Structural Evidence That Brain Cyclic Nucleotide Phosphodiesterase Is a Member of the 2H Phosphodiesterase Superfamily*

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2',3'-Cyclic-nucleotide 3'-phosphodiesterase (CNP) is an enzyme abundantly present in the central nervous system of mammals and some vertebrates. In vitro, CNP specifically catalyzes the hydrolysis of 2',3'-cyclic nucleotides to produce 2'-nucleotides, but the physiologically relevant in vivo substrate remains obscure. Here, we report the medium resolution NMR structure of the catalytic domain of rat CNP with phosphate bound and describe its binding to CNP inhibitors. The structure has a bilobal arrangement of two modules, each consisting of a four-stranded β-sheet and two α-helices. The β-sheets form a large cavity containing a number of positively charged and aromatic residues. The structure is similar to those of the cyclic phosphodiesterase from Arabidopsis thaliana and the 2'-5' RNA ligase from Thermus thermophilus, placing CNP in the superfamily of 2H phosphodiesterases that contain two tetrapeptide HX(T/S)IαX motifs. NMR titrations of the CNP catalytic domain with inhibitors and kinetic studies of site-directed mutants reveal a protein conformational change that occurs upon binding.

The abundance of the enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP)EC 3.1.4.37) in the central nervous system of all mammals and some other vertebrates such as amphibians and birds has long been an enigma. This derives from the continuing failure to identify a physiological substrate for this enzyme. CNP has an apparent specificity for nucleoside 2',3'-cyclic phosphate, which it cleaves to 2'-nucleotide end products, none of which (with the exception of NADP/NADPH) are found in metabolite pools. The last 4 decades of research have failed to attribute a function to this protein, although many possibilities have been considered (extensively reviewed in Refs. 1–3). More recently, RICH, a neuronally associated homolog of CNP, has been discovered in fish (4, 5), and the catalytic active site of CNP has been investigated (6).

CNP and RICH share catalytic features with three other groups of enzymes: fungal/plant RNA ligases involved in tRNA splicing (7, 8), bacterial and archaeal RNA ligases (9) that ligate tRNA half-molecules containing 2',3'-cyclic phosphate and 5'-hydroxyl termini, and plant and yeast cyclic phosphodiesterases (CPDases) that hydrolyze ADP-ribose 1',2'-cyclic phosphate to yield ADP-ribose 1'-phosphate (at least one of these latter enzymes also hydrolyzes nucleoside 2',3'-cyclic phosphates) (10, 11). These enzymes are thought to play a role in the tRNA-splicing pathways. The x-ray structures of a CP-Dase from Arabidopsis thaliana (12–14) and, most recently, 2',5'-RNA ligase from Thermus thermophilus (15) have been determined.

Members of this enzyme superfamily occur across a vast range of organisms ranging from bacteria to mammals. It has been suggested (16) that all four classes of enzymes originated from a common ancestor because they all have two similarly spaced histidine-containing tetrapeptides; their catalytic domains have a similar size of ~200 residues with similar pattern of predicted secondary structural elements; and they all catalyze hydrolysis of either 2',3'-cyclic phosphates to 2'-phosphates or 1',2'-cyclic phosphate to 1'-phosphate. Recently, new members of this superfamily have been identified (17).

Investigations of CNP have provided a variety of observations concerning the relationship of CNP to the cytoskeleton and its localization to discrete regions of oligodendrocytes and paranodal compartments of the myelin sheath, adjacent to the axon (18–25). CNP comprises ~4% of the central nervous system total myelin protein and is most abundant in oligodendrocytes. Recently, it has been reported that CNP binds to tubulin and that it may play a role in anchoring microtubules to the plasma membrane as well as in regulating tubulin polymerization (26). A second isoform (CNP2) has also been identified, which contains a unique 20-amino acid N-terminal domain that targets the protein to mitochondria (27). Also, recent studies on CNP-null mutant mice revealed that the absence of CNP causes axonal swelling and neuronal degeneration (28). These observations underline the importance of this enzyme in brain and point to a multifaceted role for CNP in myelinationogenesis and the maintenance of the myelin-axonal interface.

