Identification of Essential Residues in 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase

CHEMICAL MODIFICATION AND SITE-DIRECTED MUTAGENESIS TO INVESTIGATE THE ROLE OF CYSTEINE AND HISTIDINE RESIDUES IN ENZYMATIC ACTIVITY*

Received for publication, October 16, 2000, and in revised form, February 1, 2001
Published, JBC Papers in Press, February 5, 2001, DOI 10.1074/jbc.M009434200

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2',3'-Cyclic nucleotide 3'-phosphodiesterase (CNP; EC 3.1.4.37) catalyzes in vitro hydrolysis of 3'-phosphodiester bonds in 2',3'-cyclic nucleotides to produce 2'-nucleotides exclusively. N-terminal deletion mapping of the C-terminal two-thirds of recombinant rat CNP1 identified a region that possesses the catalytic domain, with further truncations abolishing activity. Protein and kinetic analysis indicated that this domain forms a compact globular structure and contains all of the catalytically essential features. Subsequently, this catalytic fragment of CNP1 (CNP-CF) was used for chemical modification studies to identify amino acid residues essential for activity. 5,5'-Dithiobis-(2-nitrobenzoic acid) modification studies and kinetic analysis of cysteine CNP-CF mutants revealed the nonessential role of cysteines for enzymatic activity. On the other hand, modification studies with diethyl pyrocarbonate indicated that two histidines are essential for CNPase activity. Consequently, the only two conserved histidines, His-230 and His-309, were mutated to phenylalanine and leucine. All four histidine mutants had Kcat values 1000-fold lower than wild-type CNP-CF, but Km values were similar. Circular dichroism studies demonstrated that the low catalytic activities of the histidine mutants were not due to gross changes in secondary structure. Taken together, these results demonstrate that both histidines assume critical roles for catalysis.

* This work was supported by a grant from the Medical Research Council of Canada (MRC). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a studentship from the MRC and the Fonds pour la Formation de Chercheurs et l’Aide à la Recherche.

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optic nerve, particularly during nerve regeneration (29, 31), in contrast to expression of CNP by oligodendrocytes, both cells share some biological features, such as process extension and rapid, abundant membrane synthesis. These similarities suggest that CNP and RICH may operate by similar physiological mechanisms involving this enzymatic activity.

As a prerequisite in our overall strategy to fully address the physiological relevance for CNPase activity in oligodendrocytes using CNPase inactive mutants for cell biological studies, we sought to identify amino acid residues critical for enzymatic activity. Although nothing is known about the active site structure and the catalytic mechanism of CNP to suggest candidate residues, recent site-directed mutagenesis studies of zebrafish RICH suggested that a conserved histidine residue in both CNP and RICH proteins was critical for enzymatic activity (29). Furthermore, there was evidence to suggest that cysteine(s) may be essential for activity, based on the observation that CNP was inactivated by inorganic mercurials (2, 3). Accordingly, in this paper, we have undertaken a study of shared motifs that comprise part of the catalytic domains of both CNP and RICH proteins and of specific cysteines and histidines within them, employing chemical modification and site-directed mutagenesis. We report here that, whereas cysteines are within them, employing chemical modification and site-directed mutagenesis. We report here that, whereas cysteines are not essential for enzymatic activity, two conserved histidine residues in CNP and RICH proteins, corresponding to His-230 and His-309 in rat CNP1, are essential for catalysis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pure 2',3'-cyclic NADP (cNADP) was prepared as described by Sogin (17) using NADP (disodium salt) from Roche Molecular Biochemicals and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) as described by Sogin (17) using NADP (disodium salt) from Roche Molecular Biochemicals and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide—carbodiimide—carbodiimide—HCl from Sigma. DEPC, DNB, MMTS, 2-AP, N-glucose-6-phosphate (monosodium salt), hydroxyamine, ampicillin, and phenylmethylsulfonyl fluoride were purchased from Sigma. Lysylase (hen egg white), glucose-6-phosphate dehydrogenase (yeast), pancreatic elastase (pig), and thrombin (human plasma) were obtained from Calbiochem. Potassium cyanide (KCN) was from Fisher, and iodoacetamide was from Promega. DNase I was from Boehringer Mannheim. As a prerequisite in our overall strategy to fully address the physiological relevance for CNPase activity in oligodendrocytes using CNPase inactive mutants for cell biological studies, we sought to identify amino acid residues critical for enzymatic activity. Although nothing is known about the active site structure and the catalytic mechanism of CNP to suggest candidate residues, recent site-directed mutagenesis studies of zebrafish RICH suggested that a conserved histidine residue in both CNP and RICH proteins was critical for enzymatic activity (29). Furthermore, there was evidence to suggest that cysteine(s) may be essential for activity, based on the observation that CNP was inactivated by inorganic mercurials (2, 3). Accordingly, in this paper, we have undertaken a study of shared motifs that comprise part of the catalytic domains of both CNP and RICH proteins and of specific cysteines and histidines within them, employing chemical modification and site-directed mutagenesis. We report here that, whereas cysteines are not essential for enzymatic activity, two conserved histidine residues in CNP and RICH proteins, corresponding to His-230 and His-309 in rat CNP1, are essential for catalysis.

