane for huDHO-ATC and 10 kDa for huDHO and ctDHOlike.

In turn, the ctDHOlike-ATC sample eluted from the first affinity step was diluted to 0.15 M NaCl and loaded onto a 5 ml HiTrap Q HP column (GE Healthcare) equilibrated in buffer B (20 mM Tris pH 8.0, 0.15 M NaCl, 5% glycerol, 1 mM DTT). The protein recovered from the flow-through was digested overnight with GST-tagged PreScission under mild rocking conditions and concentrated up to 12-20 mg ml⁻¹ in an Amicon Ultra-system with a 30 kDa cut-off membrane.

huATC was produced as reported (Ruiz-Ramos et al., 2013). The protein was eluted from the 5 ml HisTrap FF column by increasing the imidazole concentration to 300 mM. Dialysis and tag cleavage was performed as above but in buffer S (Tris HCl pH 7.0, 0.075 M NaCl, 5% glycerol, 2 mM β-mercaptoethanol). The dialyzed and cleaved sample was applied to a 5 ml Hitrap SP HP column (GE Healthcare, USA) equilibrated in buffer S. The cleaved huATC elutes at ~0.09 M NaCl in a linear gradient of salt. The fractions containing the protein were pooled and concentrated up to ~3 mg ml⁻¹ in an Amicon Ultra system with a 10 kDa cut-off membrane. Then, the sample was loaded onto a HiTrap Heparin column (GE Healthcare) equilibrated in buffer S and eluted in a linear NaCl gradient.

Proteins were further purified by size exclusion chromatography on a Superose 6 10/300 column for the two-domain proteins or on a Superdex 200 10/300 column for the DHO domains, both equilibrated in buffer B at a flow rate of 0.5 ml/min. huATC was loaded onto a Superdex 75 10/300 column equilibrated in 20 mM Tris HCl pH 7.0, 0.1 M NaCl, 2% glycerol and 1 mM DTT. Fractions containing the protein were pooled, concentrated and directly used for further studies, or alternatively, supplemented with 20% glycerol (except for huATC that is supplemented with 30% DMSO), flash-frozen in liquid nitrogen and stored at -80°C. Purification of the huDHO-ATC<sup>D2009A</sup>, huDHO<sup>1160C</sup>-ATC, huDHO<sup>51689E</sup>-ATC, huDHO<sup>16886N</sup>-ATC, ctDHOlike<sup>1160C</sup> and ctDHOlike<sup>1160C</sup>-ATC mutants were performed as for the corresponding wild-type constructs. All purification steps were carried out at 4°C. Sample purity was evaluated by SDS-PAGE and Coomassie staining. Protein concentration was measured by absorbance at 280 nm using a NanoDrop ND-1000 spectrophotometer (ThermoFisher). Bradford method was used to measure huATC concentration (Bradford, 1976).

Gel-Filtration Coupled to Multi-Angle Light-Scattering (SEC-MALS) Measurements

For molar-mass determination, 400 μl of purified protein at 1 mg ml⁻¹ were fractionated in a Superose 6 10/300 (two-domain constructs) or in a Superdex 200 10/300 (ctDHOlike and ctDHOlike<sup>1160C</sup>) column equilibrated in buffer B using an AKTA purifier at a flow rate of 0.5 ml min⁻¹. ctDHOlike-ATC and ctDHOlike<sup>1160C</sup>-ATC were also injected at ~10 mg ml⁻¹. The eluted samples were 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 analysed using the ASTRA 6 software to obtain the molar mass (Wyatt, 1993) and plotted with GraphPad.
Enzymatic Assays

DHO activity of the huDHO, huDHO-ATC and ctDHOlike-ATC domains was measured spectrophotometrically following the production of dihydroorotate by absorbance at 230 nm (230, 1.17 mM⁻¹cm⁻¹) in a Jasco-V550 spectrophotometer at 25 °C (Sander et al., 1985). Proteins (0.5 μM) were assayed in buffer containing 50 mM sodium phosphate pH 5.5, 0.15 M NaCl, 0.2 mM TCEP, 20 μM ZnSO₄, 0.1 mg ml⁻¹ BSA and 5 mM CASP, in a final volume of 100 μl (Grande-Garcia et al., 2014).

