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A tetrameric acetylcholinesterase from the parasitic nematode *Dictyocaulus viviparus* associates with the vertebrate tail proteins PRiMA and ColQ

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Abstract

Dictyocaulus viviparus causes a serious lung disease of cattle. Similar to other parasitic nematodes, *D. viviparus* possesses several acetylcholinesterase (AChE) genes, one of which encodes a putative neuromuscular AChE, which contains a tryptophan (W) amphiphilic tetramerization (WAT) domain at its C-terminus. In the current study, we describe the biochemical characterization of a recombinant version of this WAT domain-containing AChE. To assess if the WAT domain is biologically functional, we investigated the association of the recombinant enzyme with the vertebrate tail proteins, proline-rich membrane anchor (PRiMA) and collagen Q (ColQ), as well as the synthetic polypeptide poly-L-proline. The results indicate that the recombinant enzyme hydrolyzes acetylthiocholine preferentially and exhibits inhibition by excess substrate, a characteristic of AChEs but not butyrylcholinesterases (BChEs). The enzyme is inhibited by the AChE inhibitor, BW284c51, but not by the BChE inhibitors, ethopropazine or iso-OMPA. The enzyme is able to assemble into monomeric (G₁), dimeric (G₂), and tetrameric (G₄) globular forms and can also associate with PRiMA and ColQ, which contain proline-rich attachment domains (PRADs). This interaction is likely to be mediated via WAT-PRAD interactions, as the enzyme also assembles into tetramers with the synthetic polypeptide poly-L-proline. These interactions are typical of AChE_T subunits. This is the first demonstration of an AChE_T from a parasitic nematode that can assemble into heterologous forms with vertebrate proteins that anchor the enzyme in cholinergic synapses. We discuss the implications of our results for this particular host/parasite system and for the evolution of AChE.

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Keywords

acetylcholinesterase; cholinergic nervous system; *Dictyocaulus viviparus*; ColQ; PRiMA; poly-L-proline; evolution

1. Introduction

Acetylcholine (ACh) is the major excitatory neurotransmitter controlling motor activities in nematodes [1, 2], and the enzyme which hydrolyzes and inactivates acetylcholine, acetylcholinesterase (AChE), is thus essential for regulation of cholinergic transmission. Nematodes are known to express a number of molecular forms of AChEs which, in contrast to vertebrate AChEs, are encoded by distinct genes. Of the nematodes, most detail is available on AChEs of the free-living worm, *C. elegans*, in which three genes are known to encode distinct enzymes, with a fourth gene thought to be non-functional [3, 4]. Of these enzymes, *C. elegans* ACE-1 is an amphiphilic tetramer which associates with a hydrophobic non-catalytic subunit, whereas *C. elegans* ACE-2 and ACE-3 are GPI-linked amphiphilic dimers. The genes that encode these proteins display distinct anatomical transcription patterns in neurons and musculature, suggestive of predominantly non-redundant functions [5, 6].

The bovine lungworm, *Dictyocaulus viviparus*, is a pathogenic parasitic nematode that inhabits the trachea and main stem bronchi of cattle and causes the disease parasitic bronchitis. Homologues of *C. elegans* *ace-1* and *ace-2* have been isolated from *D. viviparus*, and their predicted proteins contain C-terminal sequences similar to the *C. elegans* enzymes, suggesting that the parasite AChEs have comparable means of membrane anchorage. Thus, *D. viviparus* AChE-1 (Dv-ACE-1) is predicted to be a tetrameric form of AChE capable of associating with a non-catalytic structural subunit [7], whereas *D. viviparus* AChE-2 (Dv-ACE-2) is predicted to be a membrane-bound glycosylphosphatidylinositol-linked (GPI-linked) dimer [8]. In addition, *D. viviparus* expresses at least two other AChEs (Dv-sACE-1 and Dv-sACE-2) which are secreted and apparently monomeric [9].