**Plasmid Description**—All recombinant CNP expression vectors were generated from the rat CNP1 and CNP2 cDNA clones (14, 32). First, a BamHI restriction site was introduced into the noncoding region of the rat CNP1 and CNP2 cDNA clones (14, 32). The authenticity of the substitutions and absence of any vector content were pooled and dialyzed overnight against phosphate-buffered saline. CNPase activity of full-length GST-CNP1 and GST-CNP1 ND mutants were examined. GST-tagged CNP1 N-terminal deletion (GST-CNP1 ND) mutants were constructed using standard protocols. For pET-15b CNP constructs, E. coli BL21 (DE3) (Stratagene, La Jolla, CA) cells were used for transformation. All proteins were expressed in the following manner. A single colony was transferred to LB medium containing 100 μg/ml ampicillin and shaken overnight at 37 °C. 2× YT medium supplemented with one-hundredth of its volume of overnight culture and grown at 37 °C to an A500 of ~1.0. Protein expression was induced by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside, and the culture was grown for an additional 6 h at 37 °C. Cells were harvested by centrifugation at 5000 × g for 15 min, and the cell pellet was stored at −80 °C until ready for use.

All operations described below were carried out at 4 °C unless otherwise indicated. GST-tagged CNP1 N-terminal deletion (GST-CNP1 ND) mutants were purified as follows. Cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA, 0.5 mM l-lysine, and 0.1 mM phenylmethylsulfonyl fluoride and stirred for 30 min. DTT (5 mM) and Triton X-100 (1%) were added to the cell suspension, followed by sonication on ice (30-s bursts, each separated by a 1-min cooling period) until the cell suspension was no longer viscous. The lysate was centrifuged at 14,000 × g for 30 min, and the soluble extract was incubated with glutathione-agarose at 4 °C for 1 h with agitation. Resin was transferred to an empty column and washed extensively with phosphate-buffered saline. Bound proteins were eluted by the addition of 3× 1 bed volume of 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 20 mM reduced glutathione. Fractions with high protein content were pooled and dialyzed overnight against phosphate-buffered saline. CNPase activity of full-length GST-CNP1 and GST-CNP1 ND mutants were measured.

Purified full-length CNP1 and CNP-CF (wild type and mutants) were obtained as follows. Cell pellet was resuspended in 50 mM sodium phosphate buffer (pH 8.0) containing 1 mM EDTA, 0.5 mM l-lysine, and 0.1 mM phenylmethylsulfonyl fluoride and stirred for 30 min. DTT (5 mM) and Triton X-100 (1%) were added to the cell suspension, followed by sonication on ice (30-s bursts, each separated by a 1-min cooling period) until the cell suspension was no longer viscous. The lysate was centrifuged at 14,000 × g for 30 min, and the soluble extract was incubated with glutathione-agarose at 4 °C for 1 h with agitation. Resin was transferred to an empty column and washed extensively with phosphate-buffered saline. Bound proteins were eluted by the addition of 7× 1 bed volume of 50 mM sodium phosphate, pH 7.5, 500 mM NaCl, and 20 mM imidazole, and fractions with high protein content were pooled. His6-tagged full-length CNP1 was dialyzed against 50 mM sodium phosphate, pH 7.0, and 150 mM NaCl, before it was used for elastase proteolysis experiments and Kcat and Vmax determinations. Purified wild-type and mutant CNP-CF were further treated.
in the following manner. To cleave the His tag, eluate from the Ni²⁺-nitrilotriacetic acid column was dialyzed against thrombin cleavage buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 5% (v/v) glycerol, and 2.5 mM CaCl₂) in the presence of thrombin (10 units per 1 liter of culture preparation). Following overnight cleavage, the protein sample was passed through a Preparative nickTOE buffers (40 mM sodium phosphate, pH 7.0, containing 1 mM EDTA) to further digest the His tag. Flow-through fractions containing cleaved CNP-CF were pooled and dialyzed overnight against storage buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 1 mM DTT) and stored at −80 °C.

All purified protein preparations were analyzed by Coomassie Blue staining and separation of proteins by SDS-polyacrylamide gel electrophoresis. The identity of the proteins was confirmed by appropriate Western blot analysis for GST tag, 6× His tag, or CNP.