ATC activity of huATC, huDHO D1686N-ATC and ctDHOlike-ATC was assayed by a colorimetric method (Prescott and Jones, 1969) adapted to a 96-well plate format (Eise and Hervé, 1990). Reactions were carried out in 50 mM Tris-acetate pH 8.3 and 0.1 mg ml⁻¹ BSA in a final volume of 150 μl as previously reported (Ruiz-Ramos et al., 2016). Proteins at varying concentrations (0.02 – 0.16 μM) were pre-incubated with 10 mM Asp for 10 min in a water bath at 25 °C. The reaction was triggered by adding 5 mM CP and stopped after 1, 3, 5, 10, 15 min with 100 μl of colour solution. The plate was shielded with film, heated at 95 °C for 15 min, and kept in the dark at room temperature for 30 min before measuring the absorbance at 450 nm in a Victor 1420 Multilabel Counter (Perkin Elmer).

Data analysis of both enzymatic assays was done with GraphPad.

Crystallization

Crystallization screenings were performed at 18 °C using a Cartesian MicroSys robot (Genomic Solutions) and the sitting-drop vapour-diffusion method in 96-well MRC plates (Hampton). Nanodrops consisting of 0.2 μl protein solution plus 0.2 μl reservoir solution were equilibrated against 70 μl of reservoir. Initial screening involved different protein concentrations and the commercial crystallization screens JCSPG+, PACT, Pro-Complex, pHClear and pHClear II Suites (Qiagen), Wizard II (Molecular Dimensions) and Crystal Screen (Hampton). Initial hits obtained from purified ctDHOlike-ATC at ~5 mg ml⁻¹ were further optimized in MRC 48-well sitting-drop plates (Hampton). Well-diffracting diamond-shaped crystals appeared after 10-15 days in 0.8 M succinic acid pH 7.0 and also in 0.5 M ammonium sulphate, 0.1 M NaCl and 0.1 M Tris pH 8.5 and reached a maximum size of 142 x 100 x 100 μm³. Wing-shaped crystals grew after 2-3 weeks in 20% PEG 20000 and 0.1 M citric acid pH 4.5 with a maximum size of 200 x 50 x 10 μm³. In this last condition, only one crystal diffracted below 4 Å. Cryo-protection was reached by directly soaking the crystals in a solution containing the mother liquor supplemented with 25% or 20% glycerol for diamonds and wing-shaped crystals, respectively. Crystals were flash-frozen in liquid nitrogen. CP-bound crystals were obtained by 10 min soaking in cryo-protecting solution plus 2 mM CP.

Data Collection and Structure Determination

X-Ray diffraction data were collected at XALOC (ALBA, Barcelona) and ID30B (ESRF, Grenoble) beamlines using Pilatus 6M detectors. For each set, a total wedge of 180° of data was collected with 1° oscillation and 1 s exposure per frame. Data processing and scaling were performed with XDS (Kabsch, 2010) and AIMLESS (Winn et al., 2011). Crystallographic phases were determined by molecular replacement using PHASER (McCoy et al., 2007) and huATC (PDB: 5G1N) and huDHO (PDB: 4C6M) as search models. ctATC and ctDHOlike models were built with Coot (Emsley et al., 2010) and refined with REFMAC (Vagin et al., 2004) or PHENIX (Adams et al., 2010). The figures of the structural models were prepared with Pymol (The PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC.).

Mass Spectrometry

SDS-PAGE bands were excised and proteins were reduced with 15 mM TCEP and carbamidomethylated with 30 mM chloroacetamide in 50 mM ammonium bicarbonate (ABC) for 45 min at 25 °C protected from light and, subsequently digested with Trypsin (1:50 enzyme / protein w/w) overnight at 37 °C. Resulting peptides were desalted by home-made C18 Empore tips and analysed by LC-MS/MS onto a Qq-TOF IMPACT Bruker. The raw files were processed using the Proteome Discoverer 1.4.0.1 software. Fragmentation spectra were searched against the Human Uniprot Database containing canonical and manual curated sequences (20599 entries), supplemented with a home-made database comprising the contaminant proteins most commonly found in our assays, using Sequest as the search engine. The precursor and fragment mass tolerances were set to 25 ppm and 0.075 Da, respectively, and up to two tryptic missed cleavages were allowed. Carbamidomethylation of cysteine was considered as fixed modification, while oxidation of methionine, and phosphorylation of serine, threonine and tyrosine and monoacetylation of lysine were chosen as variable modification for database searching. The results were filtered to 1% FDR using percolator.