The nature of the molecular forms produced by AChE, be they monomers, dimers, or tetramers, depends in large part on the nature of the carboxyl terminus of the enzyme [10]. Nematode secreted AChE monomers (AChE_S) have a truncated C-terminus, while GPI-linked AChE dimers (AChE_H) possess a hydrophobic C-terminus, which is cleaved upon the addition of the GPI anchor. Tetrameric AChE molecules have a highly conserved tryptophan-containing amphiphilic C-terminus, which mediates tetramer formation and also association with non-catalytic structural proteins. In invertebrates, including nematodes, this non-catalytic protein and its function have not been identified [4, 11, 12]. In contrast, in vertebrates, tetrameric AChE (AChE_T) associates with collagen Q (ColQ), which stabilizes the enzyme and anchors it to the basal lamina, and with the proline-rich membrane anchor (PRiMA), which is required for the intracellular processing and anchoring of AChE in the plasma membrane of neurons [13–16]. This association is mediated by an interaction between the tryptophan (W) amphiphilic tetramerization (WAT) domain of the C-terminus of tetrameric AChE with proline-rich attachment domains (PRADs) present in ColQ and PRiMA [10]. Previously, we cloned a cDNA (*Dv-ace-1*) for a putative AChE_T from *D. viviparus* [7]. Here, we report the kinetic and pharmacological characteristics of the Dv-ACE-1 AChE enzyme, its molecular forms, and its association with the vertebrate tail proteins ColQ and PRiMA, and the synthetic polypeptide poly-L-proline. We also discuss the implications of these interactions with regard to their role in the cholinergic nervous system of *D. viviparus*.

2. Materials and Methods

2.1. Sequence analysis

Sequences were aligned with Clustal X and phylogenetic analysis by the neighbor-joining method [17]. Amphipathic α -helical properties and helical wheels were determined by the method of Zidovetzki *et al.* [18].

2.2. Expression of cDNAs for AChE, ColQ, and PRiMA; incubations with poly-L-proline

The cDNA for the AChE, Dv-ACE-1 (GenBank Accession Number DQ375489), that was cloned previously as described in Matthews *et al.* [7], was sub-cloned into the expression vector, pcDNA3.1. COS-7 monkey cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum. Cells were plated at a density of 2.5×10^6 cells/75 cm² culture flask, incubated overnight, and transferred to OptiMEM medium. FuGene was then used to transfect or cotransfect the cells with 7.8 μ g each of one or more of the cDNAs for AChE, ColQ, and PRiMA. The cells were then incubated for 48 h at 37°C before the medium was removed and the cells were extracted in high ionic strength (HIS) buffer (10 mM NaHPO₄, pH 7, 1 M NaCl, 1% Triton X-100, 1 mM EDTA). Extracts were centrifuged at 20,000g for 20 min and the supernatants were assayed for ChE activity [19].

Poly-L-proline (Sigma, St. Louis; molecular weight 1,000–10,000) experiments were done in two different ways. In some experiments, poly-L-proline was added to cultures when they were transfected with *Dv-ace-1* and enzyme was harvested 48 h later. In other experiments, poly-L-proline was incubated for 3 h with Dv-ACE-1 that was previously extracted from cells with low salt (LS) buffer (10 mM NaHPO₄, pH 7, 1 mM EDTA).

2.3. Measurement and analysis of AChE activity and inhibition

AChE activity was measured according to the method of Ellman *et al.* [20] as modified by Doctor *et al.* [21] in 100 mM NaHPO₄, pH 7, 0.3 mM DTNB, 167 mM NaCl, and 258 μ M Triton X-100. Acetylthiocholine (ATCh), butyrylthiocholine (BTCh), and propionylthiocholine (PTCh) were used as substrates at various concentrations; for pharmacological analyses and assays of sucrose gradients, the concentration of ATCh was 1 mM.

The kinetic parameters K_m , K_{ss} , b , and V_{max} , were determined as described by Radić *et al.* [22] and Kaplan *et al.* [23] using Sigma Plot to fit the data to a modified version of the Michaelis-Menten equation:

$$v = \left(\frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}} \right) \left(\frac{V_{max}}{1 + K_m/S} \right)$$

K_{ss} is the dissociation constant for binding of substrate to a second site on the enzyme. The parameter, b , indicates the relative catalytic efficiency of the ternary SES complex compared to SE. If $b < 1$, the enzyme shows substrate inhibition; if $b > 1$, the enzyme shows substrate activation, and if $b = 1$, Michaelis-Menten kinetics is observed.

Values of IC₅₀ for the inhibitors used were determined by incubating enzymes with various concentrations of drug for 20 min and then assaying for enzyme activity in the presence of ATCh. SigmaPlot was then used to fit the data to a three-parameter logistic function, yielding IC₅₀ values. Since classical diagnostic differential inhibition alone was being examined, it was not necessary to determine k_i or K_I values for the inhibitors [24–26].

