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# Molecular cloning of the myelin specific enzyme 2′,3′-cyclic-nucleotide 3′-phosphohydrolase

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We describe the isolation of cDNA clones for bovine brain 2′,3′-cyclic-nucleotide 3′-phosphohydrolase (CNPase, EC 3.1.4.37), the third most abundant protein in central nervous system myelin. The cDNA encodes the complete protein (400 amino acids) and hybridizes to a major size species of mRNA in bovine brain tissue, approx. 2.7 kb in size. CNPase mRNA levels do not appear to be affected in quaking dysmyelinating mutant mice. The sequence reveals probable sites for CNPase phosphorylation by cAMP-dependent protein kinase and a region of homology with haemocyanin.

CNPase; Myelin; cDNA sequence

## 1. INTRODUCTION

2′,3′-cyclic-nucleotide 3′-phosphohydrolase (CNPase) was the first enzyme to be unequivocally characterized as a component of the myelin membrane, previously myelin was thought to be enzymatically inert [1]. Although the enzyme converts 2′,3′-cyclic nucleotides to 2′-nucleotides in vitro, 2′,3′-cyclic nucleotides have not been found at significant levels in any mammalian tissues. CNPase appears as two subunits that vary from 44 to 47 kDa when run on SDS-polyacrylamide gels, both of which exhibit enzymic activity [2]. The two subunits have nearly identical amino acid compositions and peptide patterns [3]. The CNPase upper subunit is phosphorylated in a cAMP-dependent manner by an endogenous myelin kinase both in vitro [2] and in vivo [4]. Rapid increases in the enzyme level occur during myelination [5] which suggests that the enzyme may be involved in the myelination process.

To begin investigating the role of CNPase in the development of the myelin sheath, as well as genetic factors involving demyelinating diseases and dysmyelinating disorders, we have identified and sequenced cDNAs corresponding to the CNPase protein.

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## 2. MATERIALS AND METHODS

*Staphylococcus aureus* V8 protease, thermolysin (type X) and trypsin were from Miles, Sigma and Worthington, respectively; radiochemicals from Amersham; and acetonitrile from Rathburn Chemicals. Other reagents and enzymes were from Sigma, Boehringer Mannheim and New England Biolabs.

### 2.1. *Purification and partial protein sequencing of CNPase*

CNPase was purified from bovine brain white matter [6]. Enzymic digests (trypsin and

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*Staphylococcus aureus* V8 protease, 1:4, w/w, 1 h at 37°C, 0.1% ammonium bicarbonate, thermolysin, as for latter but 4 h at 50°C) were resolved on an HPLC reverse-phase column (PLRP-S300 A°, Polymer Laboratories, England) by a linear gradient of 0.1% trifluoroacetic acid at 1% acetonitrile/min, using UV detection at 220 nm. Selected peaks were either sequenced manually [7] or applied to a gas-phase microsequenator.

### 2.2. *Synthetic oligodeoxyribonucleotide synthesis and analysis of cDNA clones*

Two oligonucleotide probes were synthesized: a 17-mer of 64-fold redundancy (5′-TCNAC$^{G}_{A}$-TC$^{G}_{A}$TTNGGCCA-3′, where 'N' denotes a mixture of C, T, A and G) and a 'best guess' 38-mer (5′-GAGCAGCAG$^{C}_{T}$TGGCCCT$^{G}_{G}$TGGCCCAAT-GATGTGGACAA-3′). The probes were 5′-end labelled [8] and $2 \times 10^5$ plaques of a bovine brain λgt10 cDNA library [9] were screened [8,10].

CNPase DNA fragments were cloned into M13 mp18 and mp19 vectors and sequenced by dideoxy chain termination [11].

### 2.3. *RNA isolation and Northern blot analysis*

RNA was isolated from fresh mouse and bovine tissue [12]. Total RNA was electrophoresed on a 1% agarose gel in 6.5% (v/v) formaldehyde, blotted onto Hybond-N (Amersham), hybridized to cDNA clone C8.0 which had been 'oligolabelled' to $10^8$ cpm/μg [13], followed by washing and exposure to X-ray film.

