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Cloning, Expression, and Properties of a Nonneuronal Secreted Acetylcholinesterase from the Parasitic Nematode *Nippostrongylus brasiliensis**

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We have isolated a full-length cDNA encoding an acetylcholinesterase secreted by the nematode parasite *Nippostrongylus brasiliensis*. The predicted protein is truncated in comparison with acetylcholinesterases from other organisms such that the carboxyl terminus aligns closely to the end of the catalytic domain of the vertebrate enzymes. The residues in the catalytic triad are conserved, as are the six cysteines which form the three intramolecular disulfide bonds. Three of the fourteen aromatic residues which line the active site gorge in the Torpedo enzyme are substituted by nonaromatic residues, corresponding to Tyr-70 (Thr), Trp-279 (Asn), and Phe-288 (Met).

High level expression was obtained via secretion from *Pichia pastoris*. The purified enzyme behaved as a monomeric hydrophilic species. Although of invertebrate origin and possessing the above substitutions in the active site gorge residues, the enzyme efficiently hydrolyzed acetylthiocholine and showed minimal activity against butyrylthiocholine. It displayed excess substrate inhibition with acetylthiocholine at concentrations over 2.5 mM and was highly sensitive to both active site and "peripheral" site inhibitors. Northern blot analysis indicated a progressive increase in mRNA for AChE B in parasites isolated from 6 days postinfection.

Acetylcholine (ACh)[1] is the major excitatory neurotransmitter which regulates motor functions in the nematode *Caenorhabditis elegans* (1), and cholinergic motor neurons have been identified in both free-living (1, 2) and parasitic (3, 4) nematode species. In contrast to vertebrates (5) and insects (6), *C. elegans* possesses multiple genes which encode acetylcholinesterases (AChEs). Genetic studies originally defined three separate genes, *ace-1*, *ace-2*, and *ace-3* (7–9) which encoded different catalytic forms of AChE (10), although recent data resulting from cDNA cloning indicate that *C. elegans* possesses at least four separate genes for AChE (11).

In addition to possessing cholinergic motor neurons, many parasitic nematodes secrete AChE into the culture medium when maintained *in vitro* (12–14). A nonneuronal origin for these enzymes has been confirmed by enzyme- and immunohistochemistry, which demonstrated that they are synthesized by and secreted from specialized amphidial and secretory glands (13, 15–17). This remarkable phenomenon is exhibited predominantly by nematodes which inhabit the gastrointestinal tract of their vertebrate hosts and has provoked much discussion on the putative physiological role of these enzymes (18, 19).

Analysis of the AChE secreted by the human hookworm *Necator americanus* indicated that the enzyme existed as a nonamphiphilic dimer (G_2^{na} form) (20), whereas both G_1^{na} and G_2^{na} forms were identified in secreted products of *Trichostrongylus colubriformis*, a nematode parasite of sheep (21). We are utilizing *Nippostrongylus brasiliensis*, the adult stages of which colonize the jejunum of rats, as a model system in which to study AChE secretion and have recently purified and characterized three variants of secreted AChE (22). These variants (designated A, B, and C) are all G_1^{na} forms, with apparent masses of 74, 69, and 71 kDa, respectively, when resolved by SDS-polyacrylamide gel electrophoresis. Substrate and inhibitor specificities defined them as true AChEs rather than pseudocholinesterases. Although they show broadly similar enzymatic properties, they can be distinguished by subtle differences in molecular mass, inhibitor profiles, and excess substrate inhibition and display distinct and characteristic mobilities on nondenaturating PAGE (22).

These forms are differentially expressed by *N. brasiliensis* during residence in the small intestine of the rat. Thus, parasites which have entered the duodenum from the stomach by three days postinfection exclusively secrete isoform A. Following moulting to the adult stage and migration to a more distal position in the jejunum however, a switch to expression of forms B and C occurs, with form B progressively becoming the most abundant secreted enzyme (22–24). In this report, we have derived amino-terminal amino acid sequence of form B and utilized this to isolate a full-length cDNA clone. High level expression of a recombinant form of the enzyme was obtained via secretion from the methylotrophic yeast *Pichia pastoris*. The unusual primary structure of the enzyme is discussed in relation to the enzymatic properties of the recombinant enzyme

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF052508.

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[1] The abbreviations used are: ACh, acetylcholine; AChe, acetylcholinesterase; PAGE, polyacrylamide gel electrophoresis; ES, excretory/secretory; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair(s); ASCh, acetylthiocholine; BuSCh, butyrylthiocholine; iso-OMPA, tetraisopropyl pyrophosphoramide; BW284c51, 1,5-bis(4-allyldimethylammoniumphenylpentan-3-one dibromide.

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and to the structure and properties of AChEs from other organisms.

EXPERIMENTAL PROCEDURES

Parasites—N. brasiliensis were isolated from the small intestine of male Sprague-Dawley rats between days 3 and 10 postinfection as described previously (24). Excretory/Secretory (ES) products were collected from culture supernatants as described (22, 24), concentrated in Centricon 10 microconcentrators (Amicon 4205), washed into 50 mM phosphate-buffered saline, pH 7.4, and the protein concentration determined via the Bradford assay.

Cloning and Sequencing—We utilized a 5′ primer tailored to the 6 amino-terminal amino acids of AChE B (DDGPTV) and containing a *Not*I restriction site (5′-atgcggccgcGA(T/C)GA(T/C)GGNCCNACNGT-3′), and a 3′ primer based on the conserved sequence flanking the active site serine residue (FGESAG) linked to a *Xho*I restriction site (5′-atctcgagCCNGCN(C/G)(A/T)(C/T)TC(T/G/A)CC(A/G)AA-3′) to amplify a fragment by RT-PCR which would putatively encode the amino-terminal region of AChE B (lowercase indicates nucleotides added for cloning purposes). A standard 100-μl PCR reaction was utilized, using 30 ng of cDNA, 500 ng of each primer, and 30 cycles of: 94 °C for 30 s, 54 °C for 1 min, 72 °C for 2 min. This procedure yielded a product of 580 bp, which was subcloned into pBluescript II (Stratagene) and sequenced to confirm that it encoded a cDNA fragment which was homologous to AChEs. We then utilized this fragment to screen a cDNA library constructed in λZAP (Stratagene) and isolated several clones, which were sequenced on both strands by dideoxy chain termination following subcloning into pBluescript.

