Assembly of human C-terminal binding protein (CtBP) into tetramers

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Running title: Assembly of tetrameric CtBP

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Keywords: CtBP, transcription coregulator, tetrameric assembly, NAD(H), MALS, Cancer target, dehydrogenase, structural biology, crystallography

ABSTRACT

C-terminal binding protein 1 (CtBP1) and CtBP2 are transcriptional coregulators that repress numerous cellular processes, such as apoptosis, by binding transcription factors and recruiting chromatin-remodeling enzymes to gene promoters. The NAD(H) linked oligomerization of human CtBP is coupled to its co-transcriptional activity, which is implicated in cancer progression. However, the biologically relevant level of CtBP assembly has not been firmly established, nor has the stereochemical arrangement of the subunits above that of a dimer. Here, multi-angle light scattering (MALS) data established the NAD\textsuperscript{+} and NADH-dependent assembly of CtBP1 and CtBP2 into tetramers. An examination of subunit interactions within CtBP1 and CtBP2 crystal lattices revealed that both share a very similar tetrameric arrangement resulting from assembly of two dimeric pairs, with specific interactions likely being sensitive to NAD(H) binding. Creating a series of mutants of both CtBP1 and CtBP2, we tested the hypothesis that the crystallographically observed interdimer pairing stabilizes the solution tetramer. MALS data confirmed that these mutants disrupt both CtBP1 and CtBP2 tetramers, with the dimer generally remaining intact, providing the first stereochemical models for tetrameric assemblies of CtBP1 and CtBP2. The crystal structure of a subtle destabilizing mutant suggested that small structural perturbations of the hinge region linking the substrate- and NAD-binding domains are sufficient to weaken the CtBP1 tetramer. These results strongly suggest that the tetramer is important in CtBP function and the series of CtBP mutants reported here can be used to investigate the physiological role of the tetramer.

C-terminal binding proteins (CtBP) 1 and 2 are paralogous transcriptional co-regulators that modulate numerous cellular processes by binding transcription factors and recruiting chromatin remodeling enzymes such as histone deacetylases, methyl transferases and demethylases to targeted promoters (1-3). CtBP1 was first identified through interactions with the C-terminal region of the adenovirus E1A oncoprotein and the ability to modulate E1A transforming activities (4,5). CtBP co-
transcriptional function is important in normal embryogenesis, as it is a regulator of the epithelial to mesenchymal transition (EMT) and is integral in proper fetal cell differentiation. Knockout experiments in mice reveal distinct roles for CtBP1 and CtBP2 in development, with the loss of CtBP2 embryonically lethal whereas CtBP1-null mice are small but the majority survive (6). Alternate splice forms of CtBP1 and 2 also have non-nuclear roles, including membrane trafficking (7).

Both CtBP paralogues have been implicated as global repressors of the epithelial phenotype and of apoptotic pathways (1) and numerous lines of evidence implicate human CtBP in cancer progression. CtBP is a corepressor of genes including tumor suppressive pro-apoptotic factors (Bik, Noxa), cytoskeletal/cell adhesion molecules (keratin-8, E-cadherin) and cell-cycle inhibitors (2,8). CtBP has also been found to act as a coactivator of growth and metastasis-related genes (Tiam1, MDRI, certain Wnt target genes), which facilitate the epithelial to mesenchymal transition (EMT) (9-11). Consistent with its role in repression of apoptotic pathways and activation of growth and metastasis, CtBP is upregulated in a number of cancer tissues including colorectal cancer (12), melanoma (13), metastatic prostate cancer (14), esophageal squamous cell carcinoma (15), ovarian cancer (16), and breast cancer (17,18). Strikingly, elevated levels of CtBP in tumor tissue have been correlated with poorer survival in breast cancer (19), ovarian cancer (16) and hepatocellular carcinoma (20). Recent results add to evidence of a link between CtBP and cancer progression by showing increased survival of APC<sup>min/+</sup> mice when CtBP2 levels are lowered by CtBP2<sup>+/−</sup> heterozygosity (21).

CtBP is unique among transcription factors in the incorporation of a D-isomer specific 2-hydroxyacid dehydrogenase (D2-HDH) domain, which reduces or oxidizes substrates using the coenzyme NAD(P)<sup>+</sup>/NAD(P)H (22,23). The best substrate identified to date for CtBP is MTOB, 4-methylthio 2-oxobutyric acid (aka 2-keto-4-methylthiobutyrate) (24), although whether this is a physiologically relevant substrate is unknown. While evidence indicates that catalytic activity is not required for some CtBP activities (8,25), mutant studies suggest that catalytic activity of CtBP can be important for Drosophila melanogaster development (26).

Regulation of gene expression through the oligomerization of transcriptional factors is an important paradigm (27,28). In the case of CtBP, substantial evidence exists that oligomerization is linked with NAD(H) binding (3,25,29-32) and dimer-destabilizing mutants have been found to inhibit transcriptional function (33-36). Assembly of CtBP has primarily been considered in terms of dimers, as NADH bound CtBP crystal structures reveal a predominant dimer with extensive interactions between subunit pairs (23,37,38). There is, however, evidence for assembly of CtBP into tetramers (29-31) at least when the full C-terminus is present. Here we present multi-angle light scattering (MALD) data showing that the predominant form of CtBP1 and CtBP2 when bound to NAD(H) is tetrameric, with tetramers forming even in the absence of the full C-terminus. Moreover, our mutant data provides strong evidence that the solution tetramer is very similar to the tetrameric assembly observed within crystals of the minimal dehydrogenase domains for both CtBP1 and CtBP2. Furthermore, the crystal structure of the CtBP1 A123V mutant reported here suggests that small perturbations in the flexible hinge region are capable of destabilizing the CtBP tetramer.