## 3. RESULTS

The homologies of the 3 isolated positives implied by restriction maps (fig.1), were confirmed by cross-hybridization experiments. Many regions were sequenced in several clones, and the entire coding region was sequenced on both strands. AUG codons found in frame at positions 13, 73 and 250 (fig.2) are all embedded in the sequence fitting the consensus for initiation codons [14]. We feel, however, that the putative initiation codon is at position 73, since the amino acid content predicted from this cDNA sequence is most similar to that determined for the CNPase protein [15]. The open reading frame thus encodes a protein of 400 amino acids with a molecular mass of 44875 Da. Additional peptide sequence data from CNPase agreed with the sequence translated from the cDNA, and confirmed the identity of the cDNA clones. The sequence reveals no significant regions of hydrophobicity that could serve as membrane-spanning portions. A computer search of the National Biomedical Research Foundation (NBRF) data bank revealed a similarity with a portion of the α-chain of Japanese horseshoe crab haemocyanin [16]. This similarity (~70%, corresponding to residues 32–47 of the haemocyanin α-chain) was judged to be of statistical significance by the program RDF [17]. The predicted sequence reveals several putative recognition sequences for protein kinase A [18].

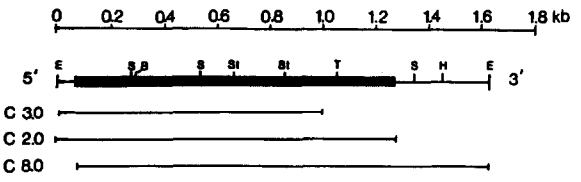


Fig.1. A partial restriction map for the CNPase cDNA clones (E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sau*3A; St, *Stu*I; T, *Taq*1). The open reading frame is shown as a thick line. The CNPase cDNA clones C2.0, C3.0 and C8.0 are represented below the restriction map.

Northern blots of total RNA from bovine and mouse brain tissue indicate that CNPase appears to be encoded by a single mRNA of ~2.7 kb in length (fig.3A,B). The more intense hybridization signal obtained for corpus callosum RNA (fig.3A, lane 2) confirms the glial cell origin of the CNPase mRNA. Even though quaking mice have been

Fig.2. The complete nucleotide sequence and deduced amino acid sequence of bovine brain CNPase. Underlined amino acids represent the peptides generated by enzymic digests. All predicted amino acids agree with those determined by peptide sequencing. Putative protein kinase A phosphorylation sites are boxed. The region of homology with haemocyanin is marked by a dotted line.

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                                                                    Met
    CCCCTCCTCATCATGAGTAGAGGCTTCTCCCGAAAGAGCCAGACGTTCCTGCCCAAGGTCTTCTTCCGCAAA ATG
                                                                     75

Ser Ser Ser Gly Ala Lys Asp Lys Pro Glu Leu Gln Phe Pro Phe Leu Gln Asp Glu Glu
TCA TCC TCA GGG GCC AAG GAC AAG CCG GAG CTG CAG TTT CCC TTC CTG CAG GAT GAG GAG
                                                                    135

Thr Val Ala Thr Leu Gln Glu Cys Lys Thr Leu Phe Ile Leu Arg Gly Leu Pro Gly Ser
ACG GTG GCC ACG CTG CAG GAG TGC AAG ACG CTC TTC ATC CTG CGA GGC CTG CCT GGG AGC
                                                                    195

Gly Lys Ser Thr Leu Ala Arg Phe Ile Val Asp Lys Tyr Arg Asp Gly Thr Lys Met Val
GGC AAG TCC ACG CTG GCC CGG TTC ATC GTG GAC AAG TAC CGG GAT GGC ACC AAG ATG GTG
                                                                    255

Ser Ala Asp Ser Tyr Lys Ile Thr Pro Gly Ala Arg Gly [Ser] Phe Ser Glu Glu Tyr Lys
TCT GCC GAC AGC TAC AAG ATC ACC CCT GGC GCC CGG GGA TCC TTC TCT GAG GAG TAC AAG
                                                                    315

Gln Leu Asp Glu Asp Leu Ala Ala Cys Cys Arg Arg Asp Phe Arg Val Leu Val Leu Asp
CAG CTG GAC GAG GAC CTG GCT GCC TGT TGC CGC CGG GAT TTC CGG GTC CTG GTG CTG GAT
                                                                    375

Asp Thr Asn His Glu Arg Glu Arg Leu Glu Gln Leu Phe Glu Leu Ala Asp Gln Tyr Gln
GAT ACC AAC CAT GAG CGG GAG CGG CTG GAG CAG CTC TTT GAG CTG GCC GAC CAG TAC CAG
                                                                    435

Tyr Gln Val Val Leu Val Glu Pro Lys Thr Ala Trp Arg Leu Asp Cys Ala Gln Leu Lys
TAC CAG GTG GTG CTG GTG GAG CCC AAG ACG GCC TGG CGG CTG GAC TGT GCC CAG CTC AAG
                                                                    495

