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Determinants of substrate specificity of a second non-neuronal secreted acetylcholinesterase from the parasitic nematode *Nippostrongylus brasiliensis*

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We recently reported on a non-neuronal secreted acetylcholinesterase (AChE B) from the nematode parasite *Nippostrongylus brasiliensis*. Here we describe the primary structure and enzymatic properties of a second secreted variant, termed AChE C after the designation of native AChE isoforms from this parasite. As for the former enzyme, AChE C is truncated at the carboxyl terminus in comparison with the *Torpedo* AChE, and three of the 14 aromatic residues that line the active site gorge are substituted by nonaromatic residues, corresponding to Tyr70 (Ser), Trp279 (Asn) and Phe288 (Met).

A recombinant form of AChE C was highly expressed by *Pichia pastoris*. The enzyme was monomeric and hydrophilic, and displayed a marked preference for acetylthiocholine as substrate. A double mutation (W302F/W345F, corresponding to positions *290* and *331* in *Torpedo*) rendered the enzyme 10-fold less sensitive to excess substrate inhibition and two times less susceptible to the bis quaternary inhibitor BW284C51, but did not radically affect substrate specificity or sensitivity to the 'peripheral site' inhibitor propidium iodide. In contrast, a triple mutant (M300G/W302F/W345F) efficiently hydrolysed propionylthiocholine and butyrylthiocholine in addition to acetylthiocholine, while remaining insensitive to the butyrylcholinesterase-specific inhibitor iso-OMPA and displaying a similar profile of excess substrate inhibition as the double mutant. These data highlight a conserved pattern of active site architecture for nematode secreted AChEs characterized to date, and provide an explanation for the substrate specificity that might otherwise appear inconsistent with the primary structure in comparison to other invertebrate AChEs.

Keywords: acetylcholinesterase; nematode; *Nippostrongylus brasiliensis*.

Acetylcholinesterases (AChEs) terminate transmission of neuronal impulses by rapid hydrolysis of acetylcholine (ACh) [1]. In nematodes, as in many organisms, ACh is the major excitatory neurotransmitter that regulates motor functions [2]. In addition to this well-defined role for AChE, many parasitic nematodes differ from free-living species in synthesizing AChEs in specialized secretory glands and expelling the enzymes to the external environment [3,4]. This unusual behaviour is exhibited primarily by parasites that inhabit the alimentary tract of their host. Despite much speculation on the physiological function of these enzymes, a biological role for this behaviour has yet to be established [5,6].

The adult stages of *Nippostrongylus brasiliensis*, which colonize the jejunum of rats, secrete three monomeric nonamphiphilic (G_1^{na}) variants of AChE designated A, B and C, with apparent masses of 74, 69 and 71 kDa, respectively [7]. We have recently cloned one of these variants, AChE B [8]. The recombinant enzyme was a G_1^{na} form as expected, and behaved as a true AChE, with minimal activity towards butyrylthiocholine (BuSCh). However, analysis of the primary structure of the enzyme in comparison with other invertebrate AChEs [9–11] and site-directed mutagenesis of several vertebrate enzymes [12–14] suggested that it would be expected to show substrate specificity intermediate between AChEs and butyrylthiocholinesterases (BuChEs), particularly with regard to the substitution of an aromatic residue at position *288* in the *Torpedo* enzyme with methionine. We could not explain these properties, but suggested that the replacement of Phe*290* and Phe*331* by Trp might restrict the size of the acyl pocket or block access of BuSCh to this site [8].

We have now cloned and expressed a second variant of secreted AChE from *Nippostrongylus brasiliensis*, termed AChE C. The key features of the primary structure and enzymatic properties of the enzyme are similar to those of AChE B, and therefore we have used site-directed mutagenesis to address the question of the unexpected substrate specificity. For clarity, the numbering of residues in the *Torpedo* enzyme is given in italics.

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Abbreviations: AChE, acetylcholinesterase; ASCh, acetylthiocholine; BuChE, butyrylcholinesterase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); BuSCh, butyrylthiocholine; ES, excretory/secretory products; PSCh, propionylthiocholine.

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Note: the nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number AF194408.

Note: amino-acid residues in italic type indicate they are part of the *Torpedo* sequence; those in Roman type are part of the parasite sequence.

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MATERIALS AND METHODS

Parasites

N. brasiliensis were isolated from the small intestine of male Sprague-Dawley rats 4 days post-infection. Excretory/secretory (ES) products were collected from culture supernatants as described [7] and used for nondenaturating gel electrophoresis.