RESULTS

Multi-angle light scattering (MALD) shows that both CtBP1 and CtBP2 assemble into tetramers in the presence of NADH and NAD<sup>+</sup>

We investigated the oligomeric state of CtBP1 and CtBP2 using multi-angle light scattering (MALD) linked with size
exclusion chromatography (SEC). Our initial experiments were carried out using the minimal dehydrogenase domain constructs that we had previously used for crystallization, CtBP1 (28-353) and CtBP2 (31-364) (37). In contrast with a previous report (30), our SEC-MALS experiments on CtBP lacking the full C-terminus showed molecular weights considerably larger than dimers in the absence of sufficient NADH (Fig. 1A). SEC-MALS results for CtBP oligomerization as a function of NADH concentration are shown in Fig. 2A and Table 1. Fitting of the molecular mass dependence on NADH concentration (see Experimental Procedures) yields an EC_{50} value for the effect of NADH promoting tetramer formation of about 275nM for CtBP1 (28-353) and 180nM for CtBP2 (33-364) (Table 1). The EC_{50} value will likely be similar to the dissociation constant for NADH binding, however the linkage between NADH binding and tetramer assembly suggests that the dimeric and tetrameric forms of CtBP will bind NADH with different affinities, precluding accurate estimates of dissociation constants with the present data.

The MALS molecular mass estimates indicate heterogeneous mixtures, corresponding primarily to dimers and tetramers of CtBP, but also monomers in some cases. Given that light scattering provides weight average molecular mass estimates (M_w) (39), one can calculate approximate fractions of dimers and tetramers, assuming those are the only species contributing to the light scattering (see Experimental Procedures). Based on these assumptions, CtBP2 (31-364) is approximately 18% tetramer in the absence of NADH and rises to about 47% tetramer in the presence of 10μM NADH. In contrast, CtBP1 (28-353) appears to be a mixture of monomer and dimer in the absence of NADH, but is about 46% tetramer in the presence of 10μM NADH. Our findings of significant tetramer formation in the absence the full C-terminus contrast with an earlier report (30); this likely results from the higher protein concentration used in our experiments. (The loading concentration of 2μM in the Madison et al. experiments (30) is substantially lower than our loading concentrations of ~20μM and will become even more diluted during the ~50ml run through the SEC column before CtBP elution compared with the ~10ml run before CtBP elution in our SEC-MALS experiments.)

Given previous findings suggesting the importance of the C-terminal residues for tetrameric assembly (30), we investigated constructs that included the full C-terminus - CtBP1 (28-440) and CtBP2 (31-445) (Fig. 1B). Our results show that both assemble with masses indicative of predominantly tetramers in the presence of micromolar levels of NADH (Fig. 2B and Table 2). Assuming a simple mixture of dimers and tetramers, CtBP2 (31-445) in the absence of added NADH is about 29% tetramer and plateaus to 75% tetramer at micromolar levels of NADH. CtBP1 (28-440), shows only 15% tetramer in the absence of added NADH, but this plateaus to 79% tetramer with sufficient NADH. Thus, in agreement with Madison et al. (30), our experiments suggest significantly stronger tetramers form in the presence of the full C-terminus. Our results also suggest that the affinity of CtBP1 for NADH is slightly higher in constructs with the C-terminus (EC_{50} ~50nM) compared to those lacking the final ~85 residues (EC_{50} ~275nM), however for CtBP2, the differences are smaller (EC_{50} ~180nM vs. ~110nM).

We also investigated the effect of NAD^+ on CtBP tetramer formation (Fig. 2B). For CtBP1 (28-440) we observed an EC_{50} for NAD^+ of 140 nM compared with NADH of 50 nM. For CtBP2 (31-445), the EC_{50} values are almost identical for NAD^+ (98nM) and NADH (107nM). Notable from the curves in Fig. 2B are steeper slopes for NADH dependent tetramer assembly compared with the slopes for NAD^+ assembly, which is reflected in the fitted Hill coefficients (Table 2). This suggests potentially greater cooperativity in NADH-linked assembly of CtBP, which could be relevant for the response of CtBP to...
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coenzyme binding. Although the fitted EC50 values are indirect measurements of binding affinity, our results are consistent with similar affinities for NAD+ and NADH as reported by Madison et al. (30). Our apparent affinities for NADH are similar to that reported by Goodman and colleagues (40-42), but our results are inconsistent with much lower affinity reported for NAD+ (40).

Tetramer models based on crystal lattices of CtBP1 and CtBP2

Observation of tetramer formation even for the crystallized minimal dehydrogenase domain suggested that the crystals grown from high CtBP concentrations might show assemblies related to the tetramers detected in our SEC-MALS experiments. Examination of the CtBP crystal lattices reveals strikingly similar tetrameric arrangements for both CtBP1 and CtBP2, despite very different crystal lattices (Fig. 3). CtBP1 crystallizes with one subunit in the asymmetric unit of hexagonal space group P6322 (23,43). Three mutually perpendicular intersecting crystallographic 2-fold axes generate the D2 symmetric tetramer shown in Fig. 3A. The CtBP2 crystal lattice has eight subunits per asymmetric unit (37). These eight subunits are assembled into two tetramers as shown in Fig. 3B & C. The remarkable similarity of these three tetramers is evident, which is confirmed by structural alignment of the full tetramers. The two CtBP2 tetramers align with a root mean square (RMS) deviation of 0.441Å (for 8083 atom pairs) and the CtBP1 tetramer aligns to each CtBP2 tetramer with RMS values of 0.733Å (for 7925 atom pairs) and 0.711Å (for 7776 atom pairs). By far the most extensive interface occurs between two subunits that form the dimer first identified in CtBP1 by Kumar et al. (23), which buries surface areas of 2715-2924Å2 in CtBP1 and CtBP2. Contacts between two dimers are more limited, with pairs of subunits burying surface areas of 806-857Å2. (Buried surface area calculated with PDBePISA (44).) Thus, the tetramer is a dimer of dimers, encompassing an extensive intradimer interface and a less extensive interdimer interface.

CtBP subunits comprise an NAD-binding domain (residues 125-319 for CtBP1 and 131-325 for CtBP2) and a discontinuous substrate-binding domain (residues 28-120 and 320-353 for CtBP1; 34-126 and 333-359 for CtBP2). These two domains are connected at residues 320-321 and also by a hinge region including residues 121-124 (CtBP1) and 127-130 (CtBP2), which contributes to the interdimer contacts.

Evident upon examining the CtBP subunit arrangement is a short helix in the NAD binding domain (helix αE, as defined by Kumar et al. (23)) whose projection into the solvent in dimeric CtBP would appear to be unfavorable. The tetramer buries this short helix in a pocket between αD and αE helices of a partner subunit (Fig. 3), potentially providing a more favorable environment.