**Enzyme and Protein Assays—**CNPase activity was measured using cNADP as substrate, according to the spectrophotometric coupled enzyme assay procedure described previously (17). This assay measures the rate of hydrolysis of cNADP to NADP, which is coupled to the dehydrogenation of glucose-6-phosphate catalyzed by glucose-6-phosphate dehydrogenase. Briefly, the assay mixture (1 ml) consisted of 100 mM MES, pH 6.0, 30 mM MgCl₂, 5 mM D-glucose-6-phosphate, 5 μg of D-glucose-6-phosphate dehydrogenase, and 2.5 mM cNADP. After the addition of CNP variants to initiate the reaction, the assay was carried out at 25 °C using a Beckman DU-600 spectrophotometer fitted with a thermostatically controlled cuvette holder. CNPase activity was determined by monitoring the formation of NADPH at 340 nm using an extinction coefficient of 3400 M⁻¹ cm⁻¹ for the formation of the reaction product, N-carboxyimidazole (37). For histidine modification, CNP-CF (2 μM) in 100 mM sodium phosphate buffer, pH 6.5, was incubated with DEPC (5.5–3.1 mM) at 22 °C. The final concentration of ethanol in reaction mixtures never exceeded 4% (v/v) of the total volume and was shown not to have any effect on enzymatic activity. At various time intervals, aliquots were removed, and residual CNPase activity was measured as described above except that the enzyme assay mixture additionally contained 20 mM imidazole to quench unreacted DEPC. To examine substrate protection against DEPC inactivation, CNP-CF was preincubated with or without 50 mM 2'-AMP in 100 mM sodium phosphate buffer, pH 6.5, for 10 min prior to inactivation with DEPC. Stoichiometry of N-carboxyethylhydroxylamine in CNP-CF (2 μM), treated with 0.5 μM DEPC, was determined by monitoring the time-dependent increase in absorbance at 240 nm. The control containing the same components but without DEPC was used to blank the absorbance. The number of modified histidines was calculated using an extinction coefficient of 3400 M⁻¹ cm⁻¹ (37). The stoichiometry of histidine modification was correlated with enzyme activity by monitoring in a parallel experiment the time-dependent loss of activity.

**Hydroxylamine Reversal—**Hydroxylamine stock solution was initially adjusted to pH 7.0 using NaOH. Reactivation of DEPC-inactivated enzyme with hydroxylamine was assessed by incubating CNP-CF (2 μM) in 100 mM sodium phosphate buffer, pH 6.5, with 1 mM DEPC for 2.5 min at 22 °C until enzyme activity decreased to 9% of its original activity. The reaction was rapidly quenched with 10 mM imidazole, pH 7.0. Hydroxylamine was then added to a final concentration of 0.5 mM. Aliquots were removed at every half hour, and residual enzyme activity was measured. In the control reaction using unmodified enzyme, hydroxylamine did not affect enzyme activity.

**RESULTS**

**Mapping the CNPase Catalytic Domain—**Two separate lines of evidence indicated that the CNPase catalytic domain is located within the C-terminal region comprising two-thirds of the polypeptide. Previous sequence alignment analyses of CNP and RICH proteins revealed that both proteins are highly homologous only within this region (28, 29) (Fig. 1). This also matched the amino acid sequence of a CNPase active ~30-kDa proteolytic fragment generated by pancreatic elastase digestion of bovine CNP1 from myelin (30) (see Fig. 1). To map further the catalytic domain of CNP within its primary amino acid sequence, various rat CNP1 N-terminal deletion (ND) mutants were generated and expressed as recombinant GST fusion proteins in E. coli (Fig. 2). Purified full-length and mutant proteins were assayed for CNPase activity. As expected, GST-CNP1 ND-150, which corresponds to the C-terminal two-thirds region, exhibited activity identical to the full-length GST-CNP1 and the same buffer or 10 mM DTT as Residual activities and absorbance at 410 nm were routinely monitored until no further changes were observed. Following this, 0.2 mM DTNB was added to the treated samples, and its effect on CNPase activity was monitored. The addition of KCN to native CNP-CF did not have any detrimental effects on enzymatic activity.

**DEPC Inactivation—**DEPC was freshly prepared prior to each experiment by diluting the stock solution with anhydrous ethanol. DEPC concentration was determined by reaction with imidazole and monitoring increase in absorbance at 240 nm using an extinction coefficient of 3400 M⁻¹ cm⁻¹ for the formation of the reaction product, N-carboxyimidazole (37). For histidine modification, CNP-CF (2 μM) in 100 mM sodium phosphate buffer, pH 6.5, was incubated with DEPC (0.5–3.1 mM) at 22 °C. The results of the CNPase activity studies are shown in Table I. Digestion of the CNPase active ~30-kDa proteolytic fragment generated by elastase digestion was similar to that prior to
protease treatment (38–40). In addition, limited proteolysis of CNP using other proteases (trypsin and pronase) also yielded CNPase active fragments of similar sizes compared with the elastase-generated fragment (40). The fact that the C-terminal two-thirds of CNP is enzymatically active and resistant to limited proteolysis suggested that this region forms a tightly folded globular structure, which contains all of the necessary molecular components for enzymatic activity. Accordingly, the recombinantly expressed truncated fragment of CNP could then be used for chemical modification studies to simplify analysis. In order to address this question, purified CNP1 ND-150 and full-length CNP1 were treated with pancreatic elastase (1:100, w/w) for 18 h at 22 °C, and the peptide fragments were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). Complete proteolysis was attained under these conditions, since longer incubation did not alter the gel band patterns. Proteolysis of full-length and truncated CNP1 both yielded identical 30 kDa polypeptides. The proteolytic fragment was slightly smaller than CNP1 ND-150, since extraneous residues at the C terminus were removed, based on the amino acid sequence of elastase-generated bovine CNP1 fragment (30) (see Fig. 1). As shown in Table I, $K_m$ and $k_{cat}$ values were similar for both protein samples prior to and after elastase treatment. These results confirm that the carboxyl-terminal two-thirds of CNP is both a catalytic and a compact structural domain. Consequently, this truncated fragment of CNP1, herein referred to as CNP-CF (for CNP catalytic fragment), was ex-
pressed recombinantly and was used for chemical modification analyses.