Cloning, sequencing and site directed mutagenesis

A lambda ZAP cDNA library was screened with a cDNA probe encoding AChE B, and clones sequenced by dideoxy chain termination following *in vivo* excision with Exassist helper phage. As a result, a cDNA clone was isolated that encoded a protein with an amino-acid sequence distinct from that of AChE B. This protein was designated AChE C. Alignment of AChE C with AChEs from other sources was performed using the CLUSTALW programme [15]. Single, double and triple mutants of AChE C were generated for M300G (Phe*288* in *Torpedo*), W302F (Phe290 in *Torpedo*), and W345F (Phe*331* in *Torpedo*) using the Quickchange site-directed mutagenesis kit (Stratagene), by conversion of ATG to GGG, and TGG to TTT, respectively. Mutations were confirmed by sequencing prior to expression as secreted proteins in *Pichia pastoris*.

Expression in *Pichia pastoris*

The cDNA clones encoding wild-type and mutant AChE C were ligated into *Pst*1/*Xba*l-digested pPICZαB and plasmids linearized with *Bst*XI. Competent *P. pastoris* were transfected with the constructs, which encoded the mature proteins with an N-terminal signal peptide provided by the prepro sequence of the α-mating factor of *Saccharomyces cerevisiae*, and a C-terminal polyhistidine tag. Recombinant yeast were grown and expression induced as previously described [8]. Culture supernatants were harvested at different time points postmethanol induction and analysed for AChE activity and protein content. Wild-type and mutant AChE C were purified from culture supernatants of transformed *P. pastoris* by nickel-agarose chromatography (Qiagen) as previously described [8] and the purity of the enzyme determined by SDS/PAGE.

Sucrose density centrifugation

Sedimentation analysis was performed in 2–20% sucrose gradients (11 mL, with a 0.5 mL cushion of 50% sucrose) in NaCl/Pi in the presence or absence of 1.0% Triton X-100. The purified recombinant enzyme was applied to the gradient and centrifuged for 16 h at 36 000 r.p.m. 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 *E. coli* alkaline phosphatase (6.1 S) were included as internal standards.

Denaturing and nondenaturing electrophoresis

SDS/PAGE was performed on 12% polyacrylamide gels followed by staining with Coomassie brilliant blue. Purified recombinant AChE C, AChE B and ES products were resolved under nondenaturing conditions by electrophoresis in 7.5% polyacrylamide gels in Tris/Borate/EDTA buffer, pH 8.0, and enzyme activity visualized by the method of Karnovsky and Roots [16].

AChE activity: substrate and inhibitor specificities

AChE activity was determined according to Ellman *et al*. [17]. The standard incubation mixture contained 1 mM acetylthiocholine (ASCh) iodide as substrate in the presence of 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2) 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 calculated from the extinction coefficient of Nbs_2 [17]. One unit of activity is defined as 1 μmol of substrate hydrolysed per min at 20 °C. The K_m values for ASCh, butyrylthiocholine (BuSCh), and propionylthiocholine (PSCh) of wild-type and mutant enzymes were determined by linear regression from plots of $1/V$ against $1/S$, utilizing substrate concentrations in the range 0.01–1 mM (i.e. below excess substrate inhibition). The substrate inhibition constant (K_{ss}) values were determined by plotting $1/V$ against S, utilizing a range of substrate concentrations between 5 and 30 mM for wild type and 30 and 80 mM for mutant AChE. Inhibition constants (K_i) for BW284C51 and propidium iodide were determined using two fixed inhibitor and six ASCh concentrations between 0.01 and 1.0 mM. Assays were carried out in triplicate in the presence or absence of inhibitor. Kinetic constants were determined using GRAPHPAD PRISM 2.0 software (Graphpad, San Diego, CA).

RESULTS

Cloning and structural features of AChE C

In order to isolate cDNAs encoding alternative forms of secreted AChEs, we screened a lambda ZAP cDNA library with a full-length clone for AChE B [8]. Several clones were isolated by this procedure that differed significantly in nucleotide sequence from *AChE B*. The longest clone was 1848 bp, containing a single ORF of 560 amino acids, a consensus polyadenylation site (AATAAA) and a poly(A) tail (GenBank™ accession number AF194408). The amino-acid sequence of the predicted mature protein was 90% identical to AChE B, 28% identical to *Caenorhabditis elegans* ACE-1 [11] and 35% identical to *Torpedo californica* AChE [18]. Figure 1 depicts the alignment of AChE C with AChE B of *N. brasiliensis*, ACE-1 of *C. elegans* and the T subunit of *T. californica* AChE. By analogy with the N-terminal sequence of mature AChE B, the protein is numbered from the first aspartate residue. Although lacking an initiator methionine, the protein has a largely hydrophobic N-terminal stretch of 22 amino acids similar to that of AChE B and indicative of a signal peptide. Serine, glutamate and histidine residues are present in positions corresponding to the catalytic triad of AChEs [19]. Six cysteine residues are found in positions that correspond to those responsible for the three conserved intramolecular disulphide bonds, but as for AChE B, an additional two cysteine residues are present at positions 231 and 262. The protein is truncated at the C-terminus in comparison to AChEs from other sources, and there are two consensus sequences for N-linked glycosylation at positions 124 and 376.