Expression in P. pastoris—N. brasiliensis-secreted AChE B was expressed in *P. pastoris* via the expression plasmid pPICZα (Invitrogen). The nucleotide sequence encoding the mature enzyme was amplified from cDNA clone C43 using the sense primer 5′-tatactgcagATGATG-GTCCGACGGTGGTG-3′ and the antisense primer 5′-attatctagaTTGC-CTTTAGCTTCATCAAT-3′. The fragment was cloned into pPICZαB cut with *Pst*I and *Xba*I, and the reading frame was verified by partial sequencing of the recombinant plasmid, which was then linearized with *Sac*I. The construct was integrated into competent *P. pastoris* X-33 via homologous recombination according to the instructions of the manufacturer (Invitrogen), and transformants were confirmed by PCR with oligonucleotide primers homologous to pPICZαB sequences flanking the insertion site. The construct thus encoded the mature AChE B protein with an amino-terminal signal peptide provided by the pre-pro sequence of the α-mating factor of *Saccharomyces cerevisiae* and with a carboxyl-terminal polyhistidine tag. Recombinant yeast were grown in BMGY medium (1% yeast extract, 2% peptone, 0.1 M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 400 ng ml^{-1} biotin, 1% glycerol) to saturation (2 days), and expression was induced in BMMY medium (same as BMGY, but with glycerol replaced by 1% methanol) at 30 °C. Culture supernatants were harvested at different times post-methanol induction and analyzed for AChE activity.

Purification of Native and Recombinant AChE—The native enzymes were purified from ES products of worms isolated 8 days postinfection by affinity chromatography on 1-methyl-9-[Nβ-(ε-aminocaproyl)-β-aminopropylamino]-acridinium bromide hydrobromide-Sepharose 4B (a kind gift of Prof. I. Silman) as described previously (22). Amino-terminal amino acid sequence of the major AChE isoform (form B) was obtained following resolution by 10% SDS-PAGE and blotting onto a polyvinylidene difluoride membrane (Bio-Rad) and was performed by Severn Biotech (Kidderminster, UK). The recombinant AChE was purified from culture supernatants of transformed *P. pastoris* by nickel-charged chelating Sepharose chromatography. Chelating Sepharose (Amersham Pharmacia Biotech) was charged with 1% $NiSO_4$, washed with distilled water and equilibrated with 50 mM sodium phosphate, pH 8.0, containing 300 mM sodium chloride (buffer A). The yeast culture supernatant was clarified by centrifugation at 25,000 × *g* for 10 min at 4 °C, concentrated on an Amicon cell, and dialyzed against buffer A. The dialysate was loaded onto the column at a flow rate of 40 ml h^{-1}, which was subsequently extensively washed with buffer A and then buffer A plus 10 mM imidazole. The enzyme was then eluted from the column with buffer A plus 200 mM imidazole, and the purity of the enzyme was determined by SDS-PAGE.

Denaturing and Nondenaturing Electrophoresis—SDS-PAGE was performed on 12% polyacrylamide gels followed by staining with Coomassie Brilliant Blue. Purified recombinant AChE and ES products were resolved under nondenaturing conditions by electrophoresis in 7.5% polyacrylamide gels in Tris-borate-EDTA (TBE) buffer, pH 8.0, and enzyme activity was assayed by the method of Karnovsky and Roots (25).

Sucrose Density Centrifugation—Sedimentation analysis was performed in 2–20% sucrose gradients (11 ml, with a 0.5-ml cushion of 50% sucrose) in phosphate-buffered saline in the presence or absence of 1.0% Triton X-100. The purified enzyme was applied to the gradient and centrifuged for 16 h at 36,000 rpm at 4 °C in an SW41 rotor (170,000 × *g*). Fractions (0.25 ml) were collected and assayed for AChE activity. Bovine liver catalase (11.3 S) and *Escherichia coli* alkaline phosphatase (6.1 S) were included as internal standards.

Northern Blotting—Total RNA was isolated from parasites as described previously (26). For Northern blots, RNA was denatured in glyoxal, 10 μg per track separated on 1.2% agarose gels and transferred to nylon filters. Equal loading of RNA was confirmed by ethidium bromide staining of gels prior to blotting and methylene blue staining of filters following transfer. Filters were hybridized with the full-length C43 cDNA probe labeled by random priming in 5× SSC, 0.25 mg ml^{-1} salmon sperm DNA, 0.25% SDS, 0.25% dried milk powder at 65 °C with 10^6 cpm ml^{-1} of probe, and blots were washed in 0.1× SSC, 0.25% SDS at 65 °C.

AChE Activity and Substrate and Inhibitor Specificities—AChE activity was determined by the method of Ellman (57) with 1 mM acetylthiocholine (ASCh) iodide as substrate in the presence of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) in 100 mM sodium phosphate, pH 7.0, at 20 °C. The reaction was monitored by measuring the absorbance at 412 nm, and hydrolysis of ASCh was calculated from the extinction coefficient of 5,5′-dithiobis(2-nitrobenzoic acid) (27). One unit of AChE is defined as 1 μmol of substrate hydrolyzed per min at 20 °C. The K_m value for ASCh was determined by linear regression from plots of $1/V$ against $1/S$, utilizing substrate at a range of concentrations between 25 and 200 μM (*i.e.* below substrate inhibition). Inhibition constants (K_i) for eserine, BW284C51, propidium iodide, and gallamine were determined using two fixed inhibitor and six ASCh concentrations (0.05–0.20 mM). Assays were carried out in triplicate in the presence or absence of inhibitor. Kinetic constants were determined using Graphpad Prism 2.0 (San Diego, CA).