Kinetics of CNPase Inactivation by DTNB—It was previously reported that incubation of CNP with inorganic mercurials resulted in complete inactivation, implicating the potential involvement of cysteine residue(s) in CNPase activity (16, 41–43). To determine if these residues are important for enzymatic activity, inactivation kinetics, using DTNB as a cysteine-modifying agent, were characterized. DTNB inactivated CNP-CF in a time- and dose-dependent manner, and the rate of inactivation followed pseudo-first-order kinetics (Equation 1). Loss of enzymatic activity was fully restored by treatment with DTT, indicating that modification of either cysteine inactivated to a lesser extent than C314S mutant or wild-type enzyme. This indicated that modification of either cysteine was modified when the enzyme was completely inactivated (Fig. 5A; open circles).

To determine whether DTNB modification is active site-directed, the ability of 2'-AMP, a product inhibitor (45, 46), to protect against DTNB inactivation was tested. A high concentration of 2'-AMP was used to obtain maximum occupancy of the active site, since DTNB also acts as a reversible and competitive inhibitor with a $K_I$ value similar to that of 2'-AMP ($K_I = 500 \mu M$). Incubation of elastase-digested CNP-CF with 2'-AMP afforded extensive protection against DTNB inactivation and cysteine modification (Fig. 5B). Rate constants for inactivation and cysteine modification were both reduced 5-fold. The fact that one less cysteine was modified in the presence of 2'-AMP suggested that this cysteine may be located in or near the active site and could be essential for activity.

*Site-directed Mutagenesis of Cysteines in CNP-CF and Chemical Modification of Mutants*—To confirm results of the chemical modification studies and to identify essential cysteine(s), Cys-231, Cys-236, and Cys-314 were individually mutated to both serine (structurally similar to cysteine) and alanine (absence of hydrophilic functional group). Recombinant CNP-CF mutant proteins were expressed, purified, and assessed for enzymatic activity in the same manner as the wild-type protein (Table II). Contrary to the chemical modification results, none of the mutants exhibited sufficient differences in its kinetic parameters compared with the wild-type enzyme to indicate that cysteines assumed an important role for enzymatic activity.

In an effort to identify the two DTNB-sensitive cysteines, purified cysteine-to-serine CNP-CF mutants were digested with elastase to generate the slightly smaller proteolytic resistant fragment for DTNB titration experiments. Under denaturing conditions, two free sulfhydryl groups were determined for all of the mutants (Table III). In the native state, approximately only one cysteine residue could be detected for the C236S and C314S mutants, suggesting that the two DTNB-sensitive cysteines must be Cys-314 and Cys-236, respectively. Of these two modifiable cysteines, Cys-236 is protected from DTNB inactivation in the presence of 2'-AMP, since only 0.3 cysteine was modified in the C314S mutant. DTNB modification under the conditions of inactivation for wild-type CNP-CF was carried out for C236S and C314S mutants to determine whether one or both DTNB modified cysteines were responsible for inactivation (Table IV). After 30 min, both mutants were inactivated to constant levels, albeit the C236S mutant was inactivated to a lesser extent than C314S mutant or wild-type enzyme. This indicated that modification of either cysteine caused loss of enzymatic activity. CNPase activity was fully restored by treatment with DTT.

Loss of enzymatic activity by DTNB could be caused by incorporation of a large functional group, such as TNB, at a cysteine within or near the active site, thereby sterically hindering substrate binding and/or catalysis. Additionally, modification may induce conformational changes leading to the for-
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FIG. 4. Inactivation of CNP-CF by DTNB. A, CNP-CF (3–4 μM) in 50 mM sodium phosphate, pH 7.0, and 1 mM EDTA was incubated at 25 °C with either 0 ( ), 0.1 (○), 0.25 (▲), 0.50 (▲), 0.75 (▲), or 1.0 (□) mM DTNB. Aliquots were withdrawn at various time intervals for determining residual activities. Results were plotted as a logarithm of residual activity versus time, yielding straight lines, the slopes of which represent the pseudo-first-order rate constants of inactivation (k\text{inact}). B, double reciprocal plot of k\text{inact} versus DTNB concentration from which Kd and k\text{act} values were calculated.

FIG. 5. Stoichiometry of DTNB-mediated inactivation and protection by 2-AMP. A, correlation between number of cysteines modified by DTNB and residual activity of CNP-CF ( ) and CNP-CF elastase proteolytic fragment ( ). Both proteins (3–4 μM) were treated with 0.025 mM DTNB at 25 °C. Aliquots were removed at intervals and assayed for enzymatic activity. The number of modified cysteines was calculated from the differential absorbance at 410 nm in a parallel experiment under identical conditions. B, protection of CNP-CF elastase proteolytic fragment from DTNB inactivation and modification. CNP-CF elastase proteolytic fragment (4 μM) was preincubated for 10 min at 25 °C in the absence ( ) or presence ( ) of 50 mM 2'-AMP, prior to DTNB inactivation.