The arrangement of subunits in the crystallographically observed CtBP tetramers has intriguing features as a potential model for the observed solution tetramer. Interdimer contacts are formed from the NAD-binding domain along with the hinge region between the NAD-binding and substrate-binding domains. Strikingly, each subunit projects its C-terminus towards the interdimer interface, suggesting that the presence of an additional ~85 C-terminal residues may provide interactions to stabilize the tetrameric form, consistent with our MALS results and the results of Madison et al. (30). Additionally, bound NAD(H) coenzymes are in closer proximity across the interdimer interface than across the intradimer interface, suggesting that the interdimer interface may be sensitive to NAD(H) binding. Finally, an Arg side-chain (Fig. 3E) provides a direct link between an NAD(H) bound in one subunit with a subunit across the interdimer interface, as discussed below. Thus, the crystallographically observed tetramer provides a detailed and plausible model for the solution tetramer.

Mutational investigation of tetramer model
Based on the tetramer model described above, we designed a series of site-directed mutants for both CtBP1 and CtBP2 to test the hypothesis that the observed interdimer interface stabilizes the solution tetramer. Three groups of residues were investigated, all of which should primarily impact the interdimer interface: 1) A nonpolar interaction along the two-fold axis relating two dimers that involves Leu 221 (215 in CtBP1) from the short αE helix (23) of each subunit (Fig. 3D). The atoms from the leucine side-chains come within 3.6 Å of each other in CtBP1 and are 3.8-4.8 Å from each other in the lower resolution CtBP2 structure. We mutated this leucine to the larger Tyr. 2) A particularly intriguing interaction involves Arg 190 (184 in CtBP1) (from the amino end of αD(23)) which forms ionic hydrogen bonds with the NAD(H) phosphate in its own subunit and the main-chain carbonyl of Asp 215 (209 in CtBP1) across the interdimer interface (Fig. 3E). To test the need for a charged Arg that can donate hydrogen bonds to both groups, we mutated this residue to a Gln. 3) The third group includes residues in the hinge between the NAD and substrate binding domains of CtBP (Fig. 3F). The contacts involving the hinge regions were interrogated with mutations of Ser 128 (Ala 122 in CtBP1) to Thr, to test the contact itself and of Ala 129 (Ala 123 in CtBP1) to the larger Val and Leu, to test if bulkier residues at this position could alter the hinge conformation and destabilize the interdimer interface. Mutants for both CtBP1 and CtBP2 were constructed in the context of the full C-terminus, purified and subjected to SEC-MALS to investigate the effect on tetramer formation.

Results of SEC-MALS experiments, run with 10µM NADH, on the CtBP2 mutants are shown in Fig. 4A and Table 3. The dimeric molecular mass for the CtBP2 (31-445) construct is 98.0kDa. Wild-type CtBP2 ran as mostly tetrameric in these experiments, with a MALS molecular mass measured to be 185kDa. All six putative interdimer destabilizing mutants eluted at a similar volume (Fig. 4A), with MALS determined molecular masses of 94-103kDa. Thus, our results are fully consistent with these mutants destabilizing the tetramer, but maintaining their dimeric assemblages, as predicted from the crystallographic tetramer model.

The SEC traces for the CtBP1 mutants are more complex than those for CtBP2 (Fig. 4B). SDS-PAGE gels demonstrate that the C-terminal region of CtBP1 is more susceptible to proteolytic cleavage than that of CtBP2. An SDS-PAGE gel of the samples used in this experiment indicated that all mutants showed some proteolysis in the C-terminal region. All mutants except for R184Q displayed a major band at a full-length size of 47kDa, whereas the major band for R184Q was at a molecular mass of about 41kDa, consistent with a sequence ending at approximately residue 378. (The CtBP1 crystals discussed below, grown from constructs expressing the full C-terminus, were found to have been proteolyzed just after residue 378.) The greater C-terminal proteolysis for CtBP1, compared to CtBP2, suggests that the C-terminus may be more flexible in CtBP1. Such increased flexibility, along with partial proteolysis, may contribute to the observed greater variation in elution volumes for CtBP1 mutants as the SEC elution depends upon overall protein shape rather than strictly on the molecular mass. (The C-terminal 86 amino acids are the least conserved, with only a 51% sequence identity between human CtBP1 and CtBP2, compared with 89% identity in the crystallized minimal dehydrogenase domain.) The MALS molecular mass values show smaller variations, providing more accurate estimates of the oligomeric state. The four CtBP1 mutants of residues projecting into the interdimer interface show MALS molecular masses of 84-100kDa (Table 3). These are in the range expected for dimers of CtBP1 (28-440) of 94kDa and those for partially proteolyzed species (28-378) of 82kDa. These results, thus, support those from CtBP2 indicating that the interdimer interface is similar to that observed in the crystallographic tetramer.
The most subtle mutants designed to destabilize the tetramer were of A123/129 (CtBP1/CtBP2), residues that are in the hinge region but which project their beta carbon into its subunit rather than towards the interdimeric interface. In this case, the results between CtBP1 and CtBP2 show some interesting differences. Whereas CtBP2 A129V and A129L were found to have molecular masses close to that expected for dimers and tetramers, suggesting a more subtle destabilization of the CtBP1 interdimer interface (Table 3). These results indicate that subtle conformational changes in the hinge region connecting the two domains can substantially impact tetramer stability.

**Crystal structures of CtBP1 with extended C-terminus**

Our results, complementing those of Madison et al. (30) highlight the contribution of the final ~85 residues to stabilization of the CtBP tetramer. In an attempt to investigate the C-terminal residues and also the conformational changes induced by mutation of hinge residues, we grew crystals from CtBP1 expressed with the full C-terminus for wild-type and a subtle tetramer destabilizing mutant (A123V), both in the presence of NADH. (Attempts to grow crystals of A123L, with its slightly larger side chain, were unsuccessful.)

Crystallization of expressed CtBP1 (28-440) was successful only intermittently; SDS PAGE gels revealed that those samples that successfully crystallized were intermediate in size between CtBP1 (28-440) and CtBP1 (28-353). Mass spectrometry measurements confirmed that these samples were shorter than full-length, with a predominant species encompassing residues 28-378. We therefore consider these crystal structures to be CtBP1(28-378).