2.4. Velocity sedimentation on sucrose gradients and collagenase digestion

The molecular forms of ChE were analyzed by velocity sedimentation in 5–25% isokinetic sucrose gradients prepared in 10 mM NaHPO₄, pH7, 1M NaCl, and 1 mM EDTA (with or without the non-ionic detergent Brij 97), containing 1 mg/ml bovine serum albumin. Sedimentation was in an SW 41 rotor at 30,000–37,000 rpm for times satisfying the equation $[(\text{rpm})^2 \times t (\text{h})] = 2.5 \times 10^{10}$, as described previously [24]. Apparent sedimentation coefficients were calculated relative to the sedimentation of catalase (11.3 S). Sedimentation data were plotted in two ways. For molecular forms in the presence and absence of detergent, expressed with or without PRiMA, and digested or undigested by collagenase when expressed with ColQ, profiles are presented as fractional activity of total ChE activity on the gradient as a function of sedimentation coefficient: fractional activity on gradient = (activity in a given fraction/total activity on gradient); sedimentation coefficient = (fraction number) (11.3S/fraction number of catalase peak) [27]. For the experiments performed with poly-L-proline, since there is not a shift in sedimentation coefficient but a change in peak height accompanying the association of poly-L-proline with Dv-ACE-1, data are presented as a fraction of control AChE activity on the gradient: fractional activity on gradient relative to control = [(activity in a given fraction/total control activity on gradient) (volume of control sample on gradient/volume of experimental sample on gradient)]; sedimentation coefficient = (fraction number) (11.3S/fraction number of catalase peak). For collagenase digestion, HIS extracts were adjusted to 10 mM CaCl₂ and incubated at 37°C for 1 h with or without 200 µg/ml collagenase as described previously [27].

3. Results

3.1. Sequence analysis

We compared the sequence for Dv-ACE-1 to the sequences of the other AChEs from *D. viviparus* and the AChE from *Torpedo californica*. The sequences of Dv-ACE-1 and the other AChEs from *D. viviparus* contain the catalytic triad and disulfide bonds characteristic of all ChEs; however, Dv-ACE-1 differs from the other *D. viviparus* AChEs and from vertebrate AChEs, as represented by *T. californica* AChE, in a number of ways. Vertebrate AChEs have 14 aromatic amino acid residues lining the catalytic gorge of the enzyme, while invertebrate AChEs typically have no more than 13 aromatic residues in the gorge lining (Table 1). The aromatic residue homologous to Tyr70 in *T. californica* AChE is almost always replaced by a non-aromatic amino acid, as is the case for Dv-ACE-1. Another difference between the vertebrate and invertebrate AChEs is that, in vertebrates, the acyl pocket is composed of Phe288 and Phe290 (in *T. californica*), while in invertebrates, the pocket appears to be comprised of Phe residues corresponding to Phe290 and Val400, which are Phe298 and Phe411 in Dv-ACE-1 AChE [28]. As outlined previously [29], the *D. viviparus* AChEs differ from one another at their C-termini: Dv-sACE-1 and Dv-sACE-2 have truncated C-termini; Dv-ACE-2 has a hydrophobic C-terminus, which is replaced by a GPI anchor; and Dv-ACE-1 has an amphipathic helical C-terminus similar to that of other AChE_T subunits. AChE_T has a tryptophan WAT domain consisting of seven conserved aromatic residues, including three tryptophans. In the putative WAT domain of Dv-ACE-1, six of these aromatic residues and all three tryptophans are conserved (Fig. 1). The WAT domain forms an amphipathic α -helix with a staircase arrangement of aromatic and hydrophobic residues on one side of the helix that interact with the prolines of PRADs (Figs. 1, 2; [30]). Many invertebrate WAT domains have an insertion with respect to the vertebrate sequence, and this insertion results in the preservation, but rotation, of the hydrophobic moment (μH) of the amphipathic helix of the T-peptide (Fig. 2). Met21 is replaced as Ile22 in the *D. viviparus* sequence. A phylogenetic tree of the T-peptides shows that the sequences for protostome and deuterostome AChEs and for butyrylcholinesterases (BChEs) segregate into their own clades as expected on the basis of current phylogenies (Fig. 3).