Eleven of the 14 aromatic residues that line the wall of the active site gorge in the *Torpedo* enzyme [19] are either conserved or show conservative substitutions. Two of the nonconservative substitutions, Asn289 (Trp*279*) and Met300 (Phe*288*) are identical to AChE B, whereas Tyr*70* is substituted by Ser65 rather than threonine.

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                                     1
AChE C   NSCRGLPARLLLAICILSTSAADDGPTVELSSGAKIHGVYMEVDGQTVSAYLGVPFAT--    36
AChE B      MGLPARLLLAICVFSTSAADDGPTVVLSSGTKIHGIYMDVNGQTVSAYLGVPFAT--
Ce              MRNSLLFFIFLPSILAVDLIHLHDGSPLFGEEVLSQTGKPLTRFQGIPFAEPP
Tc       MNLLVTSSLGVLLHLVVLCQADDHSELLVNTKSGKVMGTRVPVLSSHISAFLGIPFAEPP    39
                                    1
ACHE C   --AERFAKPTLTETYGKDIEAIQPSKTCFQSKDETYPGFDGAEMWNPPTDLSEDCLSLNI    94
AChE B   --AERFAMPTLTETYGGDIEALQLSKTCFQTKDETYPGFDGAEMWNPPTELSEDCLSLNI
Ce       VGNLRFKKPKPKQQWRIPLNATTPPNSCIQSEDTYFGDFYGSTMWNANTKLSEDCLYLNV
Tc       VGNMRFRRPEPKKPWSGVWNASTYPNNCQQYVDEQFPGFSGSEMWNPNREMSEDCLYLNI    99

ACHE C   WVPQN--P--DGNVIVWIYGGGFFSGSPSLALYNGSVLAARTGAVFVTVNYRVGPFGFFY   150
AChE B   WVPEN--P--DGNVIVWIYGGGFFSGSPSLALYNGSVLAGKTNAVVVNVNYRVGPFGFFY
Ce       YVPGKVDPNKKLAVMVWVYGGGFWSGTATLDVYDGRILTVEENVILVAMNYRVSIFGFLY
Tc       WVPSPR-P-KSTTVMVWIYGGGFYSGSSTLDVYNGKYLAYTEEVVLVSLSYRVGAFGFLA   157

ACHE C   LGADSK-PGNVGLLDQQTALKWIHNNIEFFKGDPSKVTLFGESAGGASVTSHLLAPDSHS   209
AChE B   LGANSKAPGNVGLLDQQTALKWIHNNIEYFKGDPSKVTLFGESAGGTSVTSHLLAPDSHS
Ce       MN-RPEAPGNMGMWDQLLAMKWVHKNIDLFGGDLSRITLFGESAGAASVSIHMLSPKSAP
Tc       LHGSQEAPGNVGLLDQRMALQWVHDNIQFFGGDPKTVTIFGESAGGASVGMHILSPGSRD   217

ACHE C   LFSKIIVNSGSIHNVWATRSPCTMLHISMKTAKALGCVENYDFKIIREAEGRCTVLGGNV   269
AChE B   LFSKIIVNSGSIHNVWATRSPCTMLHISMKTAKALGCVENYEIKPIREAEGRCTVLGADA
Ce       YFHRAIIQSGSATSPWAIEPRDVALARAVILYNAMKCGN-------------MSLINPDY
Tc       LFRRAILQSGSPNCPWASVSVAEGRRRAVELGRNLNCN-----------------LNSDE   260

ACHE C   DKIYECMKEKTPDDIQSAGNSDAIYENMLPMEWPFGPITYDDNYFKGDVRRKLFNDEFKK   329
AChE B   DTIYECMKEKTPEKIQSEGNSDAIYAEMLPMEWPFGPITYDDNYFKGEVRRKLFSGEFKT
Ce       DRILDCFQRADADALRENEW--APVRE--FGDFPWVPV-VDGDFLLENAQTSLKQGNFK-
Tc       E-LIHCLREKKPQELIDVEWNVLPFDS--IFRFSFVPV-IDGEFFPTSLESMLNSGNFK-   315

ACHE C   DVSAIFGTVKDEGTFWLPYYLSDSGFSFFPDMGSDSEANAAKIDESNYTASMQAFEGYFG   389
AChE B   DVSAIFGTVKDEGTFWLPYYLSESGFSFFPDQDSDSEANAAKIDEANYTASMQAFEGYFG
Ce       KTQLLAGSNRDESIYFLTYQLPDI----FPVADFFTKTDFIKDRQLWIKGVKDLLPRQIL
Tc       KTQILLGVNKDEGSFFLLYGAPG-----FS-KDSESK---IS-REDFMSGVKLSVPHAND   365