All CtBP crystals described to date are built from tetrameric assemblies, although they have not previously been described as such. Thus, it is not surprising that only a subtle tetramer-destabilizing mutant (A123V) successfully crystallized. The crystal structures of both wild-type and A123V mutants were determined by molecular replacement using the CtBP1-HIPP structure (28-353) (43) and refined to resolutions of 2.6 and 2.4 Å, respectively. (The resolution cutoffs were conservatively chosen.) Final crystallographic statistics are provided in Table 4.

Despite an additional 25 residues in the wild-type and A123V CtBP1 (28-378), only four additional residues are evident in the electron density maps and modeled in our final structures (Fig. 5). These results are consistent with findings on rat CtBP that the C-terminus is largely disordered (31).

The isomorphous crystal lattices for wild-type and A123V CtBP1(28-378) permit direct comparison of the structural differences using difference maps $[F_o(A123V) - F_o(\text{wild-type})]$. As is evident from Figure 6, the mutation-induced changes largely localize to the mutant site. Substitution of Val for Ala at position 123 induces movements of Glu 326 and the α5 helix (23) (which includes Glu 326) deeper into the subunit. Interestingly, the maps do not suggest significant movement of residues in the partner subunit across the tetrameric interface. This observation suggests that the tetrameric subunit arrangement is sufficiently stabilized by the CtBP1 crystal lattice that any strain induced by Val 123 is absorbed within a subunit. Our MALS results, however, clearly show that the tetramer is destabilized by the mutation, such that in solution there may be significant effects on the interaction between the partner subunits with this mutation. The observation that the homologous mutation (A129V) in CtBP2 is almost entirely dimeric suggests that the CtBP2 subunit structure is unable to accommodate the strain induced by the presence of the Val 129.

**DISCUSSION**

Assembly of protein components is an important theme in transcriptional regulation (45-47). A central element of this process is often the oligomerization of one or more
individual protein components contributing to transcriptional complex assembly (27,28). In the case of CtBP, strong evidence exists for the role of NAD(H) in both co-transcriptional activity and oligomerization (3,23,25,29,32,33). Previously published results have provided evidence that CtBP can assemble to dimers and larger species (3,25,29-32), but have not provided a quantitative relationship for the NAD(H) dependence of tetramer assembly nor a stereochemical model of the CtBP tetramer.

The results presented here demonstrate a clear relationship between NAD(H) binding (with an affinity in the 100nM range) and CtBP assembly into tetramers. Our mutant data provide strong evidence that subunits in the solution tetramer are arranged similarly to the tetrameric assemblages present in previously determined crystal structures of human CtBP1 and CtBP2 (23,37) and rat CtBP/BARS (38). This tetramer is assembled from the pairing of two dimers to form NAD(H) sensitive interdimer contacts primarily using the NAD-binding domain of CtBP. In this tetramer, the closest approach of NADH molecules across the intradimer interface is over 30 Å, but less than 15 Å across the interdimer interface (see Fig. 3); this closer approach supports the idea that the interdimer interface would be more sensitive to NADH binding than the intradimer interface. Moreover, the interaction between NADH across the interdimer interface to the main-chain carbonyl of residue 209/215 (CtBP1/CtBP2) mediated by Arg 184/190 (Fig. 3E) provides an ionically mediated contact across the interdimer interface that should be directly sensitive to NAD(H) binding.

Although NAD(H) stabilizes the CtBP tetramer, some tetramer formation is evident even in the absence of NAD(H) from our experiments. The ability to form tetramers in the absence of NAD(H) allowed Nardini et al. (48) to grow crystals and determine the structure of the NAD(H) binding impaired G172E mutant rat CtBP1/BARS protein by using seeding with NADH bound CtBP crystals. These crystals, which are isomorphous to the human CtBP1 crystals described above, have a tetrameric form despite no bound coenzyme. This result shows the ability of NAD(H) free CtBP to form tetramers, which can be stabilized by lattice interactions triggered by the crystal seeding. The strength of lattice interactions to stabilize the tetramer is also evident in our A123V crystal structure, which, despite having a tetramer-stabilizing mutant, maintains tetramer contacts, by absorbing mutant-induced strain within a subunit.

Previous investigations into the role of assembly in CtBP have investigated the activity of mutants specifically designed to disrupt the CtBP dimer (33-36). Our results strongly suggest that the CtBP dimer is required for the assembly of a CtBP tetramer formed from the pairing of two CtBP dimers. Therefore, it is likely that dimer destabilizing mutants will also destabilize CtBP tetramers. Thus, previous work showing the importance of dimers is fully consistent with an important role for tetramers.

Our results suggest that the tetramer, assembled as observed in crystal lattices, may be important for CtBP co-transcriptional activity. Importantly, there is an observed correlation between elevated levels of CtBP and poor prognosis in breast cancer (19), ovarian cancer (16) and hepatocellular carcinoma (20) as well as the observation of elevated CtBP levels in other cancers (12-15). Increased levels of CtBP will not only raise the concentration of co-transcriptional factor but also increase the proportion of CtBP that assembles into tetramers, thus potentially increasing its transcriptional activity. Tetramer destabilizing mutants identified here provide important tools for dissecting the role of CtBP tetramers in co-transcriptional function and cancer progression. Moreover, our model of the tetrameric assembly provides stereochemical details for the first time, that can be used with structure-based drug design approaches for development of novel CtBP tetrameric destabilizing inhibitors.
EXPERIMENTAL PROCEDURES

Expression and mutagenesis of CtBP1 and CtBP2

The expression and purification procedures were adapted and optimized from earlier studies (37,43). The ligated, purified plasmid containing the desired CtBP construct was transformed into Z-competent BL21(DE3)RIL E. coli cells. A single clonal colony was then grown in a starter culture of LB broth overnight at 37°C. The starter culture was used to inoculate between three and six 1L cultures grown in Research Products International Terrific Broth using 50mL starter per liter. Cultures were grown at 37°C while shaking at 150RPM and induced with IPTG, at a final concentration of 0.2mM, after reaching OD_{600} between 0.800 and 1.00. The temperature was reduced to 30°C at the time of induction and the cells were harvested four hours later. The cells were pelleted by centrifuging for 20 minutes at 4700 RPM, and resuspended in 10 mL harvesting buffer (pH 7.6; 0.1 M NaCl; 0.05 M Tris-HCl; 0.2 mM EDTA) per liter of culture. One tablet of EDTA-free complete Mini (Roche Diagnostics) protease inhibitor cocktail was added per liter of culture and the cells frozen at -80°C. Site-directed mutants were created using the QuikChange protocol (Stratagene) using the modified approach of Liu and Naismith (49).