Here, we describe the structure of the brain CNP catalytic domain as determined by NMR and show that it is highly similar to the plant CPDase and the archaeabacterial RNA...
ligase despite low overall similarity in amino acid sequence. This work brings us a step closer to understanding the function of CNP and its evolutionarily conserved enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The catalytic fragment of CNP (CNP-CF, residues 164–378) was subcloned into pET15b (Novagen, Madison, WI) and expressed in the *Escherichia coli* expression host BL21(DE3) (Stratagene) as a His-tagged fusion protein. The protein was purified by immobilized metal affinity chromatography on a Ni^2+/-H^1001-loaded chelating Sepharose column (Amersham Biosciences). Isotopically labeled CNP-CF was prepared from cells grown on minimal M9 medium containing [15N]ammonium chloride and/or [13C]glucose (Cambridge Isotopes Laboratory, Andover, MA). For the backbone assignments, partially deuterated triple-labeled (2H, 15N, 13C) CNP-CF was produced by expressing the protein in 90% D2O- and 10% H2O-containing minimal M9 medium. The N-terminal His tag was cleaved from CNP-CF by overnight dialysis with thrombin (Amersham Biosciences) at 1 unit/mg of fusion protein at room temperature. Benzamidine-Sepharose and Ni^2+/-H^11001-loaded chelating Sepharose were used to remove thrombin and the His tag peptide from CNP-CF. The resulting 219-amino acid protein contained four extraneous residues from the His tag. The sequence composition of purified CNP-CF was confirmed by mass spectrometry.

CNP catalytic fragment mutants were created by overlap extension PCR using the Expand High Fidelity PCR system (Roche Diagnostics) (29). The mismatched oligonucleotide sequences used to generate the mutants were as follows (only the sense oligonucleotides are listed): T232A (ACA to GCA), 5'-GTG CGT CAC TGT **GCA** ACC AAA TTC TGT-3'; D237V (GAC to GTC), ACC AAA TTC TGT **GTC** TAC GGG AAG GCC-3'; G276A (GGG to GCA), 5'-CCC AAG ACA GCT **GCA** GCC CAG GTG GTG-3'; A308G (GCT to GGA), 5'-CCA GGG AGC CGA **GGA** CAT GTC ACC CTA-3'; T311A (ACC to GCG), 5'-AGC CGA GCT CAC **GTC** GGG AAG GCC-3'; Q322A (CAG to GCC), 5'-GTG CAG CCA GTG **GCC** ACA GGC CTT GAC-3'; G324A (GGC to GCG), 5'-CCA GTG CAG ACG **GCC** CTA GAC-3'; and Y376A (TAC to GCT), 5'-TTC ACG GGG **GCT** TAT GGG TGA GGA TCC ATT AT-3. The boldface underlined sequences correspond to the mutated codons. The authenticity of the substitutions and the absence of any undesired mutations were confirmed by sequence analysis. The CNP-CF histidine mutants (H230L and H309L) were generated as previously described (6).

**NMR Spectroscopy**—NMR resonance assignments of the catalytic fragment of CNP were determined previously (30). All NMR experiments were recorded at 310 K. NMR samples were 1–2 mM protein in 50 mM sodium phosphate buffer, 0.15 M NaCl, 1 mM diithiothreitol, and 0.1 mM sodium azide at pH 6.0. Attempts to use Pf1 phage or compressed polyacrylamide to obtain residual dipolar coupling constraints were not successful. Nuclear Overhauser effect correlation spectroscopy (NOESY) constraints for the structure determination were obtained from **15N**-edited NOESY (mixing time of 100 ms) and **13C**-edited NOESY (2H phosphodiesterase superfamily).

