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Crystal structures of human CtBP in complex with substrate MTOB reveal active site features useful for inhibitor design

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Abstract

The oncogenic corepressors C-terminal Binding Protein (CtBP) 1 and 2 harbor regulatory D-isomer specific 2-hydroxyacid dehydrogenase (D2-HDH) domains. 4-Methylthio 2-oxobutyric acid (MTOB) exhibits substrate inhibition and can interfere with CtBP oncogenic activity in cell culture and mice. Crystal structures of human CtBP1 and CtBP2 in complex with MTOB and NAD⁺ revealed two key features: a conserved tryptophan that likely contributes to substrate specificity and a hydrophilic cavity that links MTOB with an NAD⁺ phosphate. Neither feature is present in other D2-HDH enzymes. These structures thus offer key opportunities for the development of highly selective anti-neoplastic CtBP inhibitors.

Keywords

Cancer target; Dehydrogenase; NADH; Transcriptional coregulator

1. Introduction

The paralogous transcription coregulators C-terminal Binding Proteins (CtBP) 1 and 2 are critical modulators of numerous cellular processes; overexpression of both has been linked to multiple human cancers. CtBP1 was initially identified due to its interaction with the C-terminal region of the adenovirus E1A oncoprotein and modulation of E1A transforming activities [1,2]. CtBP functions as a transcriptional regulator by tethering chromatin remodeling proteins, such as histone deacetylases, histone methyl transferases, and histone demethylases, to DNA bound transcription factors [3,4]. Alternative splice forms of CtBP 1 and 2 also have non-nuclear functions in the Golgi and at synapses [5]. CtBP 1 and 2 are unique among transcription factors in the incorporation of a D-isomer specific 2-hydroxyacid dehydrogenase (D2-HDH) domain, which reduces or oxidizes substrates

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utilizing coenzyme NAD(P)⁺/NAD(P)H [6]. The D2-HDH family (Fig. S1), which is not otherwise involved in transcriptional regulation, includes bacterial D-lactate dehydrogenase (D-LDH) and D-hydroxyisocaproate dehydrogenase (D-HicDH) and, importantly for the development of inhibitors specific for human CtBP, the human enzymes glyoxylate reductase / hydroxypyruvate reductase (GRHPR) and phosphoglycerate dehydrogenase (PHGDH) [7–10]. CtBP recruitment of coenzyme NAD⁺ or NADH induces dimerization[11], an event necessary for transcriptional repressor activity, linking enzymatic function and transcriptional activity [12]. With a reported 100 fold higher affinity for NADH than NAD⁺ [13], CtBP may be able to respond to the redox state of the cell, increasing repressor function when stimuli, such as hypoxia and high extracellular glucose levels, increase the NADH/NAD⁺ ratio [14,15].

Mounting evidence implicates CtBP repressor function in human cancer. CtBP corepression activity targets pro-apoptotic factors (Bik, Noxa), cytoskeletal/cell adhesion molecules (keratin-8, E-cadherin)[16], and tumor suppressors (p16^{INK4a}, p15^{INK4b})[4], facilitating the epithelial to mesenchymal transition (EMT), conferring resistance to apoptosis and promoting metastasis and oncogenesis [17]. CtBP is targeted for degradation by individual or combined effects of multiple tumor suppressors including APC [18,19], HIPK2[20], JNK1[21], and ARF [22]. Consistent with these cellular effects of CtBP, overexpression of CtBP is observed in the majority of human colon, prostate, ovarian, and breast cancers [23–27].

A substrate for CtBP catalysis, 4-Methylthio 2-oxobutyric acid (MTOB) antagonizes CtBP transcriptional regulation [23,27,28]. MTOB induced apoptosis through displacement of CtBP2 from the *Bik* promoter in HCT116 colon cancer cells [23]. Administering MTOB in mouse xenograft models resulted in decreased tumor burden and prolonged survival for MTOB treated mice compared with untreated mice [23]. Additionally, MTOB evicted CtBP from target promoters in breast cancer cell lines, shifting phenotypic indicators (E-cadherin/vimentin) from mesenchymal to a more epithelial phenotype [27]. Although high MTOB concentrations (mM) are required for (substrate) inhibition of CtBP, MTOB's clear inhibitory effect on cancer cells provides proof of principle that small molecules could be developed to effectively treat cancers specifically regulated by CtBP activity.

Crystal structures of both CtBP 1 and 2 have been reported [29–31], but none are in complex with potential substrates. We report here crystal structures for both human CtBP1 (28–353) and CtBP2 (33–364) minimal dehydrogenase domains in ternary complexes with coenzyme NAD⁺ and ligand MTOB. These structures reveal unique active site CtBP features, including a prominent tryptophan and a hydrophilic channel, which may be useful for the design of highly selective inhibitors.