AChE C   KSSKAIEILKDGFKDLGDEETMYRD---GVARFVGDLFFTCNLVEFVDHVAPKIDEAYMY   446
AChE B   KSSKAIEILKEGFKDLGDVQTMYRD---GVARFVGDFFFTCNLVEFVDHVTQKISEAYMY
Ce       KCQLTLAAVLHEYEPQ-DLPVTPRDWINAMDKMLGDYHFTCSVNEMALAHTKHGGDTYYY
Tc       LGLDAVTLQYTDWMDD-NNGIKNRD---GLDDIVGDHNVICPLMHFVNKYTKFGNGTYLY   421

AChE C   YFKARSSANPWPKWMGVMHGYEIEYEFGYPFINSAAYKEADKDQTISKEFMQLIKEFVEN   506
AChE B   YFKARSSANPWPKWMGVMHGYEIEYEFGYPFINSTAYKEVDKDRTISEEFMQLIKEFVKN
Ce       YFTHRASQQTWPEWMGVLHGYEINFIFGEPLN-QKRFNYTDEERELSNRFMRYWANFAKT
Tc       FFNHRASNLVWPEWMGVIHGYEIEFVFGLPL--VKELNYTAEEEALSRRIMHYWATFAKT   479

AChE C   GK---------FDGKWPKYNEEGKVMVIEDGTSS-----SIEDKNIQQKYCQIINEARKA   552
AChE B   GK---------FDDEWPKYEG-GKVMVIEDDASR-----KIEDKDIQQKYCKIINDARQA
Ce       GDPNKNEDGSFTQDVWPKYNS-VSMEYMNMTVESSYPSMKRIGHGPRRKECAFWKAYLPN
Tc       GNPNEPHS---QESKWPLFTT-KEQKFIDLNTEP-----MKVHQRLRVQMCVFWNQFLPK   530

AChE C   VIDEAKGN                                                      560
AChE B   LIDEAKGN
Ce       LMAAVADVGDPYLVWKQQMDKWQNEYITDWQYHFEQYKRYQTYRQSDSETCGG
Tc       LLNATETIDEAERQWKTEFHRWS-SYMMHWKNQFDHYSRHESCAEL                575
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Fig. 1. Alignment of *N. brasiliensis* AChE C with other AChEs. The derived amino-acid sequence of *N. brasiliensis* AChE C was aligned with that of AChE B, ACE-1 from *C. elegans* (Ce), and the T subunit of *T. californica* (Tc). *T. californica* AChE is numbered from N-terminus of the mature protein (marked '1'), and AChE C by analogy with the N-terminal sequence of AChE B [8]. The residues in the catalytic triad (Ser-His-Glu) are indicated with an asterisk, and the position of two potential sites for N-linked glycosylation marked with squares. Cysteine residues at positions known to be involved in intramolecular disulphide bonds are indicated by solid triangles, whereas open triangles depict the two additional cysteines in the *N. brasiliensis* enzymes. 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* AChEs, whereas open circles indicate nonaromatic residues.

Table 1. Kinetic parameters of wild-type and mutant enzymes. K_m (mM), K_{ss} (mM), k_{cat} (min^{-1}) and k_{cat}/K_m ($M^{-1} \cdot min^{-1}$) for acetylthiocholine (ASCh), propionylthicholine (PSCh) and butyrylthiocholine (BuSCh) were determined as described in Materials and methods. Figures represent the mean ± SD of three independent experiments. ND, not determined.

Substrate	Wild-type	W302F, W345F	M300G, W302F, W345F
ASCh			
K_m	0.14 ± 0.01	0.20 ± 0.02	0.30 ± 0.03
K_{ss}	1.31 ± 0.21	13.15 ± 4.50	18.30 ± 2.67
k_{cat}	1.10 ± 0.23 × 10^5	1.46 ± 0.30 × 10^5	4.01 ± 0.41 × 10^5
k_{cat}/K_m	7.86 × 10^8	7.30 × 10^8	13.37 × 10^8
PSCh			
K_m	0.23 ± 0.02	0.15 ± 0.02	0.12 ± 0.04
K_{ss}	2.10 ± 0.56	7.29 ± 3.24	10.31 ± 2.54
k_{cat}	1.01 ± 0.17 × 10^5	1.11 ± 0.28 × 10^5	1.21 ± 0.19 × 10^5
k_{cat}/K_m	4.39 × 10^8	7.40 × 10^8	10.08 × 10^8
BuSCh			
K_m	ND	ND	0.12 ± 0.03
K_{ss}	ND	ND	ND
k_{cat}	ND	ND	0.38 ± 0.04 × 10^5
k_{cat}/K_m	ND	ND	3.17 × 10^8

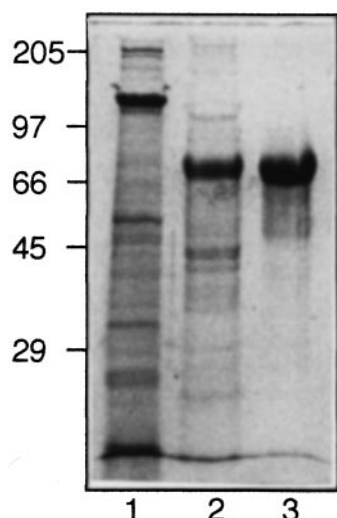

Fig. 2. Expression and purification of recombinant AChE C. Proteins secreted by *Pichia pastoris* transfected with cDNA encoding *N. brasiliensis* AChE C are shown here, resolved by SDS/PAGE under reducing conditions (lane 2), alongside the purified enzyme (lane 3), and proteins secreted by wild type yeast (lane 1). The gel was stained with Coomassie blue, and molecular mass markers are shown in kDa.