RESULTS

cDNA Cloning of Secreted AChE B—We purified native AChEs (forms B and C) from ES products of worms isolated 8 days postinfection by affinity chromatography on 1-methyl-9-[Nβ-(e-aminocaproyl)-β-aminopropylamino]-acridinium bromide hydrobromide-Sepharose 4B, resolved the enzymes by SDS-PAGE, and blotted them onto a polyvinylidene difluoride membrane. This procedure resulted in the determination of just six amino acids of amino-terminal sequence of the major isoform (form B) as DDGPTV. This information allowed us to design a primer that was utilized in combination with another to the conserved sequence flanking the active site serine residue of AChEs (FGESAG) to amplify a cDNA fragment of approximately 580 bp by RT-PCR, using RNA from parasites isolated 5 days postinfection. This cDNA fragment was sequenced and showed significant homology to cholinesterases from diverse sources and was, therefore, used to screen a cDNA library constructed in λZAP. Several clones were isolated by this procedure and sequenced. The sequence of the longest clone (C43, 1799 bp) is shown is Fig. 1. It contains a short (12 bp) 5′-untranslated region, a single open reading frame of 1680 nucleotides, and a 3′-untranslated region of 107 bp, containing a consensus polyadenylation site (AATAAA, underlined) and a poly(A) tail. Analysis of the amino acid sequence encoded by C43 predicts a signal peptide of 19 residues, defined by the amino-terminal sequence of the native protein (underlined). The primary structure of the mature protein exhibits clear similarities to cholinesterases from numerous species, including 30% identity to ACE-1 of *C. elegans* (28) and 35% identity to *Torpedo californica* AChE (29).

Structural Features of N. brasiliensis Secreted AChE B—The most pertinent features of AChE B defined by the primary structure are illustrated in Fig. 2 in which it is aligned with that of *C. elegans* ACE-1 (28) and the T subunit of *T. californica* AChE (29). The residues in the catalytic triad, Ser-193, Glu-342, and His-466 (Ser-200, Glu-327, and His-440 in *Torpedo*),

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TTCGGCACGAGG ATG GGA CTT CCC GCT AGA TTA CTC CTG GCA ATA TGC GTC TTC TCG ACG
              M   G   L   P   A   R   L   L   L   A   I   C   V   F   S   T    16

TCG GCG GCT GAT GAT GGT CCG ACG GTG GTG CTT TCC TCG GGA ACA AAA ATT CAT GGA
 S   A   A   D   D   G   P   T   V   V   L   S   S   G   T   K   I   H   G    35

ATA TAC ATG GAT GTA AAC GGA CAA ACG GTG AGC GCC TAC CTA GGT GTT CCA TTT GCT
 I   Y   M   D   V   N   G   Q   T   V   S   A   Y   L   G   V   P   F   A    54

ACG GCG GAG AGG TTT GCG ATG CCG ACG TTA ACC GAA ACC TAC GGC GGA GAT ATT GAA
 T   A   E   R   F   A   M   P   T   L   T   E   T   Y   G   G   D   I   E    73

GCT CTA CAG CTT TCA AAA ACC TGT TTC CAA ACC AAA GAC GAA ACG TAC CCG GGA TTT
 A   L   Q   L   S   K   T   C   F   Q   T   K   D   E   T   Y   P   G   F    92

GAT GGT GCT GAA ATG TGG AAT CCA CCA ACG GAG CTT TCA GAA GAT TGT CTG AGT TTG
 D   G   A   E   M   W   N   P   P   T   E   L   S   E   D   C   L   S   L   111

AAC ATC TGG GTC CCG GAA AAT CCC GAT GGC AAT GTG ATT GTT TGG ATT TAT GGA GGA
 N   I   W   V   P   E   N   P   D   G   N   V   I   V   W   I   Y   G   G   130

GGT TTC TTC AGT GGT TCC CCG TCG CTT GCA CTT TAC AAT GGA TCC GTG CTG GCC GGT
 G   F   F   S   G   S   P   S   L   A   L   Y   N   G   S   V   L   A   G   149

AAA ACG AAC GCA GTA GTT GTG AAT GTC AAC TAT CGA GTT GGA CCA TTC GGC TTC TTC
 K   T   N   A   V   V   V   N   V   N   Y   R   V   G   P   F   G   F   F   168

TAC CTC GGA GCC AAC TCA AAG GCA CCT GGA AAT GTT GGT TTG CTG GAT CAG CAA ACT
 Y   L   G   A   N   S   K   A   P   G   N   V   G   L   L   D   Q   Q   T   187

GCA CTG AAA TGG ATT CAT AAT AAC ATA GAA TAC TTC AAA GGA GAT CCT AGC AAG GTT
 A   L   K   W   I   H   N   N   I   E   Y   F   K   G   D   P   S   K   V   206

ACG CTT TTC GGT GAA TCG GCT GGT GGC ACC TCT GTC ACG TCT CAT CTG CTG GCC CCC
 T   L   F   G   E   S   A   G   G   T   S   V   T   S   H   L   L   A   P   225

GAC AGC CAC AGC TTG TTC AGC AAA ATC ATT GTT AAT AGC GGA TCT ATA CAT AAT GTA
 D   S   H   S   L   F   S   K   I   I   V   N   S   G   S   I   H   N   V   246

TGG GCG ACT AGA AGT CCA TGT ACT ATG CTA CAC ATC TCA ATG AAA ACA GCG AAG GCA
 W   A   T   R   S   P   C   T   M   L   H   I   S   M   K   T   A   K   A   265

CTA GGA TGT GTC GAA AAT TAC GAG ATC AAA CCC ATC AGA GAA GCA GAG GGT CGA TGC
 L   G   C   V   E   N   Y   E   I   K   P   I   R   E   A   E   G   R   C   282

ACT GTA TTG GGA GCA GAC GCT GAT ACG ATT TAC GAG TGC ATG AAG GAG AAG ACA CCG
 T   V   L   G   A   D   A   D   T   I   Y   E   C   M   K   E   K   T   P   301

GAG AAG ATT CAG AGC GAG GGC AAC TCT GAC GCG ATA TAT GCT GAG ATG CTT CCG ATG
 E   K   I   Q   S   E   G   N   S   D   A   I   Y   A   E   M   L   P   M   320

GAG TGG CCA TTT GGT CCG ATC ACC TAC GAC GAT AAC TAC TTC AAG GGA GAA GTA CGG
 E   W   P   F   G   P   I   T   Y   D   D   N   Y   F   K   G   E   V   R   339

CGA AAG CTC TTC AGC GGT GAG TTC AAG ACG GAC GTG TCG GCG ATC TTC GGA ACG GTG
 R   K   L   F   S   G   E   F   K   T   D   V   S   A   I   F   G   T   V   358