3.2. Kinetic and pharmacological characterization

To confirm the nature of the ChE activity of recombinant Dv-ACE-1, we measured its kinetic characteristics with the substrates ATCh, PTCh, and BTCh, as substrate specificity can be used to classify ChE. Classically, in the vertebrates, AChEs hydrolyze ATCh preferentially, PTCh appreciably, and BTCh little, if at all; in contrast, BChEs prefer BTCh as a substrate [29]. Invertebrates appear to express only AChE. However, while many invertebrate AChEs, such as the AChEs from the nematode *Nippostrongylus brasiliensis*, possess the vertebrate AChE substrate specificity pattern [29, 31–33], other invertebrate AChEs, such as those from the nematodes *Caenorhabditis briggsae*, *Caenorhabditis elegans*, and *Steinernema carpocapsae*, show substantial activity with BTCh in addition to ATCh and PTCh [4, 34]. Dv-ACE-1ChE maximally hydrolyzes the substrates ATCh, PTCh, and BTCh at a ratio of 100:78:5 (Fig. 4a; Table 2). These substrate specificity data are consistent with quantitative data for the AChEs from *N. brasiliensis*, but contrast with those for the AChEs from *Caenorhabditis* spp. and *S. carpocapsae*. Dv-ACE-1 shows substrate inhibition with ATCh and PTCh, characteristics of AChE, but not with BTCh, with which it shows substrate activation, a characteristic of BChE (Fig. 4A; Table 2). Overall, these data suggest that the enzyme is an AChE. ChE activities can also be characterized on the basis of ‘diagnostic’ inhibitors, which preferentially inhibit either AChE or BChE. Thus, we further analyzed specific activities by investigating inhibition of Dv-ACE-1 by physostigmine, which inhibits all ChEs; BW284c51, which inhibits AChEs preferentially; and ethopropazine and iso-OMPA, which inhibit BChEs preferentially. Physostigmine and BW284c51 inhibited the enzyme, both at sub-micromolar concentrations, while ethopropazine and iso-OMPA only inhibited the enzyme at much higher concentrations of inhibitor (Fig. 4B; Table 3). These data confirm that Dv-ACE-1 is an AChE.

3.3. Molecular forms of AChE in the absence and presence of ColQ, PRiMA, and poly-L-proline

As sequence analysis indicated that Dv-ACE-1 contains a WAT domain in a T-peptide C-terminus ([7]; Figs. 1 and 2; Table 1) and thus appears to be AChE_T, we predict that it should be able to form monomers, dimers, and tetramers; and furthermore interact with the PRAD-containing proteins ColQ and PRiMA, as well as poly-L-proline [10]. To investigate if these interactions do occur, we analyzed the molecular forms of the enzyme by velocity sedimentation on sucrose gradients in the presence and absence of the nonionic detergent Brij 97. Velocity sedimentation on sucrose gradients is the established method of choice for separating, classifying, and quantifying the various molecular forms of both vertebrate and invertebrate ChEs [3, 27, 34–38]. Following velocity sedimentation, amphiphilic monomers and dimers (G_1^a and G_2^a : 4S and 6S in the presence of Brij 97) of Dv-ACE-1 were identified (Fig. 5A, B). In addition, nonamphiphilic tetramers (G_4^{na} ; 11S) were observed in extracts and media. It should be noted that G_1^a and G_2^a are not easily resolved on gradients, but are identified by the shift in their sedimentation coefficients to higher values in the absence of detergent [39, 40]. In comparison to extracts, the proportion of tetramers was observed to be greater in the enzyme secreted into media. It was also identified that Dv-ACE-1 was capable of associating with the PRAD domains of PRiMA and ColQ. For PRiMA, the interaction was detected by a shift in the sedimentation coefficient of the G_4 form from 11S to 9S in the presence of Brij 97 (G_4^a ; Fig. 6A), consistent with the interaction of the hydrophobic transmembrane region of PRiMA with the detergent [15]. For ColQ, the interaction was revealed by a peak at 16S [A_{12}]; most likely due to the addition of the large collagenic tail, which is cleaved by collagenase to produce lytic G_4 (Fig. 6B) [41]. Synthetic poly-L-proline also associated with Dv-ACE-1 to promote the formation of tetramers (11S). This effect was observed in a dose-dependent manner when the cultures were treated with the polypeptide for 48 h upon transfection with cDNA for the catalytic subunit (Fig. 7A), or when poly-L-proline was incubated with Dv-ACE-1 in cellular extracts (Fig. 7B).