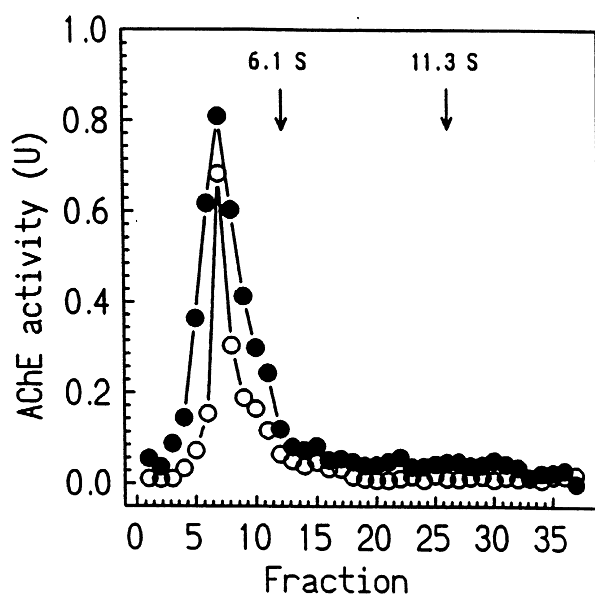

Fig. 3. Sedimentation analysis. Purified recombinant AChE C was resolved by sucrose density centrifugation in the presence (○) or absence (●) of 1.0% Triton X-100, and fractions assayed for AChE activity, The positions of internal sedimentation standards bovine liver catalase (11.3 S) and *E. coli* alkaline phoshatase (6.1 S) are indicated.

Expression and properties of recombinant enzyme

AChE C was next expressed as a secreted protein in *P. pastoris*. Approximately 50 mg total protein was secreted per litre of culture medium by 80 h postmethanol induction, and more than half of this was accounted for by AChE C (Fig. 2). The purified enzyme had an apparent mass of 73 kDa when resolved by SDS/PAGE under reducing conditions. Sucrose density gradient centrifugation identified AChE C as a monomeric non-amphiphilic (G_1^{na}) enzyme with a sedimentation coefficient of 4.5 S (Fig. 3). When subjected to nondenaturing PAGE, it resolved as a single band that comigrated with form C of the native secreted parasite AChEs (Fig. 4, lanes 4 and 2).

Recombinant AChE C showed preferential activity towards ASCh, with inhibition at substrate concentrations over 2.5 mM (Fig. 5, panel A). The specificity constants (k_{cat}/K_m) of the enzyme for ASCh and PSCh were determined at 7.86×10^8 and 4.39×10^8 $M^{-1}\cdot min^{-1}$, respectively (Table 1). The activity against BuSCh was too low for determination of kinetic constants.

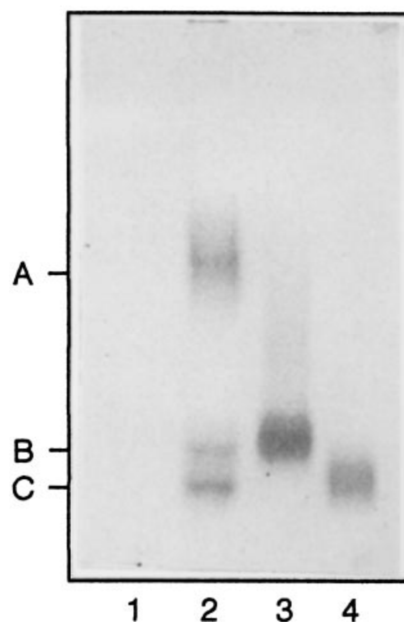

Fig. 4. Co-migration with native AChE C in nondenaturing PAGE. Purified recombinant AChE C (lane 4) was resolved by nondenaturing PAGE alongside recombinant AChE B (lane 3), native secreted proteins of adult *N. brasiliensis* (lane 2), and proteins secreted by wild-type *P. pastoris* X-33 (lane 1). AChE activity was visualized according to Karnovsky and Roots [16]. The migration of native secretory enzymes designated as forms A, B and C [7] is indicated.

Table 2. Inhibition constants for wild-type and mutant enzymes. Inhibition constants (K_i) for BW284C51 and propidium iodide were determined using two fixed inhibitor and six ASCh concentrations (0.01–1.0 mM). Assays were carried out in triplicate in the presence or absence of inhibitor. NI, no inhibition.