AAG GAC GAA GGA ACA TTC TGG CTA CCG TAC TAC CTG TCT GAG AGT GGC TTC AGT TTC
 K   D   E   G   T   F   W   L   P   Y   Y   L   S   E   S   G   F   S   F   377

TTT CCT GAC CAG GAT TCT GAC AGT GAG GCA AAC GCA GCA AAA ATA GAC GAG GCC AAC
 F   P   D   Q   D   S   D   S   E   A   N   A   A   K   I   D   E   A   N   396

TAT ACG GCA TCA ATG CAA GCT TTC GAG GGG TAC TTT GGA AAG AGC AGC AAA GCT ATA
 Y   T   A   S   M   Q   A   F   E   G   Y   F   G   K   S   S   K   A   I   415

GAG ATC CTC AAG GAA GGA TTC AAA GAT TTG GGT GAT GTA CAA ACA ATG TAT CGC GAC
 E   I   L   K   E   G   F   K   D   L   G   D   V   Q   T   M   Y   R   D   434

GGT GTT GCT CGA TTC GTT GGA GAC TTC TTC TTT ACC TGC AAT TTG GTG GAA TTT GTC
 G   V   A   R   F   V   G   D   F   F   F   T   C   N   L   V   E   F   V   453

GAT CAT GTC ACC CAG AAG ATT AGC GAA GCC TAC ATG TAC TAC TTC AAA GCA CGA TCT
 D   H   V   T   Q   K   I   S   E   A   Y   M   Y   Y   F   K   A   R   S   472

TCC GCA AAC CCA TGG CCA AAA TGG ATG GGA GTC ATG CAC GGA TAC GAG ATC GAA TAT
 S   A   N   P   W   P   K   W   M   G   V   M   H   G   Y   E   I   E   Y   491

GAA TTC GGA TAT CCT TTC ATC AAC TCC ACT GCT TAC AAG GAA GTA GAC AAG GAT CGA
 E   F   G   Y   P   F   I   N   S   T   A   Y   K   E   V   D   K   D   R   510

ACA ATC TCC GAG GAG TTC ATG CAA CTA ATC AAG GAA TTC GTA AAG AAC GGA AAG TTT
 T   I   S   E   E   F   M   Q   L   I   K   E   F   V   K   N   G   K   F   529

GAT GAC GAA TGG CCG AAA TAT GAG GGA GGA AAG GTA ATG GTG ATT GAG GAC GAC GCT
 D   D   E   W   P   K   Y   E   G   G   K   V   M   V   I   E   D   D   A   548

TCG AGA AAG ATC GAA GAC AAA GAC ATC CAG CAG AAA TAT TGC AAA ATC ATT AAT GAT
 S   R   K   I   E   D   K   D   I   Q   Q   K   Y   C   K   I   I   N   D   567

GCC AGA CAA GCT CTA ATT GAT GAA GCT AAA GGC AAC TAG TTTCCATTCAAATCGGGACGACAG
 A   R   Q   A   L   I   D   E   A   K   G   N   STOP                         579

TATCGACCGAAATGATACAGCCTGTGATAAATCTTTCTATGCCGCAGCGAACAATAAATGATTCATAAACAAAAAAA
AAA
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FIG. 1. **Nucleotide and derived amino acid sequence of cDNA encoding the secreted AChE B of *N. brasiliensis*.** The amino acid sequence is numbered from the initiating methionine, and the amino-terminal sequence of the mature secreted protein is *underlined*. Residues predicted to comprise the catalytic triad of the enzyme are *circled*, and three potential sites for *N*-linked glycosylation are *boxed*. A consensus sequence for polyadenylation (AATAAA) in the 3′-untranslated sequence is also *underlined*.

are conserved. Six cysteine residues are present (62–89, 247–276, and 428–543) that define the position of the three intramolecular disulfide bonds conserved in cholinesterases (67–94, 254–265, and 402–521; Refs. 30, 31), and an additional two cysteine residues are present in positions 232 and 263 in the *N. brasiliensis* sequence. The protein is truncated at the carboxyl

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                          1
Nb   ---MGLPARLLLAICVFSTSAADDGPTVVLSSGTKIHGIYMDVNGQTVSAYLGVPFAT--   36
Ce   -------MRNSLLFFIFLPSILAVDLIHLHDGSPLFGEEVLSQTGKPLTRFQGIPFAEPP
Tc   MNLLVTSSLGVLLHLVVLCQADDHSELLVNTKSGKVMGTRVPVLSSHISAFLGIPFAEPP   39
                         1

Nb   --AERFAMPTLTETYGGDIEALQLSKTCFQTKDETYPGFDGAEMWNPPTELSEDCLSLNI   94
Ce   VGNLRFKKPKPKQQWRIPLNATTPPNSCIQSEDTYFGDFYGSTMWNANTKLSEDCLYLNV
Tc   VGNMRFRRPEPKKPWSGVWNASTYPNNCQQYVDEQFPGFSGSEMWNPNREMSEDCLYLNI   99

Nb   WVPEN--P--DGNVIVWIYGGGFFSGSPSLALYNGSVLAGKTNAVVVNVNYRVGPFGFFY  150
Ce   YVPGKVDPNKKLAVMVWVYGGGFWSGTATLDVYDGRILTVEENVILVAMNYRVSIFGFLY
Tc   WVPSPR-P-KSTTVMVWIYGGGFYSGSSTLDVYNGKYLAYTEEVVLVSLSYRVGAFGFLA  157

Nb   LGANSKAPGNVGLLDQQTALKWIHNNIEYFKGDPSKVTLFGESAGGTSVTSHLLAPDSHS  210
Ce   MN-RPEAPGNMGMWDQLLAMKWVHKNIDLFGGDLSRITLFGESAGAASVSIHMLSPKSAP
Tc   LHGSQEAPGNVGLLDQRMALQWVHDNIQFFGGDPKTVTIFGESAGGASVGMHILSPGSRD  217