### TABLE II

**Kinetic parameters of the catalytic domain mutants of CNP1**

Kinetic parameters were determined as described under "Experimental Procedures." Results shown are the average of two determinations ± S.D.

| CNP-CF       | Km (cNADP) | k_{cat} | k_{cat}/Km |
|--------------|------------|---------|------------|
|              | μM         | s⁻¹     | μM⁻¹ s⁻¹   |
| Wild type    | 237 ± 13   | 1195 ± 18 | 5.0        |
| C231S        | 473 ± 13   | 825 ± 8  | 1.7        |
| C231A        | 231 ± 11   | 968 ± 13 | 4.2        |
| C236S        | 379 ± 14   | 1107 ± 14 | 2.9       |
| C236A        | 354 ± 33   | 1476 ± 45 | 4.2       |
| C314S        | 241 ± 8    | 594 ± 5  | 2.5        |
| C314A        | 333 ± 14   | 1116 ± 15 | 3.4       |
| C397S        | 297 ± 8    | 1132 ± 10 | 3.8       |
| H230F        | 119 ± 7    | 1.15 ± 0.02 | 0.010   |
| H230L        | 104 ± 4    | 1.16 ± 0.01 | 0.011   |
| H309F        | 100 ± 2    | 1.35 ± 0.01 | 0.013   |
| H309L        | 98 ± 3     | 1.27 ± 0.01 | 0.013   |

Activity, indicating that all of the surface-exposed cysteines were stable thiocyanate-derivatives (data not shown). Complete chemical modification of wild-type and mutant CNP-CF with the smaller thiol-reacting reagent, MMTS, resulted in minor activity losses, ranging from 20 to 42% of initial activity (Table IV). These results demonstrate that the loss of CNPase activity is attributable to the steric effects of DTNB modification of Cys-236 and Cys-314.

**Kinetics of CNPase Inactivation by DEPC**—It was recently reported that histidine(s) may play an important role in CNPase activity; mutation of a histidine in the zebrafish CNP homolog, z-RICH, corresponding to His-309 in rat CNP1, completely abolished enzymatic activity (29). Consequently, selective chemical modification with DEPC was pursued to examine the potential involvement of histidine(s) in CNP.

CNP-CF incubated with DEPC at pH 6.5 and 22 °C resulted in a time-dependent loss of CNPase activity (Fig. 6A). However, DEPC is unstable in aqueous solutions, and in order to correct for its decomposition, the inactivation data were fitted using Equation 2 (52),

\[
\ln \frac{A(t)}{A_0} = -k_t k_e I_o (1 - e^{-kt})
\]

where A(t) is the residual activity at time t, I_o is the initial concentration of DEPC, k\text{t} is the pseudo-first-order rate constant for the reaction of CNP-CF with DEPC, and k\text{e} is the first-order rate constant for DEPC hydrolysis. A plot of the

mation of a less active enzyme. Recovery of enzymatic activity following replacement of the TNB moiety with a smaller functional group, such as a cyanide or thiomethyl group, has previously been used to demonstrate the nonessential role of cysteines and steric sensitivity to DTNB modification (47–51). Reaction of DTNB-inactivated wild-type and mutant CNP-CF with 80 mM KCN resulted in significant recovery of activity (Table IV). Further addition of DTNB did not cause loss of
natural log of residual activity against effective time [(1 - e^{-kt})/k] at different DEPC concentrations yielded straight lines, indicating that inactivation followed pseudo-first-order kinetics. The pseudo-first-order rate constant varied linearly as a function of DEPC concentration with a second-order rate constant of 0.41 mM^{-1} min^{-1} (Fig. 6B). The linear curve intersected at the origin, indicating that the chemical modification is the result of a simple, irreversible bimolecular process (53). Although DEPC reacts selectively with histidine residues, it can also react with other nucleophilic amino acid residues, such as cysteine and tyrosine, as well as with primary amino groups (37, 54, 55). Consequently, it was necessary to rule out the possibility that modification of a residue other than histidine causes inactivation under the conditions used for the DEPC inactivation experiment. Treatment of DEPC-inactivated CNP-CF with 0.5 mM hydroxylamine at pH 7.0 and 22 °C resulted in complete recovery of CNPase activity within 1 h. This suggested that DEPC inactivation was not due to the modification of reactive lysine, arginine, or cysteine residues, since hydroxylamine cannot cleave carbethoxy adducts from these residues. In addition, inactivation is not attributable to irreversible conformational changes or bicarbethoxylation of histidines, since neither possibility can be reversed by hydroxylamine. Difference spectra of native and DEPC-treated CNP-CF revealed a peak with an absorption maximum at 240 nm, characteristic of N-carbethoxyhistidines (data not shown). Furthermore, O-carbethoxylation of tyrosine residues can also react with other nucleophilic amino acid residues, such as cysteine and tyrosine, as well as with primary amino groups (37, 54, 55). Consequently, it was necessary to rule out the possibility that modification of a residue other than histidine causes inactivation under the conditions used for the DEPC inactivation experiment. Treatment of DEPC-inactivated CNP-CF with 0.5 mM hydroxylamine at pH 7.0 and 22 °C resulted in complete recovery of CNPase activity within 1 h. This suggested that DEPC inactivation was not due to the modification of reactive lysine, arginine, or cysteine residues, since hydroxylamine cannot cleave carbethoxy adducts from these residues. In addition, inactivation is not attributable to irreversible conformational changes or bicarbethoxylation of histidines, since neither possibility can be reversed by hydroxylamine. Difference spectra of native and DEPC-treated CNP-CF revealed a peak with an absorption maximum at 240 nm, characteristic of N-carbethoxyhistidines (data not shown). Furthermore, O-carbethoxylation of tyrosine residues can also react with other nucleophilic amino acid residues, such as cysteine and tyrosine, as well as with primary amino groups (37, 54, 55).