Purification of CtBP1 and CtBP2

Cells were thawed slowly on ice and then lysed in a Microfluidics Corporation model 1109 cell disrupter. 35 mg of Roche Diagnostics DNase I, 500µL 2M MgCl₂ and 500µL 40 mM CaCl₂ were added per 100mL lysate. The lysate was then gently stirred at 4°C for 30 minutes before the insoluble fraction was pelleted by centrifuging at 19,000RPM for 45 minutes. The supernatant was then mixed with 8 mL HisPur™ Ni-NTA Resin (Thermo Scientific), and gently stirred at 4°C for two hours to allow CtBP to bind to the resin.

The bead-supernatant mixture was placed in a BioRad Econo-Column® at 4°C and the soluble fraction was allowed to flow through. The beads were then cleaned with five column volumes of wash buffer (0.0625 M Tris:HCl pH 7.4; 0.375 M NaCl; 0.05 M imidazole; 0.625 mM EDTA; 1.0 mM DTT), followed by six column volumes of wash buffer supplemented with an additional 1.7 M NaCl. Another two column volumes wash buffer was passed over the beads before six column volumes of wash buffer supplemented with 0.5% Triton-X 100 was added. An additional two column volumes of wash buffer again followed. CtBP was eluted from the beads using three column volumes of wash buffer supplemented with 250 mM imidazole. The eluent was collected and dialyzed overnight in SnakeSkin® Dialysis Tubing (Thermo Scientific) to remove imidazole. The dialysis buffer consisted of 50 mM Tris:HCl pH 7.7, 300 mM NaCl, 5 mM EDTA, 2 mM DTT and 10% glycerol. The protein was then concentrated by centrifuging at 5000 RPM in an Amicon® Ultra-15 10K centrifugation column (Millipore). Protein concentration was measured by UV absorbance at 280nm using an Ultraspec 2100 pro by Amersham Biosciences.

The protein sample was further purified by size exclusion chromatography. The FPLC (ÄKTAprime plus by GE Healthcare) and size exclusion column (Highload™ 16/60 Superdex™ 200 prep grade) were equilibrated with “FPLC Buffer” (50mM Tris:HCl pH 7.7, 300 mM NaCl, 5 mM EDTA, 2 mM DTT). The sample was prepared by adding 1.5 mM NADH to the concentrated protein solution; the solution was then centrifuged at 8000RPM for six minutes at 4°C to remove any small insoluble fraction. The flow rate was set to 1 mL/min and 62 fractions of 2 mL each were collected the appropriate fractions were concentrated in an Amicon® Ultra-15 10K centrifugation column.

SEC-MALS for NAD(H)-Dependent Oligomerization Studies

Four different CtBP constructs were tested in NAD(H)-dependent oligomerization MALS experiments: CtBP1...
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28-353, CtBP1 28-440, CtBP2 31-364, and CtBP2 31-445 were expressed and purified as described above. Protein samples were prepared for SEC-MALS by diluting CtBP stocks to 1.0 mg/mL in “FPLC buffer” supplemented by the desired concentration of NAD(H). The protein samples were filtered by in a Costar 0.22 µm Spin-X column at room temperature. 100µL protein samples were injected into the SEC-MALS instrument. The MALS system consisted of a Dawn Helios-II MALS detector (Wyatt), an Optilab T-rEX differential refractive index detector (Wyatt), and the 1260 Infinity HPLC system (Aligent) with a Wyatt Corporation 0.78 x 30 cm HPLC column with 500 Å pore size was used. In later experiments, a TSKgel G3000SWxl column (Tosoh Bioscience) was substituted, but the effect of the column on the quality of the data obtained was negligible. For the wild-type constructs with the full C-terminus, measurements at given NADH and NAD+ concentrations were carried out in triplicate (Table 2 and Fig. 2B).

The data for the dependence on molecular mass as a function of NAD(H) concentration (Fig. 2) were fit with Prism 7(GraphPad Software, Inc.) to the equation

\[ Y = L + (U - L)/(1 + 10^\log(CE50 - x)^n) \]

where L and U are the lower and upper Mw plateaus, respectively, x is the concentration of NAD(H) in nM, and n is the Hill coefficient.

The molecular mass obtained from light scattering from a heterogeneous mixture of protein molecules is the weight average molecular weight (M_w): M_w = \( \Sigma N_i M_i^2 / \Sigma N_i M_i \) where N_i is the molar or fractional concentration (39). Assuming a mixture of just CtBP dimers and tetramers allows simplification to M_w = [F_T(2M_D)^2 + (1-F_T)M_o^2]/[F_T(2M_D) + (1-F_T)M_o], where M_D is the dimeric molecular weight and F_T is the fraction of CtBP in the tetrameric form. Rearranging this equation, we obtained estimates of the fraction tetramer from F_T = (M_w-M_D)/(3M_D-M_w).

SEC-MALS for Mutant Studies

The mutant SEC studies were carried out as described above using a TSKgel G3000SWxl column (Tosoh Bioscience) working in concert. The instruments were equilibrated in FPLC buffer supplemented with 10 µM NADH. Protein samples consisted of 1 mg/mL CtBP in FPLC buffer. The sample was filtered using 0.22 µM Cellulose Acetate Costar® Spin-X Centrifuge Tube Filters at 8000RPM for one minute at room temperature. 100 µL of sample solution was injected into the MALS system, with a flow rate of 1.0 mL/min. Data were analyzed using the ASTRA software package by Wyatt.