![Structure of the catalytic fragment of CNP](https://www.jbc.org/)

**Fig. 1. Structure of the catalytic fragment of CNP.** a, the backbone superposition of the 20 lowest energy structures. The superposition was done using regions Phe^169–His^150 and Val^228–Leu^372. b, ribbon representation of CNP-CF showing locations of conserved residues from tetrapeptide HX/T/S/I motifs. C-term and N-term, C and N termini, respectively. c, very similar topology of CNP-CF (upper), *A. thaliana* CPDase (middle), and *T. thermophilus* RNA ligase (lower) from the 2H phosphodiesterase superfamily.
Solution Structure of CNP

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TABLE I

Structural statistics for the CNP catalytic fragment

| Restrains for structure calculations | Total restraints used |
|-------------------------------------|-----------------------|
| Total NOE restraints                 | 2338                  |
| Intraresidual                       | 1925                  |
| Sequential (i-j) = 1                | 763                   |
| Medium range (1 < |j| < 5)              | 523                   |
| Long range (|i-j| ≥ 5)         | 264                   |
| Hydrogen bond restraints             | 375                   |
| Dihedral angle restraints            | 87                    |
| Distance deviations (Å)              | 0.021 ± 0.0007        |
| Dihedral deviations                  | 1.055 ± 0.0566*       |
| Deviations from idealized geometry   |                       |
| Bonds (Å)                           | 0.0922 ± 0.0001       |
| Angles                               | 0.4107 ± 0.0062*      |
| Improvers                           | 0.3929 ± 0.0105*      |

* Root mean square.

CNP Belongs to the Superfamily of 2H Phosphodiesterases—We determined the structure of the catalytic fragment of rat brain CNP (CNP-CF), the first of a vertebrate-specific 2',3'-cyclic nucleotide 3'-phosphodiesterase (Fig. 1). The previously reported resonance assignments (30) were used to assign NOEs from 15N- and 13C-edited three-dimensional NOEY experiments. The 20 lowest energy structures of 60 calculated were chosen to represent the final ensemble. The structural statistics are shown in Table I. On average, 10.9 constraints per residue were used to calculate the CNP-CF structure. This is below the typical number of 15–20 constraints per residue in high resolution NMR structures and results from the number of unresolved overlapping NOE's and a lower sensitivity of NOEY experiments because of the relatively large protein molecular mass (24.3 kDa). The tendency of CNP-CF to aggregate also limited the protein concentration in NMR samples.

The structure shows a bilobal arrangement of two modules, each consisting of a four-stranded antiparallel β-sheet and two antiparallel α-helices located on the outer part of the modules (Fig. 1b). The first lobe consists of strands β1, β2, β6, and β7 and helices α2 and α3, whereas the second one consists of strands β3, β4, β5, and β8 and helices α1 and α4. The internal face of the modules forms a large cavity. Intense peaks in the 1H-15N HSQC spectrum of CNP-CF indicate a flexible backbone for residues from Gly208 to Lys214. Coupled with low NOE density for this region, this loop between helix α1 and strand β2 appears to be mobile.

The structure has a striking similarity to CDPase from A. thaliana (12) and to 2'-5' RNA ligase from T. thermophillus (15). Topologically, CNP-CF differs only in an extra C-terminal strand β8 that extends the antiparallel β-sheet containing strand β3 (Fig. 1c). One possible role of this β-strand is to stabilize the N and C termini on the opposite sides of the CNP domain and to position the C-terminal isoprenylation site at the membrane. The structural similarity of CNP-CF provides direct evidence that CNP belongs to the superfamily of phosphodiesterases containing dual catalytic tetrapeptide His/T/Sx motifs.

CNP-CF has a more open cavity than CDPase. This difference suggests that a larger natural ligand could exist for CNP. Sequence comparison (Fig. 2) provides possible explanations for the more closed CDPase structure. The turns between strands β3–β4 and β6–β7 in CDPase make hydrophobic contacts with each other via side chains of Phe84 and Leu168 and negatively charged ligand. Interestingly, the 2'–5' RNA ligase has hydrophobic turns and an open conformation similar to that of CNP. This conformation likely allows RNA to access the catalytic site (15).

Binding of CNP Inhibitors—To obtain more information about the active site of CNP, we titrated 15N-labeled CNP-CF...
with several compounds previously shown to inhibit CNP activity (reviewed in Ref. 1). These included orthophosphate, pyrophosphate, 2\(^{-}\)H\(^{-}\)AMP, 5\(^{-}\)H\(^{-}\)AMP, NAD, and NADP. The titrations were monitored by \(\text{H}^{15}\)N correlation spectroscopy, and shifts of amide signals as a function of ligand addition were recorded. These shifts act as a fingerprint and identify amino acid residues affected by binding (Fig. 3a).