2. Materials and Methods

2.1. Protein expression and purification

As described more fully in the Supplementary material, both CtBP 1 and 2 were expressed as His₆-tagged proteins in BL21-CodonPlus® (DE3)-RIL competent cells (Stratagene). Both proteins were purified using NiNTA beads (Qiagen) followed by size-exclusion on a

Superdex 75 column. The His₆ tag was cleaved from CtBP2 with Thrombin (Novagen) prior to the size exclusion step, but was not cleaved from CtBP1.

2.2. Crystallization of ternary CtBP2/NAD⁺/ MTOB complex

Protein (20–25 mg/mL) incubated with a 50 molar excess of MTOB overnight at 4 °C was mixed in a 2:1 ratio with buffer and crystallized by hanging drop vapor diffusion in 24 well VDX plates. The highest quality crystals were grown in buffer containing 200 mM, potassium nitrate, 15–20% PEG 3350, and 100 mM bis tris propane pH 7.0. Crystals typically grew as multiple joined plates; microseeding resulted in large single plates suitable for diffraction. Crystals were cryoprotected by submersion in mother liquor supplemented with 20% ethylene glycol for 5–10 s and then flash frozen in liquid nitrogen.

2.3. Crystallization of ternary CtBP1/NAD⁺/ MTOB complex

Protein (~10 mg/mL) was mixed with a 50 molar excess of MTOB immediately before hanging drops were setup. Bipyramidal crystals grew overnight at room temperature in 200–300 mM magnesium chloride, 0–140 mM sodium formate, 100 mM hepes pH 7.5, and 2.5 mM NAD⁺. Crystals were cryoprotected by adding well buffer solution supplemented with increasing amounts of glycerol. Once the drop containing the crystal reached 20% v/v glycerol, the crystal was moved to 30% glycerol for 5 seconds and flash frozen in liquid nitrogen.

2.4. Data Collection and Structure Solution

Diffraction data for the CtBP1/NAD⁺/MTOB and CtBP2/NAD⁺/MTOB complexes were collected on the BioCARS 14-BM-C beamline at the Advanced Photon Source of Argonne National Laboratory. The initial models were obtained by molecular replacement with PhaserMR [32]. For CtBP1, the binary complex (1MX3[29]) was used as a search model, whereas for CtBP2, individual cofactor and substrate binding domains of the binary CtBP2 complex (2OME) were used as described in the Supplementary material. Water molecules were automatically generated by Arp/Warp[33], and the structure was refined with RefMac5 [34]. All other structures were refined through PHENIX [35]. Model building between rounds of refinement was performed with Coot [36]. The atomic coordinates and structure factor amplitudes of CtBP1 and CtBP2 with bound MTOB and NAD⁺ have been deposited in the RCSB Protein Data Bank, www.rcsb.org, with accession numbers 4LCE and 4LCJ, respectively

3. Results and discussion

3.1. Overall CtBP/MTOB/NAD⁺ structures

As with previous binary crystal structures, CtBP1 crystallized with a single monomer per asymmetric unit and the molecular dyad of the physiological dimer (Fig. S2) coincident with a two-fold crystallographic axis of space group P6₄22. The CtBP2 asymmetric unit contains eight monomers, arranged as four dimeric pairs. For full crystallographic statistics, see Table 1.

MTOB binds into an active site cleft between the discontinuous substrate binding domain (28–120; 327–353) and coenzyme binding domain (125–319) (Fig. 1a). Alignment with previous binary structures and distance difference matrix plots (Supplementary data) reveal only minor differences in Ca positions upon MTOB binding to both CtBP1 and CtBP2, with rms deviations of 0.2–0.3 Å for monomer superpositions and 0.2–0.4 Å for dimer superpositions. Thus, MTOB does not induce overall tertiary or quaternary change. These results argue against ligand-linked conformational changes as driving transcriptional modulation. One possibility is that substrate turnover, generating NAD⁺ whose release may drive dimer dissociation, might underlie the transcriptional inhibition of CtBP by MTOB. An additional possibility is that, conversely, inhibition of NADH to NAD⁺ conversion at high MTOB concentrations (via substrate inhibition) may block transcriptional activities that could require cycling between dimer and monomer forms that would be expected at physiologic MTOB and NADH/NAD⁺ concentrations.