4. Discussion

From the current studies, it is clear that Dv-ACE-1 hydrolyzes ATCh preferentially and is sensitive to the AChE inhibitor, BW284c51, but not to BChE inhibitors. It was also observed that the Dv-ACE-1 produces G_1^a , G_2^a , and G_4^{na} molecular forms and that the tetramers can assemble into complexes with PRiMA, ColQ, and poly-L-proline. As prior sequence analysis indicated that the enzyme is an AChE_T possessing a WAT domain, association of this enzyme with poly-L-proline, PRiMA, and ColQ is most likely due to a WAT-PRAD interaction between the WAT domain in the T-peptide of the AChE_T and the proline-rich sequence in the PRADs of PRiMA and ColQ. This is the first demonstration of a protostome AChE_T associating with the vertebrate tail proteins PRiMA and ColQ.

While a detailed quantitative comparison is beyond the scope of this paper, and comparable quantitative data are not available for the other *D. viviparus* AChEs, we can qualitatively compare some of the kinetic and pharmacological properties of Dv-ACE-1 with Dv-sACE-1 and Dv-sACE-2, which have been characterized previously; Dv-ACE-2 has not. All three characterized nematode AChEs show a strong preference for ATCh over BTCh [9]. Although Dv-sACE-1 was not reported to show inhibition by excess substrate, while Dv-sACE-2 and Dv-ACE-1 do, it is possible that the lack of inhibition is simply due to the fact that a sufficiently high concentration of substrate was not used. We did not observe substrate inhibition of Dv-ACE-1 until concentrations of ATCh greater than 10mM were added, which was the highest concentration of substrate used with Dv-sACE-1 and Dv-sACE-2. Nevertheless, there appear to be subtle differences in substrate inhibition among Dv-sACE-1, Dv-sACE-2, and Dv-ACE-1, as indicated previously for the two soluble AChEs [9], with Dv-sACE-2 the most sensitive and Dv-sACE-1 the least sensitive. Notably, there is a difference in the structure of the peripheral site between Dv-ACE-1 and the two soluble AChEs: the replacement of the aromatic residue Trp283 in Dv-ACE-1 (Trp279 in *T. californica* AChE) by Ser315 and Gly315 in Dv-sACE-1 and Dv-sACE-2, respectively. However, it does not seem likely that this difference accounts for the properties of the three enzymes with respect to substrate inhibition. Trp279 is only modestly involved in substrate inhibition in AChE, as non-aromatic replacement only slightly decreases inhibition by excess substrate [23, 42]. However, more importantly, the Trp is present in Dv-ACE-1, which is intermediate in sensitivity to inhibition by excess substrate to the two secreted enzymes. Thus other, perhaps post-translational, differences may be involved. All three enzymes are inhibited by BW284c51 but not by iso-OMPA. However, Dv-sACE-1 and Dv-sACE-2 appear to be less sensitive to inhibition by BW284c51, than is Dv-ACE-1. This difference could be due to the replacement of the aromatic residue Trp283 by the non-aromatic residues [9]. Trp279 is an important residue for binding one of the quaternary ammonium ions of BW284c51, which is bound via this residue to the peripheral site at the top of the catalytic gorge and to the choline binding site at the bottom of the gorge via Trp84. Non-aromatic replacement of Trp279 reduces affinity of AChE for BW284c51 and other bisquaternary inhibitors [22, 23, 42, 43]. These structural and pharmacological differences could be related to different functions of neuronal and secreted AChEs in *D. viviparus*.

It is interesting that Dv-ACE-1, as an AChE from a protostome invertebrate, can associate with the vertebrate tail proteins PRiMA and ColQ, and with the synthetic peptide poly-L-proline. Previously we showed that the AChE_T from a deuterostome invertebrate, the urochordate *Ciona intestinalis*, was also capable of associating with the vertebrate tail protein ColQ [27]. The WAT domains of vertebrate AChE_Ts (e.g. *Torpedo* spp.) and the AChE_Ts of *C. intestinalis* and *D. viviparus* form amphipathic α -helices. However, although the μ H of the helix of the *D. viviparus* enzyme is preserved, its angle is different from the other two as a result of an insertion in the *D. viviparus* AChE (INS19N) with respect to

Torpedo spp. and *C. intestinalis* AChEs. Nevertheless, this insertion and change in μH angle does not prevent the association of the enzyme with the PRADs. In *T. marmorata* AChE_T, T12 Δ and M21 Δ were created to perturb the helical organization of the aromatic cluster of the WAT domain [44]. Both mutations rotate the angle of the μH comparably. However, while the T12 Δ mutation decreases the μH modestly (~20%), the M21 Δ mutation decreases it dramatically (~60%). With respect to WAT-PRAD interactions, T12 Δ decreases but does not eliminate association of the *T. marmorata* AChE with an N-terminal fragment of ColQ (Q_N), but M21 Δ abolishes the interaction completely. These data suggest it is the organization of the aromatic cluster and the staircase motif, but not its angle with respect to the catalytic subunit, which is important. In fact, there appears to be considerable flexibility of the subunits relative to the WAT domains [45]. Thus, the primary function of the helix may be to make the staircase domain accessible for interaction with PRADs.