Enzyme	BW284C51 (nM)	Propidium Iodide (μM)	Iso-OMPA
Wild-type AChE C	20.2 ± 4.0	2.31 ± 0.42	NI
W302F, W345F	40.9 ± 8.5	3.57 ± 0.65	NI
M300G,W302F,W345F	45.0 ± 3.3	4.12 ± 0.31	NI
AChE B[a]	6.4 ± 1.2	0.22 ± 0.03	NI

[a] Data for *N. brasiliensis* AChE B taken from [8].

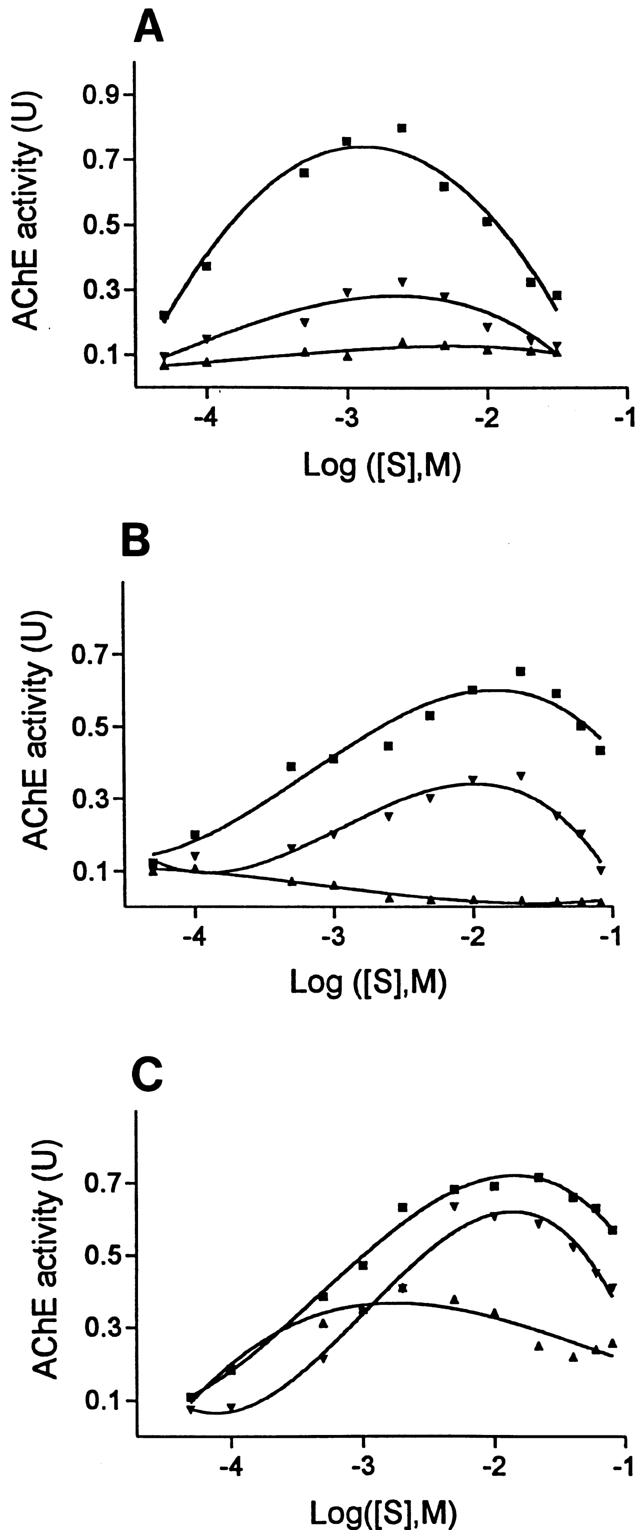

Fig. 5. Substrate specificity of wild type and mutant AChE C. The rate of hydrolysis of ASCh (■), PSCh (▼) and BuSCh (▲) were determined as described in Materials and methods, utilizing a range of substrate concentrations between 0.05 and 30 mM for the wild-type enzyme (panel A), and between 0.05 and 80 mM for the double mutant W302F/W345F (panel B), and the triple mutant M300G/W302F/W345F (panel C).

Generation and properties of mutant enzymes

AChE C thus behaved as a true AChE with minimal activity towards BuSCh, despite the substitution of an aromatic residue at position *288* in the *Torpedo* enzyme with methionine. These properties are identical to those recently described for AChE B of *N. brasiliensis* [8], and we therefore sought to resolve this apparent anomaly by site-directed mutagenesis. Both parasite enzymes contain tryptophan in place of phenylalanine at positions *290* and *331*, and based on the assumption that the larger sidechain might restrict the size of or access of substrate to the acyl pocket, we performed single and double mutations in which these residues were replaced by phenylalanine. The single mutations, namely W302F (Phe*290*) or W345F (Phe*331*), did not significantly alter the properties of AChE C with respect to substrate specificity or excess substrate inhibition (data not shown). The double mutant (W302F/345F), however, was approximately 10 times less sensitive to excess substrate inhibition with ASCh. It showed slightly enhanced activity against PSCh, although BuSCh was still inefficiently hydrolysed. In fact, a reproducible observation was that BuSCh at concentrations greater than 1 mM was hydrolysed less efficiently by the double mutant than by the wild-type enzyme, although we have no explanation for this (Fig. 5, panel B and Table 1).