Glu Lys Asn Gln Trp Gln Leu Ser Ala Asp Asp Leu Lys Lys Leu Lys Pro Gly Leu Glu
GAG AAG AAC CAG TGG CAG CTG TCA GCA GAT GAT CTG AAG AAG CTG AAG CCT GGG CTG GAG
                                                                    555

Lys Asp Phe Leu Pro Leu Tyr Phe Gly Trp Phe Leu Thr Lys Lys Ser [Ser] Ala Ala Leu
AAG GAC TTC CTG CCG CTT TAC TTC GGC TGG TTC CTG ACC AAG AAG AGT TCC GCG GCC CTC
                                                                    615

Trp Lys Thr Gly Gln Thr Phe Leu Glu Glu Leu Gly Asn His Lys Ala Phe Lys Lys Glu
TGG AAA ACT GGC CAG ACC TTC CTG GAG GAG CTG GGC AAT CAC AAG GCC TTC AAG AAG GAG
                                                                    675

Leu Arg His Phe Val Ser Gly Asp Glu Pro Arg Glu Lys Ile Glu Leu Val Thr Tyr Phe
CTG CGA CAC TTT GTC TCT GGG GAT GAG CCC AGG GAG AAG ATT GAA CTG GTC ACC TAC TTC
                                                                    735

Gly Lys Arg Pro Pro Gly Val Leu His Cys Thr Thr Lys Phe Cys Asp Tyr Gly Lys Ala
GGG AAG AGA CCG CCG GGC GTG CTG CAT TGC ACA ACC AAG TTC TGT GAC TAC GGG AAG GCC
                                                                    795

Ala Gly Ala Glu Glu Tyr Ala Gln Gln Asp Val Val Lys Lys Ser Tyr Cys Lys Ala Phe
GCT GGG GCA GAG GAG TAT GCC CAG CAA GAT GTA GTG AAG AAA TCC TAC TGC AAG GCC TTC
                                                                    855

Thr Leu Thr Ile Ser Ala Leu Phe Val Thr Pro Lys Thr Thr Gly Ala Arg Val Glu Leu
ACG CTG ACC ATC TCG GCC CTC TTC GTG ACA CCC AAG ACG ACG GGA GCC AGA GTA GAG CTG
                                                                    915

Ser Glu Gln Gln Leu Ala Leu Trp Pro Asn Asp Val Asp Lys Leu Ser Pro Ser Asp Asn
AGC GAG CAG CAG CTG GCC TTG TGG CCA AAC GAC GTG GAC AAG CTG TCT CCC TCT GAC AAC
                                                                    975

Leu Pro Arg Gly [Ser] Arg Ala His Ile Thr Leu Gly Cys Ala Gly Asp Val Glu Ala Val
CTG CCA CGG GGC AGC CGC GCA CAC ATC ACC TTG GGC TGC GCG GGT GAC GTA GAG GCC GTG
                                                                   1035

Gln Thr Gly Ile Asp Leu Leu Glu Ile Val Arg Gln Glu Lys Gly Gly Ser Arg Gly Glu
CAG ACA GGC ATC GAC CTG CTA GAG ATT GTG CGG CAG GAG AAG GGG GGC AGC CGC GGC GAG
                                                                   1095

Glu Val Gly Glu Leu Ser Arg Gly Lys Leu Tyr Ser Leu Gly Ser Gly Arg Trp Met Leu
GAG GTG GGT GAG CTC AGC CGG GGC AAG CTC TAC TCC CTG GGC AGC GGG CGC TGG ATG CTG
                                                                   1155

Ser Leu Ala Lys Lys Met Glu Val Arg Ala Ile Phe Thr Gly Tyr Tyr Gly Lys Gly Lys
AGC CTG GCC AAG AAG ATG GAG GTC AGG GCT ATC TTT ACA GGA TAC TAC GGG AAG GGC AAG
                                                                   1215

Ala Val Pro Ile Arg Ser Gly Arg Lys Gly Gly Ser Phe Gln Ser Cys Thr Ile Ile
GCC GTG CCC ATA CGC AGC GGC CGC AAG GGT GGC TCC TTT CAG TCC TGT ACC ATC ATC TGA
                                                                   1275