AChE_T tetramers have been reported to associate with a large non-catalytic subunit in the nematodes *C. elegans*, *C. briggsae*, and *Steinernema carpocapsae* [5, 12, 34, 38], although the non-catalytic proteins associated with these AChEs have not been identified. Given that Dv-ACE-1 interacts with poly-L-proline, PRiMA, and ColQ, it seems likely that the association is mediated by WAT-PRAD interactions between the WAT domain of the AChE_T subunit and the PRADs of PRiMA and ColQ or poly-L-proline itself. We therefore think that it is plausible that the interactions between AChE_Ts of nematodes with non-catalytic proteins occur via similar WAT-PRAD interactions. It would be interesting to characterize these proteins and their interactions with Dv-ACE-1. We have not been able to find any homologues of PRiMA or ColQ in the invertebrate sequence databases that we have searched. However, the recent discovery of a PRAD derived from the cytoplasmic, cytoskeletal protein lamellipodin in serum BChE in vertebrates suggests that other PRAD-containing proteins play an important role in organizing tetramers of ChEs [46]. Notably, lamellipodin is homologous to the *C. elegans* cell migration protein MIG-10 [47]. The protostome WAT sequences suggest that there may be some differences in WAT-PRAD interactions between the protostomes and deuterostomes. There are two substitutions in the staircase region of the WAT domain of protostomes: F14M and M21/22I, resulting in the loss of one aromatic residue. The functional significance of these changes, if any, is not clear at this time. However, there may be an additional aromatic residue present at another site in the WAT domain of protostomes in Tyr38. This residue actually increases the μH ~13% and potentially lengthens the domain, possibly allowing for interactions with longer PRADs. The PRADs of ColQ and PRiMA contain 8 and 14 prolines, respectively. In comparison, the PRAD of lamellipodin has 23 proline residues. The nature of the non-catalytic subunits and their functions in the nematodes remain unknown at this time, but their functions should include both the maturation of AChE_T subunits and the stabilization of tetramers [16].

Until the current study, it was not known whether Dv-ACE-1 forms tetramers that associate with a non-catalytic subunit. The nature of non-catalytic subunit partner(s) remains to be elucidated: thus far, homology sequence searches of the relevant databases (e.g. GenBank and Nembase) for homologues of either ColQ or PRiMA have yielded no molecules of significant identity to either of these proteins. The current study suggests that Dv-ACE-1 most likely utilizes the same mode of membrane anchorage as *C. elegans* ACE-1, and it would be interesting to examine whether the anatomical expression is also conserved. This was determined in *C. elegans* by the use of Green Fluorescent Protein (GFP) reporter constructs. Thus, *ace-1::GFP* expression was observed in all body wall muscle cells, the pharyngeal (pm5) muscle cells and in sensory neurons in head ganglia, whereas *ace-2::GFP* is expressed almost exclusively in neurons [5, 6].

There are two possibilities for the presence of the WAT domain and the T-peptide of AChE in both the protostome and deuterostome branches of the animal kingdom – parallel and convergent evolution [48–50]. The phylogeny is consistent with parallel evolution, suggesting that the WAT domain evolved prior to the divergence of the protostomes and deuterostomes, over 900 mya [27, 51]. The apparent deletion event in deuterostomes further supports a common heritage, with a relatively recent deletion in the chordate lineage. Although an amphipathic α -helix does not place many structural constraints on natural selection, the requirement of a preponderance of aromatic residues in the helix does, so the independent appearance of the T-peptide in protostomes and deuterostomes by convergent evolution seems unlikely. In either case, the phylogenetic distribution of the AChE_T subunit indicates that it has an ancient origin and has been conserved for a considerable period of time.