3.2. Stereochemistry of MTOB Binding

MTOB is positioned in the active site similarly to ligands in other members of the D2-HDH protein family of proteins [7,10,37]. MTOB binds between an active site loop from the substrate binding domain and catalytically conserved residues Arg and His of the coenzyme binding domain, while simultaneously positioned adjacent to the NAD⁺ nicotinamide ring (Fig. 1). Thus, the position is consistent with MTOB as a potential substrate that can be reduced by NADH.

Given the resolution of the crystallographic data (CtBP1 2.38 Å, CtBP2 2.86 Å), particular care was taken in modeling MTOB (Supplementary material). Electron density clearly allows placement of MTOB, with highest density levels at the MTOB sulfur (S8) and along the C2-C4 bond (see Fig. 1b for atom designations) in the α-keto acid moiety.

In both CtBP1 and CtBP2, the F_o-F_c maps and refined B-factors indicate that MTOB does not occupy all active sites in the crystal. For CtBP1, best crystallographic results were obtained with an occupancy of 67% (Supplementary material). For CtBP2, the quality of the 2F_o-F_c density varies across the eight monomers, ranging from excellent to poor, but in all monomers, density was present for the MTOB sulfur atom (S8) and at least some of the α-keto acid moiety (Supplementary data). Omit F_o-F_c density maps confirm correct MTOB placement in CtBP2, yielding high confidence of MTOB conformation in monomers A, C and H. The occupancies of MTOB were refined in PHENIX to 79% in monomers A and C, and these were utilized for analysis of binding stereochemistry.

MTOB forms favorable hydrogen bonds in the CtBP active site similar to those observed for substrate binding in other D2-HDHs (Table S1). The MTOB pyruvate moiety hydrogen bonding in CtBP2 more closely matches other D2-HDHs than it does in CtBP1, due to an unusual twist of the MTOB carboxylate in CtBP1 (Fig. 2). The catalytically conserved Arg266 (CtBP1)/272(CtBP2) and His315/321 from the coenzyme binding domain provide two hydrogen bonds in CtBP1 and three hydrogen bonds in CtBP2. The backbone amides in a flexible loop in the substrate-binding domain provide two more hydrogen bond donors, helping bridge the active site cleft. In CtBP1, Arg97 reaches deeper into the active site than the homologous Arg103 of CtBP2, donating a unique hydrogen bond and increased

coulombic interaction to the MTOB carboxylate. Therefore, a network of four fully conserved hydrogen bonds, with additional variable hydrogen bonds, anchors MTOB in the active sites in CtBP1 and CtBP2.

Analysis of van der Waals interactions between MTOB and CtBP (Fig. S10) reveals that the MTOB α -keto group contacts CtBP similarly to substrates in other liganded D2-DHDs. A striking difference, however, is the presence of smaller side-chains at positions Ala123/129 and Ser123/Ala130 in CtBP compared with bulky residues at homologous positions in other D2-DHDs that contact substrate (Fig. S1, blue asterisks). Outside the α -keto moiety, MTOB contacts include mostly residues previously implicated in substrate specificity of other D2-DHDs, including CtBP residues Tyr76/82, His77/83, and Met327/333, as well as Val159'/165' from the neighboring dimeric subunit. The packing of these residues prevent α -keto acids larger than MTOB from binding.

3.3. Unique active site properties

Unlike other D2-HDH enzymes, a Trp (318/324) dominates the active site in CtBP and is within van der Waals distance of all MTOB atoms outside the α -keto acid moiety (Fig. 2). This Trp has recently been implicated both as a dimerization switch in CtBP1[38] and in models of the binding of the brefeldin A (BFA) moiety in the BFA-ADP ribosyl conjugate of rat CtBP1/BARS[39]. Interestingly, the MTOB S8 atom centers over the CtBP Trp318/324 indole, rather than orienting its lone pair of electrons towards the positively charged CtBP Arg97/103, suggesting a sulfur- π interaction [40,41]. The finding that replacing the MTOB sulfur with a methylene carbon decreased enzymatic efficiency 8 fold [28], indicates that this sulfur- π interaction contributes to the substrate specificity of CtBP. Trp318/324 provides an extensive active site surface that represents an opportunity for inhibitor design as it is a characteristic not shared by other D2-DHD family members (Fig. S1, green diamond).