### Table III

| Elastase-digested fragment of CNP-CF | Cysteines present | Cysteines modified/monomer |
|-------------------------------------|-------------------|---------------------------|
|                                     |                   | Denaturing condition | Native condition | Native condition + 50 mM 2′-AMP |
| Wild type                           | 3                 | 2.68 ± 0.18             | 2.00 ± 0.07     | 1.11 ± 0.11 |
| C231S                               | 2                 | 1.92 ± 0.08             | 1.74 ± 0.03     | 0.96 ± 0.03 |
| C236S                               | 2                 | 1.91 ± 0.09             | 0.84 ± 0.02     | 0.73 ± 0.02 |
| C314S                               | 2                 | 1.83 ± 0.06             | 1.18 ± 0.03     | 0.27 ± 0.01 |

#### Method

To assess whether the chemical modification by DEPC is active site-directed, 2′-AMP was used to protect the active site from inactivation (Fig. 7A). When CNP-CF was preincubated with 2′-AMP, the rate of inactivation was significantly reduced. This suggests that the modification of one or more reactive histidine residue(s) at or near the active site is responsible for loss of CNPase activity. In order to establish the number of essential histidines, the relationship between the number of modified histidines and loss of activity was examined (Fig. 7B, filled circles). Although a total of two out of five histidine residues were modified per monomer, prolonged incubation indicated that a maximum of three residues were modified (data not shown). Since the method of extrapolating the linear portion of the curve down to complete loss of activity to derive the number of essential residues is rarely accurate (57, 58), we used Tsou’s statistical method to determine the number of essential residues, using Equation 3 (57),

\[
a_m = \frac{(p - m)}{p} \quad (\text{Eq. 3})
\]

where \( p \) represents the total number of modifiable residues, \( m \) is the number of modified residues at a given time point, \( a \) is the residual activity when \( m \) residues have been modified, and \( i \) is the number of essential residues for enzymatic activity. As shown in Fig. 7B, using \( p = 3 \), a straight line is obtained only when \( i = 2 \) (open circles), indicating that two histidine residues are essential for CNPase activity.

#### Site-directed Mutagenesis of Conserved Histidines in the CNPase Catalytic Domain

Results of the DEPC modification studies suggest that two histidines are essential for enzymatic activity. The C-terminal catalytic domain of rat CNP1 contains five histidines, two of which (His-230 and His-309) are conserved in all CNP and RICH catalytic domain sequences (Fig. 1). It seemed probable, therefore, that these two histidines are essential for enzymatic activity. To test this idea, both histidines in CNP-CF were individually mutated to phenylalanine and leucine. Both sterically conservative residues are hydrophobic and electrically neutral and thus are incapable of acting as acids or bases. All four mutant enzymes were expressed in E. coli and purified by the same procedures used for the wild-type protein. No significant differences were noticed in the expression level of the mutants or in the final yield after its purification compared with the wild-type enzyme, suggesting that the mutation did not affect protein stability. In order to compare the catalytic capabilities of mutant and wild-type CNP, kinetic parameters were measured for the enzymes (Table II). Substitution of His-230 and His-309 with phenylalanine and leucine almost completely eliminated CNPase activity. Despite the low enzymatic activity detected, all histidine mutants were amenable to kinetic analysis. Mutants exhibited very low substrate turnover; \( k_{\text{cat}} \) values were roughly 1000-fold lower than those of wild-type CNP-CF. In contrast, all mutants had slightly lower \( K_m \) values than the wild-type enzyme.

To determine whether the absence of CNPase activity in...
these mutants was due to gross conformational changes resulting from single amino acid substitutions, purified wild-type and mutant enzymes were subjected to circular dichroism spectral analysis (data not shown). Far UV spectral comparisons between wild-type and mutant proteins revealed no significant secondary structural differences, indicating that the mutation did not induce any gross conformational changes. Taken together, these results demonstrate that both conserved residues, His-230 and His-309, are essential for CNPase activity and may play a role in catalysis.