Electron Microscopy Data Collection and Image Processing

For negative staining, 300 μl of the ctDHOlike-ATC subcomplex at 0.45 mg ml⁻¹ were crosslinked with 0.05% glutaraldehyde at 4 °C for 1 h in 20 mM HEPES pH 8.0, 0.15 M NaCl and 0.5 mM TCEP. The reaction was quenched with 1 M Tris pH 8.0 for 10 min at 4 °C and applied to a Superose 6 10/300 GL column. For grids preparation, 3.5 μl of the central fraction from the main elution peak at 3.5 m3.C protected from light and, subsequently digested with Trypsin (1:50 enzyme / protein w/w) overnight at 37 °C. Resulting peptides were desalted by home-made C18 Empore tips and analysed by LC-MS/MS onto a Qq-TOF IMPACT Bruker. The raw files were processed using the Proteome Discoverer 1.4.0.1 software. Fragmentation spectra were searched against the Human Uniprot Database containing canonical and manual curated sequences (20599 entries), supplemented with a home-made database comprising the contaminant proteins most commonly found in our assays, using Sequest as the search engine. The precursor and fragment mass tolerances were set to 25 ppm and 0.075 Da, respectively, and up to two tryptic missed cleavages were allowed. Carbamidomethylation of cysteine was considered as fixed modification, while oxidation of methionine, and phosphorylation of serine, threonine and tyrosine and monoacetylation of lysine were chosen as variable modification for database searching. The results were filtered to 1% FDR using percolator.

For negative staining, 300 μl of the ctDHOlike-ATC subcomplex at 0.45 mg ml⁻¹ were crosslinked with 0.05% glutaraldehyde at 4 °C for 1 h in 20 mM HEPES pH 8.0, 0.15 M NaCl and 0.5 mM TCEP. The reaction was quenched with 1 M Tris pH 8.0 for 10 min at 4 °C and applied to a Superose 6 10/300 GL column. For grids preparation, 3.5 μl of the central fraction from the main elution peak at ~0.05 mg ml⁻¹ were applied onto a freshly glow-discharged carbon-coated 400 mesh copper EM grids and incubated 10 sec at room temperature. The grids were sequentially laid on top of three distinct 50 μl drops of MilliQ water, striped gently for 2 sec and laid on the top of two distinct 50 μl drops of 1% uranyl acetate and stained for 1 min, striped gently for 10 sec and air dried. The grids were visualized on a Tecnai 12 transmission EM (FEI, Netherlands) with Lanthanum hexaboride cathode operated at 120 keV. Images were recorded under low-dose conditions (10-20 e⁻/Å²) at 61,320x nominal magnification on a 4kx4k TVIPS TemCam-F416 CMOS camera resulting in a final pixel size at the specimen level of 2.5 Å. Particle selection was performed semi-automatically with e2boxer.py implemented in EMAN (Tang et al., 2007). 33,754 particles were used for reference-free 2D
classification and averaging with refine2d.py implemented in EMAN and CL2D from the Xmipp package (Scheres et al., 2008). A large number of class averages were produced with around 50 particles in each class. 2D averages were visually inspected and used for generating a sub-data set with best 28,937 particles.

The ctDHOlike-ATC model was built from the ctATC, ctDHOlike and human CPS-I (PDB: 5DOU) structures using Chimera (Petersen et al., 2004).

**DATA AND SOFTWARE AVAILABILITY**

All software used for this work are indicated in the Key Resources Table and in the Method Details. The coordinates and structure factors of ctATC, ctATC-CP and ctDHOlike crystal structures have been deposited in the Protein Data Bank under codes 5NNN, 5NNQ and 5NXL.