We next constructed a triple mutant (M300G/W302F/W345F) to generate an enzyme with residues in these positions identical to those of *C. elegans* ACE-1, which efficiently hydrolyses both ASCh and BuSCh [11]. The enzyme had a similar profile of excess substrate inhibition by ASCh as W302F/345F, but now readily accepted BuSCh, and had a lower K_m and enhanced activity towards PSCh (Fig. 5, panel C and Table 1). Although the K_m of the triple mutant for ASCh was approximately two-fold that of the wild type enzyme, it showed a four-fold enhancement of turnover and an overall improved catalytic efficiency with this substrate (Table 1). The properties of the single mutant M300G were not significantly altered from those of the wild-type enzyme (data not shown).

The susceptibility of wild-type and mutant AChE C to cholinesterase inhibitors was also examined. None of the enzymes were affected by the butyrylcholinesterase inhibitor iso-OMPA at concentrations up to 10 mM (data not shown), but the wild-type enzyme was highly sensitive to BW284C51, a specific inhibitor of AChEs [20] with a K_i of 20.2 nM, and the 'peripheral' site ligand propidium iodide, with a K_i of 2.31 μM, in a competitive and noncompetitive manner, respectively. Both mutant enzymes were less susceptible to inhibition by BW284C51, but showed no significant difference in inhibition by propidium iodide (Table 2).

DISCUSSION

We describe here the primary structure and properties of an acetylcholinesterase (AChE C) secreted by the parasitic nematode *N. brasiliensis*. This is the second example of this type of enzyme to be reported. Both AChE C and AChE B are maximally expressed by the parasite in the later stages of infection of the mammalian host. AChE C can be distinguished from AChE B by SDS/PAGE on the basis of its mass, and by nondenaturing PAGE by its more acidic nature, which results in accelerated migration. The recent determination of the primary structure of AChE B revealed some very unusual features [8] that are largely conserved in AChE C. AChE C is similarly truncated at the C-terminus, which aligns closely with the end of the catalytic domain of the vertebrate enzymes. The parasite

enzymes therefore do not contain sequences corresponding to the peptides of vertebrate AChEs that define hydrophobic (H) or tailed (T) subunits [21], and lack the C-terminal cysteine residue involved in intermolecular disulphide-bridge formation, thus explaining their monomeric nature. In this respect, however, they show some similarity with nonamphiphilic monomeric AChEs from snake venom [22,23]. In addition to the six cysteine residues implicated in intramolecular disulphide bridges, AChE C has two cysteines at positions 231 and 261, the former of which is predicted to lie on the molecular surface and the latter lies in an insertion of 17 amino acids (relative to *Torpedo* AChE, see Fig. 1) which is difficult to model, but most probably forms part of a loop at the surface stabilized by the second disulphide bond. It is notable that both this insertion and the additional two cysteine residues are conserved in AChE B in identical positions [8], and we are therefore in the process of determining whether these could be involved in an additional disulphide bridge.

Both the native and the recombinant enzymes are known to be glycosylated, although the sites have not been mapped. AChE C differs from AChE B in lacking a consensus sequence for N-linked glycosylation at position 480. There are therefore two consensus sequences common to both enzymes at positions 124 and 376. Of these, Asn376 would appear to be the most likely candidate for glycosylation, as Asn124 is positioned directly next to a tyrosine residue predicted to line the active site gorge (Tyr*130* in *Torpedo*). No other acetylcholinesterases appear to have consensus sequences for glycosylation at homologous sites.

The active site gorge of the *Torpedo* enzyme is lined by 14 aromatic residues that are highly conserved in AChEs from different species, and thought to contribute to a substrate guidance mechanism [19]. Eleven of these residues are either conserved or show conservative substitutions in both of the parasite secreted enzymes. Of the three nonconservative substitutions, AChE C differs subtly from AChE B in possessing a serine residue at position 65 rather than a threonine, corresponding to Tyr*70* in *Torpedo* AChE.