Titration of the catalytic fragment of CNP with orthophosphate resulted in chemical shift changes, indicating that it binds to CNP and was present in the structure determined by NMR. The biggest \(\text{H}^{1}\) and \(\text{H}^{15}\) amide chemical shift changes were observed for Thr\(^{232}\) (0.56), Thr\(^{311}\) (0.53), Gly\(^{324}\) (0.24), Val\(^{228}\) (0.21), Ala\(^{308}\) (0.17), His\(^{230}\) (0.16), Gly\(^{305}\) (0.15), and Thr\(^{323}\) (0.15). Thr\(^{232}\), Thr\(^{311}\), and His\(^{230}\) are part of the tetrapeptide motifs, which are important for the catalytic activity. This shows that the phosphate group binds in the active site. The catalytic threonines, Thr\(^{232}\) and Thr\(^{323}\), likely coordinate the phosphate moiety through hydrogen bonds. The chemical shift changes correlate with the regions of highest sequence conservation in the catalytic domains of CNP from different species (Fig. 4b).

Interestingly, CNP-inhibitor interactions were pH-dependent. The chemical shift changes upon phosphate binding were much smaller at pH 6.5 (and above) than at pH 6.0 (data not shown). The likely reason for this is deprotonation of the catalytic histidines or the phosphoryl group. This would change the electrostatic charges and interfere with hydrogen bonding to the phosphate ion. In support of this, the CNP enzymatic activity was optimal at pH 5.5–6.5 and decreased at higher pH (data not shown). Whether this reflects protonation of active-site histidine(s) or the substrate phosphoryl group remains to be determined.

The binding of AMPs resulted in a pattern of chemical shift changes very similar to that observed upon binding of phosphate (Fig. 3a). The relatively larger shifts observed for AMPs reflect the stronger binding to CNP-CF. The AMP titrations also allowed us to identify additional residues affected by binding. Located in the loop between strand\(^{2}\) and helix\(^{2}\), Cys\(^{236}\) and Asp\(^{237}\) showed minor chemical shift changes. These residues are relatively close to the tetrapeptide motifs and could participate in substrate recognition by interacting with the mobile loop Gly\(^{208}\)–Lys\(^{214}\). Speculatively, Asp\(^{237}\) could interact with either Lys\(^{212}\) or Lys\(^{214}\) to close the mobile loop upon substrate binding. However, this loop is poorly conserved among mammalian CNPs (Fig. 4b) and shows relatively small changes upon inhibitor binding (Fig. 3a), suggesting that either the loop does not function as a flap or that it needs the proper substrate for specific loop-ligand interactions.

The titration experiments also allowed us to compare binding affinities of CNP inhibitors. In the weak binding (fast exchange) regime, the signals in HSQC spectra gradually move from the unbound position to the fully bound position depending on the amount of inhibitor added. These changes can be fitted using the binding equation to estimate the dissociation constant \((K_d)\). The dissociation constants obtained from NMR titrations are shown in Table II. The results show that 3\(^{-}\)H\(^{-}\)AMP has the highest affinity for the CNP catalytic domain, followed by 2\(^{-}\)H\(^{-}\)AMP and 5\(^{-}\)H\(^{-}\)AMP. This might reflect differences in the \(pK_a\) of the phosphoryl group in the different AMPs or may be an inherent property of the CNP catalytic site. Studies at a second pH are needed to resolve the issue. Comparison of NAD and NADP shows that a terminal phosphate significantly improves binding affinity.