Crystallization and X-Ray Diffraction

Purified CtBP 28-378 WT protein was diluted to 18.0 mg/mL from 27.0 mg/mL stock with dH2O before being supplemented with 10% 15 mM NADH and 2% 100 mM tris(2-carboxyethyl)phosphine (TCEP) in dH2O. The sample was then filtered via a Costar 0.22 µm Spin-X column at room temperature. Hanging vapor diffusion drops were set up in a 1:1 ratio of protein to mother liquor with a total volume of 4 µL and incubated at 20°C. Crystals formed within 24 hours after the drops were set up but allowed to grow for several days. A single crystal with hexagonal bipyramidal morphology was observed in 100 mM HEPES buffer pH 7.5 containing 140 mM CaCl2 and 5% PEG-400. The observable dimensions along its longest axes were 260 x 400 µm. The crystal was harvested for data collection by dipping in a cryo-protectant solution consisting of mother liquor supplemented with 20% ethylene glycol. The crystal was then flash-frozen at 100K. Data were collected on a MicroMax-007-HF/Saturn 944 CCD X-ray diffraction system (Rigaku), and then processed with HKL-3000R. The structure was solved by molecular replacement, using the CtBP1 (28-353) HIPP structure (pdb ID code 4U6Q (43)) as the search molecule and refined in PHENIX version 1.11.1-2575-000. Model building between rounds of refinement was completed with Coot version 0.8.8. EL. Data
Assembly of tetrameric CtBP

tetrameric CtBP were refined to a maximum resolution of 2.60 Å.

For the CtBP1 28-378 A123V mutant, protein was diluted to 17.9 mg/mL from a 33.5 mg/mL stock with dH2O before being supplemented with 10% 15 mM NADH and 2% TCEP in dH2O. The protein sample was filtered via a Costar 0.22 µm Spin-X column at room temperature. Hanging vapor diffusion drops were set up in a 1:1 ratio of protein to mother liquor with a total volume of 4 µL and incubated at 15°C. Crystals formed between three and eight days after the drops were set up. A single crystal with hexagonal bipyramidal morphology was observed in 100 mM HEPES pH 7.5 buffer containing 80 mM MgCl2 and 4% PEG-400. The observable dimensions along its longest axes were 160 x 300 µm. The crystal was harvested for data collection after 3.5 µL cryo-protectant solution consisting of mother liquor supplemented with 20% ethylene glycol were added to the hanging drop and left to soak for 5 minutes. The crystal was then flash-frozen at 100K. Data were collected, solved, and refined as described above. Data were refined to a maximum resolution of 2.40 Å.

Liquid Chromatography Mass Spectrometry (LC/MS)

LC/MS samples were prepared by diluting CtBP1 28-440 WT stock and CtBP1 28-440 A123V stock to 2.5 mg/mL in 16.7 mM Tris:HCl pH 7.7 buffer supplemented with 100 mM NaCl and 1.67 mM EDTA. Each sample was spin-filtered in a Costar 0.22 µm Spin-X column at room temperature. LC/MS experiments were carried out by Dr. John Leszyk and Dr. Khaja Muneeruddin of the Proteomics and Mass Spectrometry core at UMass Medical School, Worcester, Massachusetts, USA.

Acknowledgements: We thank Drs. Brian Kelch and C. Robert Matthews for acquiring the Wyatt SEC/MALS instrument, Dr. Brendan Hilbert, Dr. Steven Grossman and Gordon Lochbaum for helpful discussions and Drs. John Leszyk and Khaja Muneeruddin for the LC/MS experiments. This work supported by NIH grant R01 GM119014 to WER. The diffraction data were collected on a Rigaku CCD system obtained through NIH shared instrumentation grant S10 OD012028 to CAS.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Structure factors and coordinates: The structure factors and coordinates have been deposited in the Protein Data Bank with accession codes 6CDF (CtBP1 [28-378]) and 6CDR (CtBP1 [28-378] A123V mutant).

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**Table 1.** Molecular Mass values as a function of NAD(H) concentration for crystallized constructs

|                  | [NADH] nM | Weight-Avg Mol. Mass (kDa) | Elution Peak Concentration (µM) | % Tetramer |
|------------------|-----------|-----------------------------|---------------------------------|------------|
| CtBP1 (28-353)   |           |                             |                                 |            |
| 0                | 62.0      | 0.73                        | 0                               |            |
| 10               | 64.2      | 0.73                        | 0                               |            |
| 100              | 77.4      | 0.76                        | 1                               |            |
| 500              | 103.8     | 0.95                        | 22                              |            |
| 1000             | 124.6     | 1.13                        | 46                              |            |
| 10000            | 124.5     | 1.00                        | 46                              |            |
| CtBP2 (31-364)   | 105.1     | 1.16                        | 19                              |            |
| 10               | 105.2     | 1.01                        | 18                              |            |
| 100              | 116.2     | 1.01                        | 28                              |            |
| 500              | 123.5     | 0.62                        | 36                              |            |
| 1000             | 134.8     | 0.94                        | 50                              |            |
| 10000            | 132.2     | 0.74                        | 47                              |            |

**Fitted NADH dependence**

|                  | Low M<sub>w</sub> kDa | High M<sub>w</sub> kDa | EC<sub>50</sub> nM | Hill Coefficient |
|------------------|------------------------|------------------------|-------------------|------------------|
| CtBP1 (28-353)   | 63.1 ± 4.3             | 127.7 ± 5.9            | 275 ± 90          | 1.45 ± 0.47      |
| CtBP2 (31-364)   | 105.1 ± 3.6            | 133.5 ± 4.7            | 179 ± 125         | 1.12 ± 0.70      |
Table 2. Molecular Mass values as a function of NAD(H) concentration for full C-terminal constructs. (Experiments carried out in triplicate.)