Nb   LFSKIIVNSGSIHNVWATRSPCTMLHISMKTAKALGCVENYEIKPIREAEGRCTVLGADA  270
Ce   YFHRAIIQSGSATSPWAIEPRDVALARAVILYNAMKCGN-------------MSLINPDY
Tc   LFRRAILQSGSPNCPWASVSVAEGRRRAVELGRNLNCN-----------------LNSDE  260

Nb   DTIYECMKEKTPEKIQSEGNSDAIYAEMLPMEWPFGPITYDDNYFKGEVRRKLFSGEFKT  330
Ce   DRILDCFQRADADALRENEW--APVRE--FGDFPWVPV-VDGDFLLENAQTSLKQGNFK-
Tc   E-LIHCLREKKPQELIDVEWNVLPFDS--IFRFSFVPV-IDGEFFPTSLESMLNSGNFK-  315

Nb   DVSAIFGTVKDEGTFWLPYYLSESGFSFFPDQDSDSEANAAKIDEANYTASMQAFEGYFG  390
Ce   KTQLLAGSNRDESIYFLTYQLPDI----FPVADFFTKTDFIKDRQLWIKGVKDLLPRQIL
Tc   KTQILLGVNKDEGSFFLLYGAPG-----FS-KDSESK---IS-REDFMSGVKLSVPHAND  365

Nb   KSSKAIEILKEGFKDLGDVQTMYRD---GVARFVGDFFFTCNLVEFVDHVTQKISEAYMY  447
Ce   KCQLTLAAVLHEYEPQ-DLPVTPRDWINAMDKMLGDYHFTCSVNEMALAHTKHGGDTYYY
Tc   LGLDAVTLQYTDWMDD-NNGIKNRD---GLDDIVGDHNVICPLMHFVNKYTKFGNGTYLY  421

Nb   YFKARSSANPWPKWMGVMHGYEIEYEFGYPFINSTAYKEVDKDRTISEEFMQLIKEFVKN  507
Ce   YFTHRASQQTWPEWMGVLHGYEINFIFGEPLN-QKRFNYTDEERELSNRFMRYWANFAKT
Tc   FFNHRASNLVWPEWMGVIHGYEIEFVFGLPL--VKELNYTAEEEALSRRIMHYWATFAKT  479

Nb   GK---------FDDEWPKYEGGKVMVIEDDASR-----KIEDKDIQQKYCKIINDARQAL  553
Ce   GDPNKNEDGSFTQDVWPKYNSVSMEYMNMTVESSYPSMKRIGHGPRRKECAFWKAYLPNL
Tc   GNPNEPHS---QESKWPLFTTKEQKFIDLNTEP-----MKVHQRLRVQMCVFWNQFLPKL  531

Nb   IDEAKGN                                                       560
Ce   MAAVADVGDPYLVWKQQMDKWQNEYITDWQYHFEQYKRYQTYRQSDSETCGG
Tc   LNATETIDEAERQWKTEFHRWS-SYMMHWKNQFDHYSRHESCAEL                 575
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FIG. 2. **Alignment of *N. brasiliensis*, *T. californica*, and *C. elegans* AChE sequences.** The derived amino acid sequence of the *N. brasiliensis* secreted AChE (*Nb*) was aligned with that of the T subunit of *T. californica* (*Tc*) and ACE-1 from *C. elegans* (*Ce*) using the CLUSTALW program (56). The *N. brasiliensis* and the *T. californica* AChEs are numbered from amino terminus of the mature proteins (marked "*1*"). The residues in the catalytic triad (Ser-His-Glu) are depicted with an *asterisk*, and the position of three potential sites for *N*-linked glycosylation marked with a *square*. Cysteine residues conserved at positions which are involved in intramolecular disulfide bonds are indicated by *solid triangles*, whereas *open triangles* depict the two additional cysteine residues in the *N. brasiliensis* AChE. The positions of aromatic residues that line the active site gorge in *Torpedo* AChE are marked with *circles*. *Solid circles* indicate conserved residues or conservative substitutions at these positions in the *N. brasiliensis* AChE, whereas *open circles* indicate nonaromatic residues. The protein is truncated at the carboxyl-terminus. For orientation, the *arrow* indicates the branch point for T and H subunits in *Torpedo*.

terminus by 45 residues relative to *C. elegans* ACE-1 or by 37 residues relative to the T subunit of the *Torpedo* enzyme. There are three consensus sequences for *N*-linked glycosylation, at positions 124, 377, and 480.

Eleven of the fourteen aromatic residues which line the wall of the active site gorge in the *Torpedo* enzyme (32) are either conserved or show conservative substitutions in the nematode sequence, including Trp-79 (Trp-84) which binds the quaternary ammonium group of acetylcholine in the active site (33, 34). The three residues which show nonconservative substitutions are Thr-65 (Tyr-70), Asn-290 (Trp-279), and Met-301 (Phe-288).

Expression of AChE B in N. brasiliensis—Expression of AChE B during residence of *N. brasiliensis* in the intestinal tract of the mammalian host was determined by Northern blotting, utilizing RNA from parasites recovered at different time points postinfection and the C43 cDNA as a probe. Fig. 3 demonstrates that the levels of mRNA for the secreted AChE remained relatively constant in parasites isolated between days 3 and 6 postinfection but, thereafter, rose progressively when assayed 8 and 10 days postinfection.

Expression in P. pastoris—We next expressed *N. brasiliensis* AChE B in the methylotrophic yeast *P. pastoris*, utilizing the yeast α-mating factor signal peptide to direct secretion of the enzyme, and colonies were selected that expressed high levels of AChE. In a representative example, AChE activity was detected in the culture medium assayed just 30 min after methanol induction. The concentration of total protein secreted by this recombinant yeast clone rose from approximately 6 μg ml^{-1} at 24 h to 27 μg ml^{-1} at 5 days postinduction (Fig. 4*A*), although in practice we changed the culture medium daily. Fig. 4*B*, *lane 2* shows that AChE B constituted approximately 90% of the total secreted protein. After 2 days postinduction, the medium was collected and the recombinant enzyme purified by nickel-chelating chromatography. The purified enzyme had a high specific activity when assayed against ASCh of 2,080 units mg^{-1}. It displayed an apparent mass of 69 kDa when resolved by SDS-PAGE under nonreducing conditions and one of 72 kDa under reducing conditions (Fig. 4*B*, *lane 3*), indicative of a monomeric protein with intramolecular disulfide bonds.