**Kinetic Properties of Active-site Mutants**—To define the enzymatic role of the conserved tetrapeptide motif residues, we mutated each residue individually and measured the kinetic

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### Table II: Dissociation Constants for CNP Inhibitors

| Compound | Dissociation Constant (nM) |
|----------|---------------------------|
| 3\(^{-}\)H\(^{-}\)AMP | 0.1 |
| 2\(^{-}\)H\(^{-}\)AMP | 0.2 |
| 5\(^{-}\)H\(^{-}\)AMP | 0.5 |
| NAD | 1.0 |
| NADP | 2.0 |

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**Fig. 2.** The catalytic domains of rat and human CNPs and the phosphodiesterase domain of the RICH70 protein from goldfish (g) show low sequence homology to CPDase from *A. thaliana (A. th.)* and RNA ligase from *T. thermophilus (T. th.)*. The secondary structural elements refer to rat CNP-CF. The conserved catalytic residues are indicated in **boldface**.
Fig. 3. a, chemical shift perturbation plots of the $^{15}$N-labeled catalytic fragment of CNP upon titration with KH$_2$PO$_4$ (upper left), the A$_6$ oligonucleotide (upper right), 2'-AMP (lower left), and 3'-AMP (lower right). The same regions of CNP-CF were affected with a similar magnitude of changes. b, Lineweaver-Burk plot showing hydrolysis of 2',3'-cyclic NADP (cNADP) by CNP-CF in the absence of AMP analogs (○) and in the presence of 1.5 mM 5'-AMP (■), 0.75 mM 2'-AMP (△), and 0.5 mM 3'-AMP (▲). The plots exhibit apparent $K_m$ values of 230 μM in the absence of cAMP and 591, 700, and 775 μM in the presence of 5'-AMP, 2'-AMP, and 3'-AMP, respectively. The unaltered $V_{max}$ value of ~38 μM/min indicates that the AMP analogs act as competitive inhibitors.
suggest that Thr^{232} is more critical for substrate binding, whereas Thr^{311} is more important for catalysis. Parallel studies with yeast CPDase showed that mutation of Ser/Thr residues in the tetrapeptide motifs has minor effects on enzymatic activity and suggested that the residues play a larger role in substrate recognition (16).

Interestingly, mutation of the non-active site residue Gly^{324} (helix α4) in CNP resulted in a 5-fold increase in $K_m$ and 37-fold decrease in $k_{cat}$. Mutations of other proximal residues on helix α4 such as Gln^{322} and Leu^{327} did not affect the kinetic parameters. These results agree with the NMR titration data (see below) that suggest that Gly^{324} is important for the conformation of helix α4. The other remaining mutants (D237V, G276A, A308G, and Y376A) displayed wild-type kinetic parameters, indicating that these residues are not particularly important for CNP activity.

**Inhibitor Binding Causes a Conformational Change in the CNP Catalytic Domain**—NMR titrations showed ligand-induced chemical shift changes in the residues around Gln^{324} (Fig. 3a). These residues are located in helix α4 and relatively far from the active site. This could represent a second binding site or a minor conformational change that affects these residues through contacts with strand β5 in the active site.

To distinguish between these possibilities, we compared the dissociation constants obtained by NMR for two residues, Thr^{232} and Gly^{324}, using both weak (orthophosphate) and high affinity (2'-AMP) inhibitors (Table II). Identical $K_d$ values were observed for both residues, indicating a concerted conformational change that arises from a single binding site. It is very unlikely that two distinct binding sites would demonstrate identical $K_d$ values for both substrates. In helix α4, the most affected residues are Thr^{232} and Gly^{324}. These amino acid types are less common in α-helices, and we speculate that the conformational change observed is elongation of the N-terminal part of helix α4 in the presence of bound substrate.

The CNP Catalytic Fragment Weakly Binds Hexanucleotide RNA—An interesting feature of the CNP catalytic fragment is the presence of several aromatic (Tyr^{167}, Phe^{172}, Phe^{235}, and Tyr^{52}) and positively charged (Lys^{214}, Lys^{234}, and Arg^{307}) residues in the vicinity of the active site (Fig. 5). The majority of these residues are located on the N-terminal lobe (strands β1, β2, and β7). β-Sheets with an abundance of positively charged and aromatic residues are common RNA-binding surfaces (for a review, see Ref. 37). The presence of these residues, coupled with a large binding cavity, hints that RNA may be a CNP substrate. Furthermore, many proteins from the 2F phosphodiesterase superfamily are involved in RNA-processing pathways, and some of them bind RNA (17). To test this hypothesis, we titrated CNP-CF with an RNA hexanucleotide (A₃). The