| [NADH] nM | Weight-Avg Mol. Mass (kDa) | Elution Peak Concentration (µM) | % Tetramer |
|-----------|----------------------------|-------------------------------|------------|
| CtBP1 (28-440) |                           |                               |            |
| 0         | 118.9 ± 0.5                | 1.61 ± 0.003                  | 15         |
| 25        | 125.8 ± 0.5                | 1.64 ± 0.01                   | 20         |
| 100       | 168.7 ± 0.2                | 1.28 ± 0.06                   | 66         |
| 250       | 174.2 ± 0.8                | 2.23 ± 0.08                   | 75         |
| 500       | 172.3 ± 2.7                | 2.83 ± 0.80                   | 72         |
| 1000      | 177.0 ± 1.0                | 2.57 ± 0.08                   | 79         |
| 10000     | 176.8 ± 0.8                | 2.81 ± 0.19                   | 79         |
| CtBP2 (31-445) |                           |                               |            |
| 0         | 142.2 ± 0.8                | 2.30 ± 0.08                   | 29         |
| 25        | 145.7 ± 0.7                | 2.18 ± 0.01                   | 32         |
| 100       | 160.8 ± 1.5                | 2.41 ± 0.02                   | 47         |
| 250       | 173.8 ± 2.0                | 2.49 ± 0.01                   | 63         |
| 500       | 178.5 ± 1.7                | 2.77 ± 0.64                   | 69         |
| 1000      | 180.9 ± 0.8                | 3.02 ± 0.11                   | 73         |
| 10000     | 181.7 ± 0.7                | 2.62 ± 0.09                   | 75         |

| [NAD⁺] nM | Weight-Avg Mol. Mass (kDa) | Elution Peak Concentration (µM) | % Tetramer |
|-----------|----------------------------|-------------------------------|------------|
| CtBP1 (28-440) |                           |                               |            |
| 0         | 118.9 ± 0.5                | 1.61 ± 0.003                  | 15         |
| 100       | 140.3 ± 1.3                | 1.73 ± 0.04                   | 33         |
| 250       | 150.5 ± 1.9                | 1.85 ± 0.02                   | 43         |
| 500       | 157.2 ± 1.1                | 1.82 ± 0.04                   | 51         |
| 1000      | 161.9 ± 0.7                | 2.13 ± 0.03                   | 56         |
| 10000     | 169.1 ± 0.7                | 1.87 ± 0.03                   | 66         |
| CtBP2 (31-445) |                           |                               |            |
| 0         | 142.2 ± 0.8                | 2.30 ± 0.08                   | 29         |
| 100       | 158.1 ± 0.8                | 2.42 ± 0.11                   | 44         |
| 250       | 164.6 ± 1.8                | 2.01 ± 0.13                   | 51         |
| 500       | 170.1 ± 0.6                | 2.04 ± 0.03                   | 58         |
| 1000      | 171.9 ± 0.6                | 1.79 ± 0.02                   | 61         |
| 10000     | 172.4 ± 1.5                | 1.07 ± 0.01                   | 61         |

| Fitted NAD(H) dependence | Low M<sub>w</sub> kDa | High M<sub>w</sub> kDa | EC<sub>50</sub> nM | Hill Coefficient |
|--------------------------|-----------------------|-----------------------|-------------------|-----------------|
| CtBP1 - NADH             | 118.8 ± 1.1           | 175.3 ± 0.6           | 49.7 ± 2.8        | 2.8 ± 0.2       |
| CtBP2 - NADH             | 142.1 ± 0.6           | 181.7 ± 0.5           | 107.1 ± 5.0       | 1.6 ± 0.1       |
| CtBP1 – NAD⁺             | 117.7 ± 0.8           | 170.4 ± 0.8           | 140.0 ± 8.1       | 0.85 ± 0.05     |
| CtBP2 – NAD⁺             | 142.0 ± 0.8           | 173.1 ± 0.7           | 97.6 ± 9.0        | 1.2 ± 0.2       |
**Table 3:** Molecular Mass values for CtBP Mutants

| Mutant         | Weight-Avg Mol. Mass (kDa) | Elution Peak Concentration (μM) | % Tetrmer |
|----------------|-----------------------------|---------------------------------|----------|
| CtBP2 (31-445) |                             |                                 |          |
| wild-type      | 185                         | 2.8                             | 80       |
| A129L          | 100                         | 3.3                             | 4        |
| A129V          | 105                         | 3.5                             | 1        |
| L221Y          | 102                         | 3.5                             | 2        |
| S128T          | 99                          | 3.4                             | 0.5      |
| G216N          | 94                          | 2.9                             | 0        |
| R190Q          | 94                          | 2.0                             | 0        |
| CtBP1 (28-440) |                             |                                 |          |
| wild-type      | 181                         | 2.3                             | 86       |
| A123L          | 132                         | 3.3                             | 25       |
| A123V          | 125                         | 2.3                             | 20       |
| L215Y          | 90                          | 1.8                             | 0        |
| A122T          | 100                         | 3.6                             | 3        |
| G210N          | 86.8                        | 1.4                             | 0        |
| R184Q          | 83.6                        | 2.2                             | 0        |
### Table 4: Crystallographic data collection and refinement statistics

|                        | CtBP1 (28-378) wild-type | CtBP1 (28-378) A123V |
|------------------------|--------------------------|----------------------|
| **PDB Code**           | 6CDF                     | 6CDR                 |
| **Data Collection**    |                          |                      |
| Space Group            | P6$_4$22                 | P6$_4$22             |
| a, b, c (Å), α, β, γ (°) | 88.66, 88.6, 163.91, 90, 90, 120 | 89.22, 89.22, 164.23, 90, 90, 120 |
| Bragg Spacings (Å) 1 | 50. – 2.6 (2.693-2.6)    | 50. – 2.4 (2.485-2.4) |
| R$_{merge}$            | 0.0453 (0.2695)          | 0.0655 (0.4668)      |
| I/σI                  | 30.5 (6.3)               | 27.9 (5.3)           |
| Completeness (%)       | 95.8 (88.7)              | 93.3 (99.8)          |
| Redundancy             | 7.8 (6.8)                | 12.8 (10.4)          |
| Total Reflections      | 93207 (7207)             | 193860 (15932)       |
| Unique Reflections     | 11944 (1056)             | 15090 (1537)         |
| **Refinement**         |                          |                      |
| R$_{work}$/R$_{free}$  | 0.209 / 0.243            | 0.235 / 0.258        |
| # non-hydrogen atoms   |                          |                      |
| Protein                | 2578                     | 2580                 |
| Ligands                | 48                       | 65                   |
| Solvent                | 115                      | 101                  |
| Mean B-factors (Å$^2$) |                          |                      |
| Protein                | 46.4                     | 44.2                 |
| Ligands                | 36.4                     | 38.1                 |
| Solvent                | 40.3                     | 42.1                 |
| r.m.s. deviations      |                          |                      |
| Bond lengths (Å)       | 0.003                    | 0.003                |
| Bond Angles (°)        | 0.67                     | 0.61                 |
| Ramachandran (%)       |                          |                      |
| Favored                | 95.2                     | 95.8                 |
| Allowed                | 4.8                      | 4.2                  |
| Outliers               | 0                        | 0                    |