A second unique aspect of the CtBP active site is a hydrophilic cavity between the MTOB binding site and NAD^+ . Four ordered water molecules, designated W1 through W4, fill this cavity in the CtBP1 ternary structure and link MTOB through hydrogen bonds with the phosphate group of NAD^+ (Fig. 3a). W1 forms a hydrogen bond directly with MTOB O3 and W4 forms a hydrogen bond with the NAD^+ phosphate. Two other CtBP1 structures, the human CtBP1 binary complex [29] (pdb code 1MX3) and a rat CtBP1 binary complex [30] (pdb code 1HKU), display water molecules at these four positions, while a lower resolution rat CtBP1 [31] (pdb code 2HU2) retains only water molecules at the W1 and W2 sites (Fig. 3b). Individual monomers in the binary CtBP2 structure (pdb 2OME) contain some of the conserved waters despite the low resolution relative to the CtBP1 structures (Fig. 3c). Although the low resolution of the CtBP2-MTOB complex prevents confident modeling, the $F_o - F_c$ map in our CtBP2 structure shows positive density at some positions of the water network (Fig. 3d).

The locations of the water molecules that fill the active site cavity are very similar, with the exception of position W1 (Fig. 3b,c). The precise binding location for this water molecule depends upon the occupants of the active site in various structures, with binding of ions (acetate or formate), MTOB or other water molecules dictating the location of water

binding. Interestingly, the CtBP2 MTOB carboxylate sterically clashes with a water at position W1, which may account for the lack of positive Fo-Fc density at this position (Fig. 3d). Most likely the CtBP1 MTOB carboxylate shift towards Arg97 is required for a water molecule to bind at position W1 in the CtBP1 ternary structure. Water molecules W2, W3 and W4 show less positional variation (Fig. 3b).

The observed active site cavity is present because CtBP lacks a bulky residue that is involved in substrate binding in other D2-HDHs. In these other family members, a residue with a large side chain typically occupies the homologous position to CtBP1 Ala123 or Ser124 (CtBP2 Ala129 or Ala130) in the hinge region just outside the coenzyme binding domain (Fig. S1, blue asterisks). These side chains interact with and stabilize substrate in both bacterial and human D2-HDHs. A tyrosine residue occupies the A123 position in three bacterial structures.[9,10,37]. In the human D2-HDHs PHGDH and GRHPR, similar bulky residues contact the substrate. Leu107 of GRHPR substitutes for CtBP1 S124, providing van der Waals contacts to the carboxylate of the enzymatic product, D-Glycerate [7]. In PHGDH, Asn106 substitutes for CtBP1 S124, interacting with a bound malate carboxylate [8]. These bulky side chains that fill the hydrophilic channel in other D2-HDH's prevent binding of the ordered water molecules that are observed in our CtBP structures (Fig. 4). Therefore, the cavity containing the observed water network in CtBP is a unique characteristic that may be exploited in future drug design to maximize specificity for CtBP inhibition vs. other D2HDH family members that play important roles in cellular metabolism.

The goals of our ternary MTOB/NAD⁺/CtBP structure determination were to obtain insights into the mechanism of inhibition and to determine detailed interactions that may facilitate inhibitor design. Given the lack of structural changes upon MTOB binding, mechanisms other than quaternary and tertiary rearrangements likely underlie inhibition. Based on the detailed understanding of the unusual CtBP active site cleft provided by the CtBP1/2-MTOB structures, including a prominent Trp and water channel, highly potent and specific bifunctional inhibitors can be envisioned that bind not only in the MTOB binding site and adjoining cavity, but also connect to moieties of the NAD(H) binding site. As a result, we are currently exploring compounds with aromatic character to strengthen interactions with Trp 318/324 and intend to investigate compounds capable of interfering with NADH mediated dimerization. Thus, these results provide a basis for the design of highly specific inhibitors of CtBP that could lead to therapeutically valuable compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CtBP	C-terminal Binding Protein
MTOB	4-Methylthio 2-oxobutyric acid
D2-HDH	D-isomer specific 2-hydroxyacid dehydrogenases
GRHPR	glyoxylate reductase/hydroxypyruvate reductase
PHGDH	phosphoglycerate dehydrogenase
NAD	nicotinamide adenine dinucleotide
ARF	alternative reading frame
HIPK2	Homeodomain-interacting protein kinase 2
APC	adenomatous polyposis coli

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Appendix A. Supplementary data

Supplementary Data associated with this article can be found online.

- CtBP 1 & 2 are transcriptional coregulators that have been linked to human cancers
- Crystal structures of CtBP reveal stereochemistry of binding substrate MTOB
- Two features identified, a hydrophilic channel and key Trp, are unique to CtBP
- These results provide a basis for design of specific therapeutic CtBP inhibitors

Fig. 1. Binding of MTOB to CtBP. (a) CtBP1 monomer domains; the substrate binding domain (orange) and coenzyme binding domain (green) are connected through a hinge (grey). MTOB (cyan) and NAD⁺ bind in an active site cleft formed at the interface of the domains. The interface between subunits of the physiological dimer is primarily formed between the coenzyme binding domain, including extensive contacts formed by the dimerization loop. (b) and (c) The 2Fo-Fc maps for MTOB in CtBP1 (1σ; 0.20 e-/Å³) and CtBP2 monomer A (1σ; 0.27 e-/Å³), respectively. MTOB atom labels are shown in CtBP1 (b).

