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) 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 immuno histochemistry, 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 (*G₂*) (20), whereas both *G₁* and *G₂* 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 *G₂* 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 isofrom A. Following molting 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.
and to the structure and properties of AChE 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 Centricron 10 microconcentrators (Amicon 420S), washed into 50 ml 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 NcoI restriction site (5'-atgtgacctgATGATGAGTG-3') and a 5' primer based on the conserved sequence flanking the active site serine residue (PGESAG) linked to a XhoI restriction site (5'-atctcgagCCNGCN(T/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 sequence on both strands by dyeoxy chain termination following subcloning into pBluescript. Expression in *P. pastoris—** *N. brasiliensis*-secreted AChE B was expressed in *P. pastoris* via the expression plasmid pPICZaA (Invitrogen). The nucleotide sequence encoding the mature enzyme was amplified from cDNA clone C43 using the sense primer 5'-atactcgagATGATGAGTG-3' and the antisense primer 5'-attatactgAGTTCGCTTG-3' and ligated into λZAP (Stratagene) and isolated several clones, which were sequence on both strands by dyeoxy chain termination following subcloning into pBluescript. Expression in *P. pastoris* was confirmed in BMMY medium (1% yeast extract, 2% peptone, 0.1M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 400 μg ml⁻¹). The recombinant yeast were grown in BMMY medium containing 5% (v/v) glycerol) to saturation (2 days), and expression was induced in BMMY medium (1% yeast extract, 2% peptone, 0.1M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 400 μg ml⁻¹) and enzyme activity was assayed by the method of Karnovsky and Roots (25).

**Acetylcholinesterase Activity and Substrate and Inhibitor Specificities—** AChE activity was determined by the method of Ellman (57) with 1 μM acetylthiocholine (ASch) iodide as substrate in the presence of 1 μM 5,5'-dithiobis(2-nitrobenzoic acid) in 100 μM sodium phosphate, pH 7.0, at 20 °C. The reaction was monitored by measuring the absorbance at 412 nm. The enzyme activity was calculated based on 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 Km 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 (Ki) 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 isofrom (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 (PGESAG) to amplify a cDNA fragment of approximately 550 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 in 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 consensus poly(A) tail. This procedure resulted in the determination of just six amino acids of amino-terminal sequence of the native protein (underlined). C43 predicts a signal peptide of 19 residues, defined by the consensus poly(A) tail. This procedure resulted in the determination of just six amino acids of amino-terminal sequence of the native protein (underlined).

**Structure 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-440, and His-466 (Ser-200, Glu-327, and His-440 in *T. californica*) are conserved in *N. brasiliensis* AChE B.
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.

**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.
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 mg ml⁻¹ at 24 h to 27 mg ml⁻¹ at 5 days postinduction (Fig. 4A), although in practice we changed the culture medium daily. Fig. 4B, 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. 4B, 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...
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 nonde-naturing PAGE and stained for AChE activity, and Fig. 5 demonstrates that it resolved as a fast-migrating single band 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 [AlβM]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$^{-1}$, suggesting that the estimation of specific activity for the recombinant enzyme is close to that of

2 Grigg, M. E., unpublished observations.
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 non-aromatic 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.

In *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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**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 mM (●) or 5 mM (□) 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).

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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).

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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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