Recombinant AChE B was resolved by sucrose density gradient centrifugation in a single peak at 4.5 S. There was no

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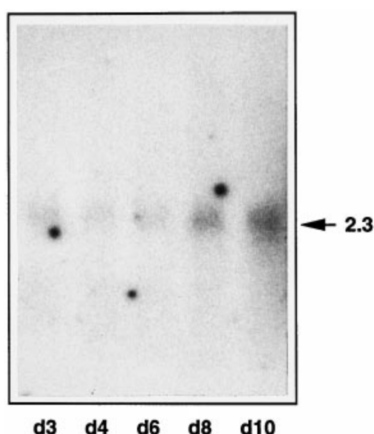

FIG. 3. **Expression of AChE B is up-regulated during residence in the intestinal tract.** Total RNA from parasites recovered from the jejunum of rats at different time points from day 3 (*d3*) to day 10 (*d10*) postinfection were subjected to analysis by Northern blotting, utilizing the C43 cDNA as a hybridization probe. A single mRNA species of 2.3 kilobases is indicated by an *arrow*. The levels of this mRNA are progressively elevated from day 8 postinfection.

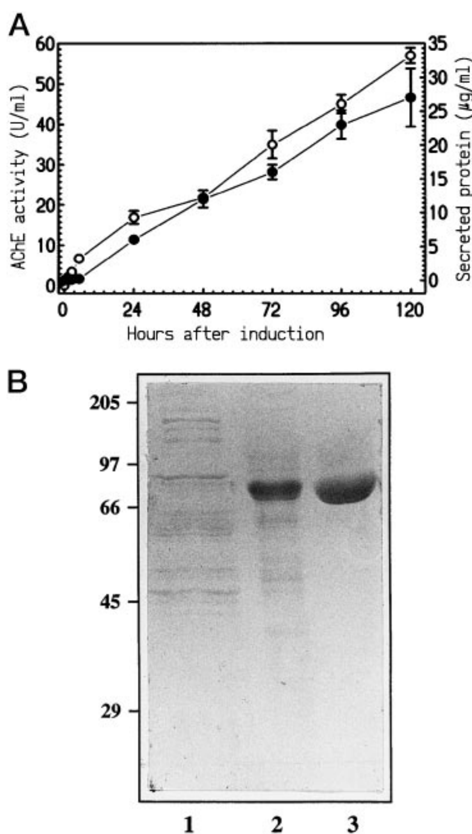

FIG. 4. **Expression of *N. brasiliensis* AChE B in *P. pastoris*.** *A*, kinetics of secretion into the culture medium following induction of expression with methanol. Total secreted protein is indicated by *closed circles*, and AChE activity is indicated by *open circles*. *B*, purification of recombinant AChE. Culture medium from *P. pastoris* X-33 wild-type cells (*lane 1*) or cells transfected with cDNA encoding *N. brasiliensis* AChE (*lane 2*), and the purified enzyme (*lane 3*) were resolved via 12% SDS-PAGE under reducing conditions. The gel was fixed and stained with Coomassie Blue. The position of molecular mass markers is shown in kDa.

shift in sedimentation in the presence of 1% Triton X-100, indicative of a monomeric nonamphiphilic (G_1^{na}) enzyme (data not shown). The purified enzyme was next subjected to nondenaturing PAGE and stained for AChE activity, and Fig. 5 demonstrates that it resolved as a fast-migrating single band

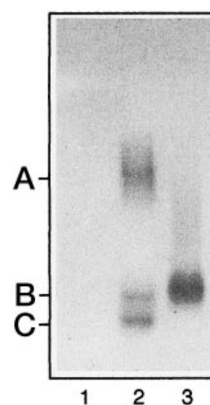

FIG. 5. **Resolution of AChE B by nondenaturing PAGE.** Secreted proteins from the culture medium of *P. pastoris* X-33 wild type cells (*lane 1*) and purified recombinant AChE (*lane 3*) were resolved by nondenaturing 7.5% PAGE alongside native secreted AChEs from parasites collected 4 days postinfection (*lane 2*). AChE activity was visualized according to Karnovsky and Roots (25). The migration of native secretory enzymes designated as forms A, B, and C (22) is indicated.

that co-migrated with form B of the native secreted parasite AChEs.

Substrate and Inhibitor Specificities—The specificity of AChE B for a range of substrates was assayed, and the results are shown in Fig. 6, demonstrating that ASCh was the preferred substrate. Thus, at a substrate concentration of 1 mM, the rate of hydrolysis of A[βM]SCh, PSCh, and BuSCh relative to ASCh were 61, 15, and 5%, respectively. Excess substrate inhibition was observed for the former substrates at concentrations exceeding 2.5 mM, and the K_m value for the enzyme was calculated at 0.23 ± 0.10 mM (n = 8).

We next assessed the susceptibility of the recombinant enzyme to cholinesterase inhibitors. Activity was unaffected by the butyrylcholinesterase inhibitor iso-OMPA at concentrations up to 10 mM (data not shown), but it was highly sensitive to eserine and BW284c51, the latter a specific inhibitor of AChEs (35). Both eserine and BW284c51 inhibited in a competitive manner, and the K_i values were calculated at 3.6 ± 1.2 and 6.4 ± 1.9 nM (n = 4), respectively. The "peripheral" site ligands, propidium iodide and gallamine, also inhibited AChE activity but in a noncompetitive manner, with K_i values of 0.22 ± 0.03 and 0.80 ± 0.07 μM (n = 3), respectively (Fig. 7).

DISCUSSION

The phenomenon of cholinesterase secretion by parasitic nematodes was first reported almost 30 years ago (12, 13, 15) and yet still presents a conundrum which awaits a satisfactory explanation. We are attempting to address the physiological significance of this process and present here a molecular characterization of one of the enzymes secreted by *N. brasiliensis*. As would be expected for a secreted protein, it is hydrophilic, and the properties of the recombinant AChE B are essentially the same as those of the native enzyme (22). The specific activity of the native enzymes purified by acridinium affinity chromatography (685 units mg^{-1}) was considerably lower than that of the recombinant enzyme purified by nickel-chelating chromatography (2,080 units mg^{-1}) however, and this is consistent with previous data (22). We attribute this to residual binding of decamethonium to the native enzymes, which can only be purified in low microgram quantities. Partial purification of native AChE B without the use of an affinity ligand gave a value of 1,450 units mg^{-1},[2] suggesting that the estimation of specific activity for the recombinant enzyme is close to that of

[2] Grigg, M. E., unpublished observations.