Fig. 2. MTOB interactions in CtBP active site shown in stereo. (a) and (b) MTOB conserved hydrogen bonds (orange) and unique hydrogen bonds (blue) in CtBP1 and CtBP2 monomer A, respectively. An additional bond (not shown) exists between MTOB O5 and R272 NH2 in CtBP2 monomer A. This bond is not conserved across all CtBP2 monomers. (c) MTOB conformation differs between CtBP1 and CtBP2. In CtBP1 (cyan) the MTOB carboxylate twists relative to CtBP2 (dark blue) to form contacts with R97. CtBP2 MTOB lacks an extensive interaction as R103 positions further away. The sulfur atoms of MTOB in both

structures position about 4 Å from W318/324, forming a sulfur-pi interaction that appears to confer MTOB specificity.

Fig. 3.

Ordered water molecules in CtBP hydrophilic active site cavity. (a) Four water molecules in CtBP1, W1-W4, form a network unique to CtBP from MTOB (cyan) to an NAD⁺ phosphate. (b) In addition to the MTOB bound structure (red), other CtBP1 structures (1MX3, blue; 1HKU, green; 2HU2, yellow) possess a nearly identical network. Position of W1 varies depending on the occupants of the active site. W2-W4 display less variation. Human CtBP1 (1MX3) possesses five waters, although B-factors of the additional water (labeled with a white “x”) suggest high mobility. (c) Individual monomers of the unbound CtBP2 structure (2OME) also possess the waters (monomer C, purple; monomer F, orange;

monomer G, green). (d) The F_o-F_c map ($0.24 \text{ e}^-/\text{\AA}^3$) of MTOB bound CtBP2 suggests the presence of waters W2-W4 in monomer A (green), monomer G (purple), and monomer H (blue). MTOB (dark blue) clashes with the W1 (red disks) when aligned to the CtBP1 structure.

Fig. 4.

Uniqueness of CtBP active site cavity. (a) MTOB (cyan) and the water network fill the CtBP1 (grey) active site cavity (blue surface). Small residue S124 (CtBP2 A130) does not contact MTOB or the active site loop, leaving a gap that allows the water network to form. (b) and (c) Related human proteins do not possess the full water network. The active site cavities for GRHPR (2WWR - green surface) and PHGDH (2G76 - purple surface) are restricted to the volume adjacent to the catalytic residues. GRHPR L107 contacts the active site loop and enzymatic product, D-glycerate. PHGDH N106 also contacts the active site

loop and a malate ion, which sits in the substrate binding site. These larger residues close the gap around the substrate/product pocket, leaving room only for waters to bind near the coenzyme phosphate group.

Table 1

Data collection and refinement statistics

	CtBP1-MTOB	CtBP2-MTOB
Data Collection		
Wavelength (Å)	0.99	0.99
Resolution range (Å) ^a	29.92 - 2.38 (2.47 - 2.38)	32.30 - 2.86 (2.96-2.86)
Space group	P6 ₄ 22	P2 ₁
Unit cell		
a, b, c (Å)	88.97, 88.97, 161.54	86.15, 140.61, 135.13
α, β, γ (°)	90, 90, 120	90, 97.87, 90
Total reflections	167249	298092
Unique reflections	15538	73354
Multiplicity*	10.7 (11.6)	4.1 (4.0)
Completeness (%)*	97.9 (99.9)	99.3 (96.9)
Mean I/sigma(I)*	22.7 (7.1)	9.6 (3.3)
Wilson B-factor	52.8	54.3
R-sym (%)*	6.5 (39.0)	9.3 (38.9)
Refinement		
R-factor (%)*	20.0 (27.7)	21.4 (30.1)
R-free (%)*	23.9 (38.9)	25.1 (33.3)
Number of atoms	2644	20058
Protein	2463	19429
Ligands	97	424
Water molecules	84	205
Protein residues	327	2646
RMS (bonds)	0.009	0.009
RMS (angles)	1.27	1.15
Ramachandran favored (%)	96	96
Ramachandran outliers (%)	0	0
Clashscore	3.79	11.14
Average B-factor	55.5	52.7
Protein average B-factor	55.9	52.9
Water average B-factor	53.5	44.3

^a highest resolution shell shown in parentheses