**DISCUSSION**

Despite prior literature on CNP (reviewed in Refs. 1–3), there is a lack of kinetic and structural information that could illuminate the biological role of this enzymatic activity. In our strategy to address the physiological relevance of its in vitro enzymatic activity, we used chemical modification and site-directed mutagenesis to identify essential residues critical for enzymatic activity, in order to ultimately generate dominant negative mutants for future expression studies in oligodendrocytes.

Two separate lines of evidence suggested that the catalytic domain of CNP might be located within the C-terminal two-thirds of the protein: 1) sequence alignment of CNP and RICH showed extensive homology only within this region (28, 29), and 2) its amino acid sequence corresponded to the enzymatically active 30-kDa peptide fragment generated by proteolysis (30). An updated full-length sequence alignment of known CNP and RICH proteins, including the recently discovered RICH protein from zebrafish (29), reveals significant homology between both proteins, in addition to a conserved C-terminal isoprenylation motif (Fig. 1). In an attempt to further delineate the primary amino acid sequence of the CNPase catalytic domain, we found that removal of the first highly conserved segment (residues 165–173) completely abolished enzymatic activity, indicating that the C-terminal two-thirds of CNP is the minimal C-terminal fragment that is enzymatically active. This region of CNP appears to contain all of the necessary molecular components for enzymatic activity, since CNP1 ND-150 and the 30-kDa proteolytic fragment generated by elastase digestion had $K_m$ and $k_{cat}$ values similar to those of full-length CNP1. Furthermore, because this truncated fragment is resistant to proteolysis by elastase, as well as to a lesser degree by other proteases (40), the catalytic domain of CNP is likely to have a tightly folded, compact globular structure. We therefore

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**FIG. 6.** Inactivation of CNP-CF by DEPC. A, CNP-CF (2.0 μM) in 100 mM sodium phosphate, pH 6.5, was incubated with either 0 (●), 0.5 (○), 1.0 (▲), 1.5 (▲), or 3.1 (□) mM DEPC at 22 °C. Samples were withdrawn at various time intervals to determine residual activities. Inactivation data were fitted to Equation 2 to obtain the pseudo-first-order rate constants of inactivation ($k_{inact}$). B, plot of the concentration dependence of $k_{inact}$ for the inactivation of CNPase activity.

**FIG. 7.** Protection of CNP-CF against DEPC inactivation by 2'-AMP and stoichiometry of inactivation. A, protection by 2'-AMP against inactivation of CNP-CF by DEPC. CNP-CF (2.0 μM), treated in the absence (●) or in the presence (○) of 50 mM 2'-AMP for 10 min, was inactivated with 1.9 mM DEPC. At fixed time intervals, aliquots were withdrawn from the mixture and assayed for CNPase activity. B, relationship between residual activity and the number of histidine residues modified by DEPC. CNP-CF (22 μM) was modified with 0.5 mM DEPC at 22 °C as described under "Experimental Procedures." At various time intervals, aliquots of the reaction mixture were withdrawn and assayed for enzyme activity. In a parallel experiment, the increase in absorbance at 240 nm was measured, from which the number of histidine residues modified were calculated. The data were analyzed in the form of a Tsou plot (57), where $i = 1$ (●), $i = 2$ (○), and $i = 3$ (▲).
used this truncated fragment of CNP for chemical modification studies.

Based on previous reports, CNPase activity was effectively inhibited by inorganic mercurials (2, 3). We investigated the importance of cysteine(s) for enzymatic activity using DTNB, a sulfhydryl-specific reagent, which allows simple and direct quantitation of modified cysteines. Incubation of CNP-CF with DTNB resulted in complete loss of enzymatic activity. Stoichiometric analysis of the CNP-CF elastase proteolytic fragment suggested that two cysteines are modified, one of which may be in or near the active site. Although these results suggest that one or both cysteines may be essential for CNPase activity, three observations contradict this: 1) mutation of each of the three candidate cysteines in CNP-CF (Cys-231, Cys-236, and Cys-314) to serine and alanine residues generated no substantial changes in kinetic parameters as compared with the wild-type enzyme; 2) treatment of TNB-modified wild-type and mutant CNP-CF with KCN resulted in recovery of enzymatic activity; and 3) wild-type and mutant CNP-CF were much less inactivated by MMTS, a smaller sulfhydryl-modifying reagent. Stoichiometric studies of the mutants showed that Cys-236 and Cys-314 are modified by DTNB, of which Cys-236 could be located in or near the active site. These results demonstrate that the addition of a large and bulky TNB group to the side chains of Cys-236 and Cys-314 caused conformational changes of the enzyme and/or steric obstruction of the active site, resulting in CNPase inactivation.