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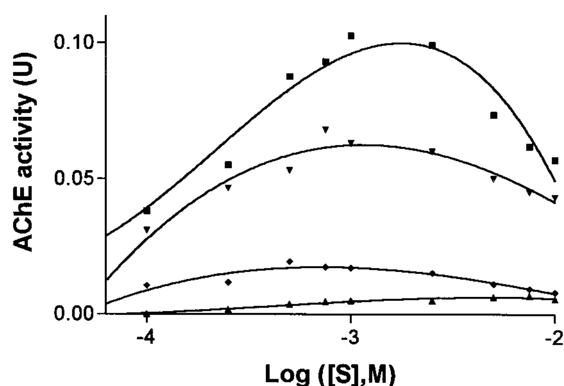

FIG. 6. **Substrate specificity of AChE B.** The rate of hydrolysis of acetylthiocholine (■), acetyl-[β-methyl]thiocholine (▼), propionylthiocholine (♦) and butyrylthiocholine (▲) were determined as described under "Experimental Procedures," utilizing a range of substrate concentrations between 0.05 and 10 mM.

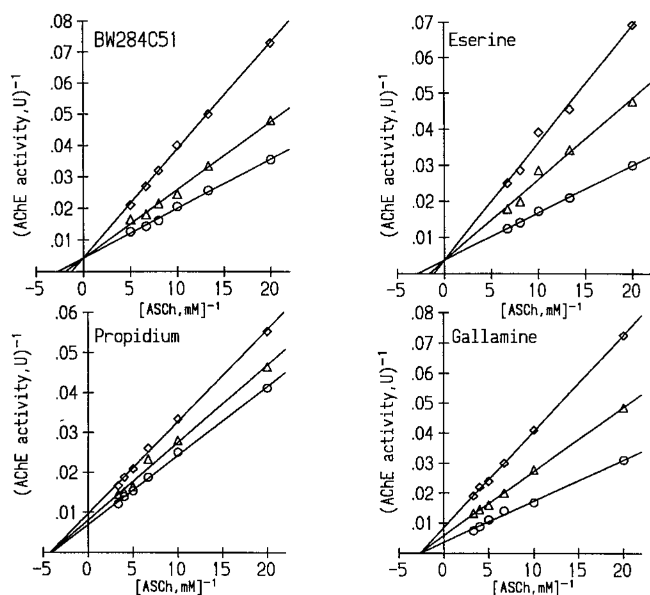

FIG. 7. **Sensitivity of AChE B to inhibitors.** Inhibition of AChE activity by eserine, BW284c51, and the peripheral site ligands propidium iodide and gallamine was assessed. Assays were performed with varying concentrations of ASCh in the absence (○) and the presence of 1 nM (△) or 5 nM (◇) eserine and BW284c51, and 0.1 μM (△) or 0.5 μM (◇) propidium iodide and gallamine. Lineweaver-Burk plots (1/*V versus* 1/*S*) are shown, indicating competitive inhibition by eserine (K_i = 3.6 ± 1.2 nM) and BW284c51 (K_i = 6.4 ± 1.9 nM) and noncompetitive inhibition by propidium (K_i = 0.22 ± 0.03 μM) and gallamine (K_i = 0.80 ± 0.07 μM).

the native enzyme.

Derivation of the primary structure of the protein from the cDNA sequence provides an explanation for its monomeric nature, as the carboxyl-terminal cysteine implicated in dimer formation is missing. In fact, the carboxyl terminus is severely truncated in comparison with vertebrate AChEs and therefore bears no resemblance to the peptides that define hydrophobic (H) or tailed (T) subunits (36). Fig. 2 illustrates that the carboxyl terminus of the parasite enzyme aligns at a position approximating to the end of the catalytic domain of *Torpedo* AChE. In this respect, the parasite enzyme is similar to AChE from *Bungarus fasciatus* venom, which is also unusual in that it is a hydrophilic monomeric enzyme with a truncated carboxyl terminus, although the carboxyl-terminus of ACE B is 12 residues shorter than that of the *Bungarus* AChE and thus lacks the basic residues associated with the latter enzyme which are cleaved on secretion (37, 38).

In many other respects, the sequence of the *N. brasiliensis* AChE B shows features consistent with AChEs from diverse species. The residues which constitute the catalytic triad are conserved, as are the six cysteine residues implicated in disulfide bond formation. An additional two cysteine residues are present in the *N. brasiliensis* sequence, however, at positions 232 and 263. The latter lies in an insertion of 17 amino acids (relative to *Torpedo*), which by analogy with the structure of the *T. californica* AChE (32) most probably forms part of a loop of 30 residues at the molecular surface stabilized by the second disulfide bond.

One of the most striking features of the structure of the *Torpedo* enzyme is the active site gorge, about 20-Å long, which penetrates halfway into the enzyme (32). Fourteen aromatic residues line a substantial part of the surface of the gorge. These residues and flanking sequences are highly conserved in AChEs from different species and are thought to delineate a substrate guidance mechanism. Eleven of these residues are either conserved or show conservative substitutions in the *N. brasiliensis* AChE. Two of the aromatic residues (Tyr-70 and Phe-288 in *Torpedo*) that are substituted in the nematode sequence by Thr-65 and Met-301 are also substituted by nonaromatic residues in mammalian butyrylcholinesterases (39) and *C. elegans* ACE-1 (28). Mutagenesis studies on *Torpedo* and human AChE have shown that Phe-288 and Phe-290 dictate substrate specificity, most probably via steric occlusion but also possibly by stabilizing the substrate in an optimal position for catalysis (34, 40, 41). It has been suggested that the intermediate substrate specificity of certain invertebrate enzymes such as *C. elegans* ACE-1 and *Drosophila melanogaster* AChE (both enzymes hydrolyze BuSCh at approximately 50% the rate of ASCh) can be explained by the substitution of Phe-288 by glycine and leucine, respectively (28, 42). Replacement of Phe-288 in *Torpedo* and human AChE by nonaromatic residues greatly enhanced the ability of these enzymes to hydrolyze BuSCh, in addition to conferring sensitivity to inhibition by iso-OMPA (34, 40, 41). It is therefore surprising that Phe-288 is replaced by a methionine residue in the *N. brasiliensis* sequence, as the enzyme shows little activity against BuSCh and no inhibition by iso-OMPA even at very high concentrations (10 mM). We assume that alternative residues must restrict substrate and inhibitor accessibility. Phe-290 and Phe-331 in *Torpedo* AChE are both substituted by Trp in the *N. brasiliensis* enzyme (Fig. 2), and it is possible that the bulkier side chain restricts the size of the acyl pocket.