1Highest resolution shell shown in parenthesis.
**FIGURES**

**Figure 1.** SEC trace and MALS molecular masses for the previously crystallized CtBP2 (31-364) construct. (A) and long (31-445) construct (B) at various levels of NADH. The continuous lines show the light scattering Rayleigh Ratio (arbitrary units) for protein elution from the SEC column and the small diamonds show the molecular mass measured by MALS measurements for the elution peaks. A) Addition of NADH to 10µM results in earlier elution from the SEC column and an increase in molecular mass from 106 kDa to 132 kDa (Table 1). B) Assembly as a function of NADH concentration is more pronounced for the CtBP construct including the full C-terminus, with molecular masses of 142 kDa in the absence of NADH increasing to 182 kDa at 10 µM NADH (Table 2). (Multiple measurements demonstrate that these results are fully reproducible.)
Figure 2. Dependence of MALS determined CtBP molecular masses as a function of NAD(H) concentration (see Tables 1 and 2). The approximate molecular masses expected for a pure dimer (left) and tetramer (right) are indicated. The data were fit with Prism 7 (see Experimental Procedures). A) MALS measured weight average molecular masses ($M_w$) for the crystallized constructs of CtBP1 and CtBP2 as a function of NADH concentration. In the absence of NADH, the $M_w$ of CtBP1 (28-353) of 62kDa indicates a mixture of monomer and dimer, this value rises to 128kDa consistent with about 46% tetramer in the presence of 10µM NADH. For CtBP2 (31-364) the $M_w$ of 106 kDa is consistent with a mixture of mostly dimer and about 18% tetramer, which rises to about 50% tetramer in the presence of 10µM NADH. B) MALS determined weight average molecular masses ($M_w$) for constructs of CtBP1 (28-440) and CtBP2 (31-445) with the full C-terminus as a function of NADH and NAD$^+$. (These experiments carried out in triplicate – error bars are shown, but are often smaller than the data point shown.) In the absence of coenzyme, CtBP1 has a $M_w$ consistent with about 15% tetramer, whereas CtBP2 has an $M_w$ consistent with about 30% tetramer. These values rise to 61 and 66% tetramer in the presence of 10µM NAD$^+$ for CtBP1 and CtBP2, respectively and to 79 and 75% tetramer in the presence of 10µM NADH for CtBP1 and CtBP2, respectively.
Figure 3. Crystallographic structures of tetramers for CtBP1 (43) and CtBP2 (37). A) Arrangement of four subunits of CtBP1 (PDB ID 4U6Q), related by crystallographic symmetry in the space group P6\(_{4}22\) form a tetramer. The main-chain trace is shown in magenta for two subunits and cyan for two subunits, except for the C-terminal residues, which are displayed in orange. A stick model of the NAD(H) is shown with yellow carbon atoms. B & C) Two crystallographically independent tetramers in the asymmetric unit of CtBP2 (PDB ID 4LCJ). Note the remarkably similar arrangement of subunits in these three crystallographically independent tetramers, each subunit of which projects its C-terminus towards a partner subunit across the interdimer interface. D, E, & F) Zoomed in regions of the interdimer contact for CtBP2 tetramer #1. Each of these regions was investigated by site-directed mutagenesis (Fig. 4).
Figure 4. SEC trace and MALS molecular masses for mutants of (A) CtBP2 (31-445) and (B) CtBP1 (28-440) in the presence of 10µM NADH. (See also Table 3.) The continuous lines show the light scattering Rayleigh Ratio (arbitrary units) for protein elution from the SEC column and the small diamonds show the molecular mass measured by MALS measurements for the elution peaks. The MALS determined weight average molecular mass ($M_w$) is shown in parentheses for wild-type and each mutant. (A) The six CtBP2 mutants show similar elution volumes and molecular masses that range from 94-103 kDa, consistent with primarily dimeric species. (B) The equivalent six CtBP1 mutants show a greater range of elution volumes that may result from partial proteolysis within the C-terminal region. Molecular masses for the four mutants whose wild-type side-chains point directly into the interdimer interface range from 84 to 100 kDa and are consistent with primarily dimeric species. The two hinge region mutants that point into the subunit, A123V and A123L have molecular masses of 126-132 kDa suggesting some tetrameric species (20-25%), but with the majority of molecules as dimeric. (MALS measurements on all mutants have been carried out multiple times and are fully reproducible.)
Figure 5. Crystal structure of CtBP1 (28-378). (A) Trace of the CtBP1 tetramer, with two subunits in magenta and two in cyan. Shown with orange carbon atoms is a stick rendition of the additional four residues observed compared with the previous CtBP1 (28-353) structures. (B) F_o-F_c difference electron density map shown in cyan at a +2σ level for the four additional residues (D354-T357) observed.
Figure 6. Crystal structure of CtBP1 (28-378) A123V compared with the structure of wild-type CtBP1 (28-378). (A) Trace of the CtBP1 tetramer with the $F_o$($A123V$) - $F_o$(WT) map contoured at $+4\sigma$ (blue) and $-4\sigma$ (red). The electron density is localized to the mutant region (black rectangles) with the exception of a negative density peak (red) near the center which appears to be an unidentified solvent molecule apparently present in the wild-type but not in the A123V structure. (B) Stereoview of the $F_o$($A123V$) - $F_o$(WT) electron density (blue $+4\sigma$, red $-4\sigma$) in the region of the mutation. Note the positive density for the two methyl carbon atoms of the mutant Val, which induces movement of E326 towards the left and upward taking with it the mainchain of helix $\alpha$5 to which it is attached. There is no evidence of significant movement in the partner subunit, despite the MALS data showing a weakening of the tetramer with the A123V mutation.