GCGTCCTGGGCCACCTGCCCCTTCTTTACAAGGGAAGGAGGAGGGGAGGGGAGCATCCCTCTGCTTGATCTTTGATTCT

TTTTTATTTTTTACTCAAAGTTAACTTTCCTGTAATTTTTAAAGACTTGTAAAATAACCTCTTTTCCTGCCCACCCCCT

TCCCCTCTAACGCTCAAGCTTCAACACAAGGGGGTGGGTAGGCGTCATTTAGGAACCTGGACCACAGTTGACCAGGCTG

GGCCAAGTTTGGCCTGGACTGGAGCCACGACCCTAAGCCCTGCTTCCAGTTACCGGGCCCCCAGGCCACTGCCCCGCAC

AAGGCTGGCTAGTGGGGAGGGGACACCCCAGCATGCAAGTAC
                                                                   1633
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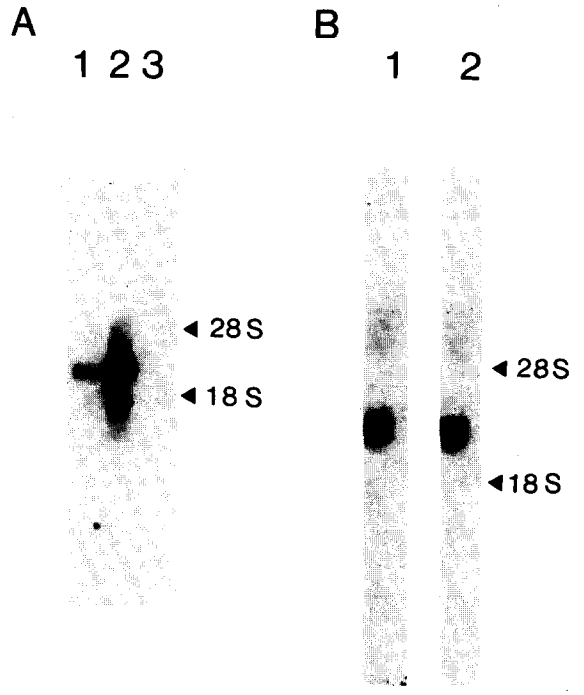


Fig.3. (A) Northern blot analysis of bovine total RNA. Lanes 1, 2 and 3 contain 5 $\mu$g of cortex, corpus callosum and liver RNA, respectively. (B) Northern blot analysis of 20 $\mu$g total RNA from quaking (qk/qk) mouse brains (lane 1) and littermate mouse brains (qk/t). Positions of 28 and 18 S ribosomal bands are indicated.

shown to have reduced levels of myelin, the size and amount of message does not appear to differ between mutant (qk/qk) and normal (qk/t) mice.

## 4. DISCUSSION

In this report we present the nucleotide and predicted amino acid sequence of the cDNA encoding the entire bovine brain CNPase protein. The predicted molecular mass, 44875 Da, compares with estimates of 44 to 47 kDa from SDS-polyacrylamide gel electrophoresis. The protein sequence shows no potential membrane spanning regions and we thus propose a model whereby CNPase is present at the cytoplasmic apposition of the myelin lamellae (the 'major dense line').

The presence of potential cAMP-dependent protein kinase phosphorylation sites is consistent with results of previous phosphorylation experiments [2]. Phosphorylation of the enzyme may be a possible control point in myelin formation.

The Japanese horseshoe crab haemocyanin is a copper-binding protein consisting of six subunits. Because only the sequence from one of the subunits has been reported in the database, we cannot evaluate the overall significance of the homology CNPase shows with this fragment. Several studies have demonstrated hypomyelination in offspring of copper-deficient mothers and an essential role for copper in myelin formation has been suggested [19].

Even though in the quaking dysmyelinating mutant myelin levels are reduced to less than 10% of control values [20], Northern blot analysis showed no apparent decrease in CNPase mRNA levels when comparing quaking mice to their littermate controls. A simple explanation consistent with this observation would be that the free polysomes on which CNPase is synthesized [21] are largely confined to the oligodendrocyte cell body and not the myelin sheath. No enrichment of CNPase mRNA was shown in the myelin mRNA fraction of rat brain [22]. The presence of one major 2.7 kb mRNA species suggests one message and it is thus probable that the two CNPase subunits differ by some posttranslational modification.

The availability of CNPase cDNA clones should allow further study into the expression and developmental regulation of this enzyme, as well as its role in myelinogenesis.

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## NOTE ADDED IN PROOF

An independent clone for CNPase has now been published (Kurihara, T. et al. (1987) J. Biol. Chem. 262, 3256–3261).

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