There are 3 consensus sequences for *N*-linked glycosylation, at positions 124, 377, and 480 in the *N. brasiliensis* sequence. Although it is not yet clear which of these sites are glycosylated in the native protein, only one of these (Asn-480) is closely aligned to a glycosylation site (Asn-557) in the *Torpedo* enzyme (43). Asn-124 is unlikely to be glycosylated as it is positioned directly after Tyr-123 (Tyr-130 in *Torpedo*), one of the aromatic residues lining the active site gorge.

N. brasiliensis AChE B is highly sensitive to inhibition by eserine and BW284c51, with K_i values of 3.6 and 6.4 nM, but is also sensitive to the peripheral site ligands propidium iodide and gallamine with K_i values of 0.22 and 0.80 μM, respectively (Fig. 7). The K_i for BW284c51 is similar to that of *Torpedo* AChE at 2 nM, whereas those for the latter inhibitors are approximately an order of magnitude lower: propidium and gallamine inhibited *Torpedo* AChE with K_i values of 2.8 and 15 μM, respectively (44). Peripheral site ligands interact with diverse subsets of residues, and recently the low sensitivity of the *B. fasciatus* AChE to these inhibitors was attributed to substitution of the aromatic residue Tyr-70 located at the entrance of the active site gorge by methionine and of an acidic residue

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(Asp/Glu) at position 285 by lysine (38). Tyr-70 is replaced by threonine in *N. brasiliensis* AChE and serine in *C. elegans* ACE-1, although both enzymes remain sensitive to peripheral site ligands: propidium inhibits ACE-1 with a K_i of 1.3 μM (28). Although our alignment and that of Arpagaus *et al.* (28) place an alanine and arginine residue at position 285 in the two nematode AChEs, both these residues are followed immediately by glutamate. Two other residues that have been implicated in inhibition by peripheral site ligands are Trp-279 and Trp-84 (Trp-286 and -86 in human AChE). Trp-279 lies at the rim of the active site gorge, and the mutant W279A shows strongly reduced inhibition by propidium (40, 34). Trp-84 is located in the active site (32) and binds the quaternary ammonium of choline (33, 34), and substitution with alanine renders the enzyme almost completely refractory to inhibition by propidium (34). A model has been proposed linking these two observations in which binding of propidium to Trp-279 induces a conformational change relayed via residues along the gorge, resulting in reorientation of Trp-84 which interferes with stabilization of substrate enzyme complexes (34). Although Trp-84 (Trp-79) is conserved in *N. brasiliensis* AChE B, Trp-279 is replaced by Asn-290 (Fig. 2), highlighting a further discrepancy in the basis for inhibition by peripheral site ligands in this enzyme.

These anomalies could be clarified by structural analysis of the *N. brasiliensis* AChE. It is possible to obtain only microgram quantities of native enzyme, but the recombinant enzyme is fortuitously expressed in *P. pastoris* at very high levels. Continuous culture for 7 days resulted in approximately 35 mg of enzyme per liter of culture medium. Expression of rat and *Bungarus* AChE in *P. pastoris* resulted in maximal levels of approximately 1 and 2 mg per liter, respectively, when cultured for the same period of time, although it was necessary to generate constructs truncated at the carboxyl terminus to exclusively produce nonamphiphilic monomers that were secreted more efficiently (45). It is unclear why the *N. brasiliensis* secretory AChE should be particularly amenable to high level expression in this system, although these are highly hydrophilic proteins that are released naturally in monomeric form (22). Moreover, they show an extremely high rate of turnover, such that adult worms have been observed to synthesize and secrete 4-fold the total body content of AChE when maintained in culture for 24 h (46). Northern blot analysis (Fig. 3) suggests that the progressive increase in parasite AChE levels during residence in the jejunum (47) is regulated primarily at the level of transcription. Moreover, the timing is consistent with a response to elements of the host immune system, as previously suggested (48, 49).

Given these observations, it is interesting to speculate on the putative role of acetylcholinesterases secreted not just by a cell but by a whole organism, as the target for these enzymes must lie in the intestinal tract of the mammalian host. One possibility lies in the suggestion that AChEs might play a role in regulating differentiation of hematopoietic cells by promoting apoptosis (50). An alternative possibility lies in the role of the enteric nervous system in regulating secretory processes of enterocytes. Nerve fibers in the mucosa terminate subjacent to the basement membrane of epithelial and enteroendocrine cells, on which muscarinic acetylcholine receptors (mAChRs) have been localized (51). Muscarinic AChR agonists evoke chloride secretion from intestinal epithelial cells, and the electrogenic flux creates an osmotic gradient resulting in passive movement of water in the lumen (52). In addition, these agonists evoke mucous secretion from goblet cells (53) and exocytosis of Paneth cells, epithelial granulocytes located at the base of the crypts of Lieberkühn (54). The granules of these cells contain a variety of antimicrobial products, including an array of pore-forming proteins termed cryptdins or crypt defensins (55), although to our knowledge these have not been tested for toxicity against macroparasites such as nematodes.

These secretory events constitute components of innate immunity which most likely contribute to expulsion of pathogens and noxious agents from the gastrointestinal tract and are regulated in part by the enteric nervous system. It is therefore an attractive proposition that AChEs secreted by nematode parasites act to inhibit secretory responses by hydrolyzing acetylcholine. The reagents generated by cloning and high-level expression of nematode secretory AChEs will help to define a biological role for these enzymes, in addition to clarifying the molecular basis for their enzymatic properties.

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