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Abbreviations

AChE	acetylcholinesterase
ATCh	acetylthiocholine
BChE	butyrylcholinesterase
BTCh	butyrylthiocholine
ColQ	collagen Q
GPI	glycophosphatidylinositol
HIS buffer	high ionic strength buffer
μH	hydrophobic moment
PRAD	proline-rich attachment domain
PRiMA	proline-rich membrane anchor
WAT domain	tryptophan amphiphilic tetramerization domain

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	Staircase domain	
<i>E. caballus</i> AChE	DTLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQD-----RCSDL----- 40	deuterostome
<i>C. familiaris</i> AChE	DTLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQD-----RCSDL----- 40	
<i>H. sapiens</i> AChE	DTLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQD-----RCSDL----- 40	
<i>F. catus</i> AChE	DTLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQD-----RCSDL----- 40	
<i>M. domestica</i> AChE	DSLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQE-----RCSDL----- 40	
<i>M. musculus</i> AChE	DTLDEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ VH ⁶ KNQ ⁷ FDHY ⁸ ---SKQE-----RCSDL----- 40	
<i>B. fasciatus</i> AChE	DNIEAEQWKA ¹ EFH ² W ³ SS ⁴ -AY ⁵ MH ⁶ KNQ ⁷ FDHY ⁸ ---NKQD-----RCSEL----- 40	
<i>E. electricus</i> AChE	ENIDAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ MH ⁶ KNQ ⁷ FDHY ⁸ ---SKQE-----RCTNL----- 40	
<i>T. nigroviridis</i> AChE	DNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ MH ⁶ KNQ ⁷ FDHY ⁸ ---SKQE-----RCTDL----- 40	
<i>T. californica</i> AChE	ETIDAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ MH ⁶ KNQ ⁷ FDHY ⁸ ---SRHE-----NCAEL----- 40	
<i>G. gallus</i> AChE	GPPEAEQWKA ¹ EFH ² W ³ SS ⁴ -SY ⁵ MG ⁶ W ⁷ RTQ ⁸ FHY ⁹ ---SRQQ-----FCATL----- 40	protostome
<i>C. familiaris</i> BChE	GNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKE-----SCAGL----- 41	
<i>F. catus</i> BChE	GNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKE-----SCAGL----- 41	
<i>H. sapiens</i> BChE	GNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKE-----SCVGL----- 41	
<i>G. gallus</i> BChE	GNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKE-----RCAGSN----- 42	
<i>E. caballus</i> BChE	GNIDAEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKE-----SCSDF----- 41	
<i>M. musculus</i> BChE	GDIDTEQWKA ¹ EFH ² W ³ SS ⁴ -NY ⁵ MD ⁶ KNQ ⁷ FDHY ⁸ ---SKKK-----ECTAL----- 41	
<i>C. intestinalis</i> AChE	SMDDEVETKWKIEFNRWT-ESMDLWDRSFKAYSDDKQS-----SCFN----- 42	
<i>C. savignyi</i> AChE	ANMDEVETKWKIEFNRWS-ESMDLWDRSFKAYSDDKQS-----SCFN----- 42	
<i>B. floridae</i> AChE A	ANITEEERQWKENFYSWRGEYMPDYSAFAFV---ERMDSKE-----RCGTSDDLE----- 50	
<i>B. floridae</i> AChE B	SDISEAERLWKEEFKLWTKQYMDKQAYKYDV---NYRD---E---GCPRESN----- 45	
<i>S. purpuratus</i> AChE 1	GDIKETEIKWKEEFKWKYTKHVMWKAFAHYI---NNKD---A---GCDGR----- 43	
<i>S. purpuratus</i> AChE 2	ANIDSEERQWKEFYQWSTKYMVDKAEFNHYL---YNKE---S---GCPNEATANETP 51	
<i>L. gigantea</i> AChE	ADISDVEREWKLOFYQWKNQYIVLWKNQFDNPL---NNYERRLQ-----TCGGTP----- 47	protostome
<i>C. teleta</i> AChE	KMSSEVEKWKQHEFHWSTWYIVLWKEIEFQYKKRIE-----GTPG----- 41	
<i>B. xylophilus</i> AChE	ADVGESFVRWKQMDRWENYINDNEFHFEQYKKHOSYRHLDM---DQCV----- 47	
<i>D. destructor</i> AChE	AEVGESFVRWKQMDRWQNDYITDQFHFQYKKYQSYRHMETR---DYC----- 47	
<i>C. elegans</i> AChE	ADVGDPLYLVKQMDKQWNEYITDQHEFEQYKKRYQTYRQSDS---ETCGG----- 48	
<i>C. briggsae</i> AChE	ADVGDPLYLVKQMDKQWNEYITDQHEFEQYKKRYQTYRQSDS---ETCGG----- 48	
<i>D. viviparus</i> AChE	ADVGDPLYLVKQMDKQWNEYITDQYHFEQYKKRYQTYRHLDS---DSCGS----- 49	
<i>L. loa</i> AChE	ADTGDQVMRWKQMDRWENYIIDWKLFEQYKKYQAYRYADSTS---GQC----- 48	
<i>M. javanica</i> AChE	ADLGESFIKWKQMDRWENDYMPENHFEQYKKRQMYRRKDEGEDNDVCG----- 51	
<i>M. incognito</i> AChE	ADLGESFIKWKQMDRWENDYMPENHFEQYKKRQMYRRKDEGEDNDVCG----- 51	