### Table II

Affinity constants for CNP-CF interactions with inhibitors as determined by NMR titration ($K_d$) and enzyme assays ($K_i$)

| Inhibitor | $K_d\,\text{mM}$ | $K_i\,\text{mM}$ |
|-----------|-----------------|-----------------|
| K$_{HPO_4}$ | 12.90 ± 1.85 (Thr^{232}) | 11.54 ± 1.95 (Gln^{324}) |
| Na$_{H_2P_2O_7}$ | 15.38 ± 2.77 (Thr^{232}) | 1.40 ± 0.14 (Thr^{232}) |
| 2'-AMP | 1.40 ± 0.14 (Thr^{232}) | 0.417 ± 0.012 |
| 3'-AMP | 0.57 ± 0.04 (Thr^{232}) | 0.239 ± 0.013 |
| NADP | 0.57 ± 0.04 (Thr^{232}) | 1.10 ± 0.14 |
| NAD | 0.52 ± 0.04 (Thr^{232}) | 1.10 ± 0.14 |
| $A_0$ | 21.19 ± 0.72 (Thr^{232}) | 0.80 |

### Table III

Kinetic parameters for the CNP-CF mutants

| CNP-CF | $K_{cat}$/NAMP | $k_{cat}$ | Relative $k_{cat}/K_m$ |
|--------|---------------|----------|-----------------------|
| Wild-type | 268 ± 6 | 928 ± 36 | 1 |
| H230L | 207 ± 20 | 0.06 ± 0.001 | 0.0006 |
| H309L | 176 ± 27 | 0.06 ± 0.002 | 0.0001 |
| T232A | 2238 ± 277 | 0.40 ± 0.005 | 0.0001 |
| T311A | 631 ± 13 | 1.4 ± 0.01 | 0.0006 |
| D237V | 403 ± 11 | 770 ± 7 | 0.54 |
| G276A | 745 ± 13 | 261 ± 30 | 0.11 |
| A308G | 335 ± 8 | 572 ± 24 | 0.49 |
| Q322A | 299 ± 36 | 659 ± 19 | 0.63 |
| G324A | 1210 ± 114 | 25 ± 6 | 0.01 |
| L327A | 487 ± 74 | 611 ± 10 | 0.37 |
| Y376A | 458 ± 42 | 930 ± 37 | 0.57 |

* cNADP, cyclic NADP.

* The $k_{cat}/K_m$ for wild-type CNP-CF is 3.5 μM$^{-1}$ s$^{-1}$. 
weak, millimolar affinity for the CNP catalytic domain, we cannot rule out 1) that CNP might bind only to double-stranded nucleic acids or to a specific RNA sequence, 2) that a 3’- or 5’-terminal phosphate might be important for binding, or 3) that the N-terminal portion of CNP may regulate binding or affect binding activity.

In considering the possible cellular functions of CNP, it is important to take into account other information such as the cellular localization of this enzyme. Both CNP isoforms are membrane-associated via isoprenylation at their C termini (21). The larger isoform of CNP, CNP2, contains a mitochondrial targeting sequence at the N terminus (27). CNP is also known to interact with tubulin (26), leading to the speculation that CNP might have a role in mRNA transport as observed in oligodendrocytes (40–42). Proteins such as myelin basic protein and carboxylic anhydrase (43, 44) and tau (45) are specifically synthesized at the periphery of the myelin-forming processes; some type of specific mRNA transport and localization machineries must exist. In addition, there are signaling molecules such as nicotinic acid-adenine dinucleotide phosphate and cADP-ribose (reviewed in Ref. 46) upon which CNP might potentially act. Future structural and enzyme studies of CNP will hopefully clarify the cellular function of this highly conserved yet enigmatic protein.

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Fig. 5. Vicinity of the catalytic site of CNP-CF. The region around the conserved HAT/SXH motifs is abundant with aromatic and positively charged residues. The proposed location of the bound phosphate ion is shown in gray.
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