As these are the only two secreted AChEs from parasitic nematodes to be cloned and sequenced thus far, it is premature to predict whether the primary structure of analogous enzymes will follow a similar pattern. All such enzymes analysed to date show a marked substrate preference for acetylcholine. The AChE secreted by the hookworm *Necator americanus* exists as a hydrophilic dimer [24], whereas both G_1^{na} and G_2^{na} forms have been identified in secreted products of *Trichostrongylus colubriformis* [25]. Nevertheless, despite the fact that the third variant of the *N. brasiliensis* secreted AChEs (AChE A) has not been fully characterized, the similarity between the other two forms in primary structure and enzymatic properties begs the question of why this parasite secretes multiple AChEs. Forms B and C are produced relatively soon after form A, and therefore sequential production of variants that would escape binding by antibodies and possible neutralization of enzyme activity is an unlikely explanation, made even more improbable by the close similarity in sequence between the two enzymes characterized to date. Given that the enzymes are secreted into the extremely hydrolytic environment provided by the intestinal tract, another possibility is that the AChEs differ in their susceptibility to proteolytic degradation, although this has not yet been assessed. It is also possible that the secreted AChEs are targetted to different sites in the jejunal mucosa, and this is currently under investigation.

Both *N. brasiliensis* AChE B and C have a methionine residue in the position corresponding to Phe*288* in the *Torpedo* enzyme. This was somewhat surprising given their minimal activity with BuSCh as substrate, as mutagenesis studies on *Torpedo* and human AChE have shown that the presence of an aromatic amino acid in this position and at *290* block access of BuSCh to the active site [12–14]. Moreover, the presence of leucine and glycine at position *288* in *Drosophila melanogaster* and *C. elegans* ACE-1, respectively, has been proposed to be responsible for the activity of these enzymes against BuSCh [10,11]. We therefore investigated the possibility that the substitution of Phe*290* and Phe*331* by Trp in the nematode enzymes might restrict the size of the acyl pocket or entrance of substrate to this site by virtue of the bulkier sidechains. As shown in Fig. 5, the double mutation of tryptophan to phenylalanine at these positions had little effect on substrate specificity. In contrast, the triple mutant (M300G/W302F/W345F) showed good activity with PSCh and BuSCh (Fig. 5 and Table 1), indicating that the collective effect of larger residues in all these three positions acted to restrict access of larger substrates. The triple mutant showed a higher K_m, but an enhanced turnover of ASCh (Table 1). Although we have no explanation for this, the larger binding pocket might increase the rate of acylation, deacylation and/or product release.

Perhaps more surprising was the effect on excess substrate inhibition, which was shifted approximately 10-fold in the mutant enzymes (Fig. 5 and Table 1). Mutation of Phe297 in mouse AChE (Phe*290* in *Torpedo*) to isoleucine has been reported to abolish substrate inhibition, in addition to reducing catalytic activity of the enzyme [13]. Single mutations of Trp302 (Phe*290* in *Torpedo*) and Trp345 (Phe*331* in *Torpedo*) in the *N. brasiliensis* AChE to Phe had no effect on substrate inhibition or specific activity (data not shown). In the double mutant (W302F/W345F), decreased sensitivity to substrate inhibition was observed, with little effect on k_{cat} or the binding of the 'peripheral' site inhibitor propidium iodide. Although these residues are not implicated in binding of propidium, Trp302 is presumably involved in substrate orientation, and both this residue and Trp345 may be involved in allosteric alterations following binding of a second substrate molecule to a lower affinity site. Mutation of both these residues to phenylalanine appears sufficient to influence these putative allosteric effects without altering catalytic efficiency of the nematode enzyme.

AChE C is sensitive to propidium iodide, although the K_i value of 2.31 μM (Table 2) is approximately 10-fold higher than that recorded for AChE B [8]. AChE C has an acidic residue (Glu) at position 295 (*285* in *Torpedo*) that has been demonstrated to be important for sensitivity of the *Bungarus fasciatus* AChE to peripheral site inhibitors [23], but as for AChE B, two other aromatic residues that contribute to this phenomenon, Tyr*70* and Trp*279* [12,14,23] are substituted by nonaromatic amino acids. Tyr*70* is replaced by serine in AChE C and threonine in AChE B, whereas both parasite enzymes have an asparagine in place of Trp*279*.

Neither the wild-type nor any of the mutant nematode enzymes reported here were susceptible to inhibition by iso-OMPA, despite the triple mutant showing reasonable activity with BuSCh, in contrast to mutagenesis studies of vertebrate AChEs [12–14]. The observed differences in activity and inhibitor profiles between the *N. brasiliensis* and vertebrate AChEs can only be satisfactorily resolved by structural analysis, which will be facilitated by the high level of expression of the nematode enzymes in *P. pastoris*.

We are currently working to determine the function of the nematode secreted AChEs, the expression of which have been demonstrated to be positively regulated by elements of the host

immune system [26,27]. We have recently identified a population of cells in the lamina propria and draining mesenteric lymph nodes of rats that express muscarinic ACh receptors in response to infection with the parasite (W. S. Russell, S. M. Henson & M. E. Selkirk, unpublished data). Identification of these cells and determination of their response to ACh will help to define the function of the nematode secreted AChEs, in addition to uncovering a possible link between the enteric nervous and immune systems.

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