We examined whether histidines assumed key roles for CNPase activity, since mutagenic studies of z-RICH revealed that mutation of His-334 to alanine resulted in loss of CNPase activity (29). Recombinant CNP-CF treated with DEPC resulted in complete inactivation. The ability of hydroxylamine to restore activity and the observed spectral changes from DEPC modification provided evidence that histidine(s) were specifically modified, as opposed to other nucleophilic residues such as tyrosine, lysine, and cysteine. Complete inactivation was concomitant with the modification of three histidines, two of which were indicated to be essential. Protection against DEPC inactivation by 2'-AMP suggested that one or both histidines are present in or near the active site. Based on these results, we expected two histidines to be invariant in the CNP and RICH proteins for which the primary sequences have been elucidated. Sequence alignment analysis shows that two residues in rat CNP1, His-230 and His-309, meet this criterion. Not surprisingly, this latter histidine corresponds to His-334 in z-RICH, which further strengthens the notion that the active sites of CNP and RICH proteins are structurally and functionally similar (see Fig. 1). Replacement of His-230 or His-309 with phenylalanine and leucine resulted in substantial loss of enzymatic activity, corroborating the conclusions drawn from chemical modification studies. All histidine mutants displayed dramatic decreases in catalytic efficiency ($k_{cat}/K_m$) by about 500-fold compared with wild-type CNP-CF, suggesting a catalytic role for both His-230 and His-309. On the other hand, the observed loss of activity may be due to conformational changes caused by the histidine substitutions. However, the fact that the histidine mutants exhibited identical parameters in $K_m$ as wild-type CNP-CF and that the circular dichroism spectra for wild-type and mutant enzymes were identical indicates that the loss of enzymatic activity is not attributed to marked secondary structural changes. This conclusion is further supported by NMR analysis (determination of three-dimensional structure of CNP-CF by NMR is in progress).2

Although there are no mechanistic studies available on CNP, RICH, or other enzymes that cleave the 3'-phosphodiester bonds of 2',3'-cyclic nucleotides to suggest specific roles for the two critical histidine residues in CNPase activity, their function may be inferred from the known catalytic mechanism of ribonuclease A (59). Although ribonuclease A catalyzes hydrolysis of cyclic phosphodiester bonds of 2',3'-cyclic phosphate RNA intermediates, this protein, unlike CNP and RICH, cleaves the phosphate ring to generate the 3'-nucleotides as end products instead. It is interesting to note that although there are no obvious sequence similarities between ribonuclease A and CNP, ribonuclease A contains two essential catalytic histidine residues: one that functions as an acid catalyst and the other as a base catalyst. Further kinetic studies are required to investigate the catalytic roles of His-230 and His-309 in CNPase activity. It will also be of interest to elucidate the structure of CNP and to compare its active site structure with those of other phosphodiesterases that cleave 2',3'-cyclic nucleotides.

A variety of observations call attention to the potential significance of CNP in the process of myelin formation. Earlier studies reported strong indications that CNP is associated with cytoskeletal elements of oligodendrocytes (7, 8, 60–62). The ability of CNP, expressed in transfected nonglial cells, to promote major alterations of cellular morphology, with the appearance of networks of filopodia and large processes reminiscent of those elaborated by oligodendrocytes, is further evidence for the interaction of CNP with cellular proteins that are normally responsible for such surface features. Moreover, we have recently shown that CNP overexpression in transgenic mice induced aberrant oligodendrocyte and myelin membrane formation during early stages of oligodendrocyte differentiation and myelination, with the appearance of redundant myelin membrane and intramyelinic vacuoles in later stages of development (9, 10). While the function of CNP is unknown, these observations suggest that CNP is part of a molecular complex that regulates and/or modulates the oligodendrocyte surface membrane expansion and migration, which lead to myelin formation.

It is intriguing that the CNPase catalytic domain of CNP and RICH proteins is highly conserved among evolutionary divergent vertebrates. In addition to the conserved regions, which presumably collectively contribute to the CNPase active site, both proteins contain a C-terminal isoprenylation motif that is conserved. Although isoprenylation has only been studied in CNP (7, 8, 12), it is probable that this motif is also functional in RICH proteins, and the function of both proteins is dependent on proper membrane and/or cytoskeletal localization. Furthermore, expression of RICH and CNP occurs prior to specific cellular events that share common characteristics, in that both retinal ganglion cells and oligodendrocytes undergo process extensions accompanied by membrane assembly during optic nerve regeneration and myelin formation, respectively. Consequently, these commonalities suggest that both proteins have similar functions that include CNPase activity.

In conclusion, we have begun to identify specific residues that are essential for CNPase activity. We have demonstrated, through chemical modification studies and site-directed mutagenesis, essential roles for His-230 and His-309 and shown that both are critical for catalysis. These mutants will subsequently be used for more extensive kinetic studies to determine the specific roles of the histidines for catalysis. More importantly, the availability of these CNPase inactive mutants now provides us with an opportunity for the first time to evaluate...

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the physiological relevance of this enzymatic activity. We are currently investigating the functional consequence of expressing these mutants as dominant negatives in oligodendrocytes.

Acknowledgments—We greatly appreciate the comments and suggestions made by Dr. J. Turnbull in the preparation of the manuscript. We also thank Vicky Kottis for expert technical assistance.

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Identification of Essential Residues in 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase: CHEMICAL MODIFICATION AND SITE-DIRECTED MUTAGENESIS TO INVESTIGATE THE ROLE OF CYSTEINE AND HISTIDINE RESIDUES IN ENZYMATIC ACTIVITY

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J. Biol. Chem. 2001, 276:14804-14813.
doi: 10.1074/jbc.M009434200 originally published online February 5, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M009434200

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