Fig. 1.

Alignment of T-peptides and WAT domains of AChEs and BChEs. Numbering of the amino acid sequences is indicated on the right and starts with the amino acids of the T-peptide C-terminus. The aromatic and hydrophobic residues of the staircase region of the WAT domain that face the prolines of PRADs are shown in red on the first line of the alignment (*E. caballus* AChE) and highlighted in yellow in the sequences. Purple highlights indicate the position of highly conserved residues of the staircase that do not face the PRAD prolines. Orange highlights denote isolated exceptions. Grey highlights indicate conserved residues outside the staircase domain. The green highlight shows the position of the conserved cysteine residue involved in intrachain disulfide bonds. Dashed lines separate vertebrate AChE and BChE. The solid line separates protostomes and deuterostomes. Species, enzymes, and accession numbers (Genbank or ENSEMBL (ENS)): *Equus caballus* AChE and BChE (ENSECAG00000006898), *Canis familiaris* AChE and BChE (XP_546946; M62411), *Homo sapiens* AChE and BChE (M55040.1; M16541), *Felix catus* AChE (AF053485.1), *Monodelphis domestica* AChE (ENSMODG00000004882), *Mus musculus* AChE and BChE (X56518.1; M99492), *Bungarus fasciatus* AChE (BFU54591), *Electrophorus electricus* AChE (AF030422.1), *Tetraodon nigroviridis* AChE (AY733039.1), *Torpedo californica* AChE (X56517), *Gallus gallus* AChE and BChE (U03472.1; AJ306928), *Ciona intestinalis* AChE (BK006073), *Ciona savignyi* AChE (BN000070.1), *Branchiostoma floridae* AChE A and AChE B (GG666612.1), *Strongylocentrotus purpuratus* AChE 1 and AChE 2 (XM_777020; XM_791571.1), *Loligo gigantea* AChE (FC760669, FC559081), *Capitella teleta* AChE (EY627216, EY551148), *Bursaphelenchus xylophilus* AChE (GU166345.1), *Dictylenchus destructor* AChE (EF583059), *Caenorhabditis elegans* AChE (X75331), *Caenorhabditis briggsae* AChE (U41846.1), *Dictyocaulus viviparus* AChE (DQ375489), *Loa loa* AChE (ADBU01000547), *Meloidogyne javanica* AChE (AF080184), *Meloidogyne incognita* AChE (AF075718.1).

Fig. 2.

Helical wheel representation of WAT domains of representative ChEs. Hydrophilic residues are circles; hydrophobic are diamonds; negatively charged are triangles, and positively charged are pentagons. Hydrophobic are green, and the amount of hydrophobicity is proportional to green to yellow shading, with zero hydrophobicity coded as yellow. Hydrophilic residues are red with the intensity of the red decreasing proportionally to the hydrophilicity. *T. californica* AChE is a vertebrate (chordate) AChE; *C. intestinalis* AChE is a deuterostome invertebrate (urochordate) AChE; *D. viviparus* AChE is a protostome invertebrate (nematode) AChE. Charged residues are light blue. Wheels according to Zidovetzki et al [18].

Fig. 3.

Phylogenetic tree of T-peptides of AChEs and BChEs. The evolutionary history was inferred using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [52] and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 [53]. Abbreviations and references found in ESTHER [54]. Species and accession numbers are the same as in Fig. 1. Dashed lines separate vertebrate AChE and BChE. Solid line separates protostomes and deuterostomes.

Fig. 4. Kinetic and pharmacological properties of Dv-ACE-1. (A) HIS extracts of COS-7 cells expressing recombinant enzyme were assayed with ATCh (▼), PTCh (○), and BTCh (●), and the data were fit as described in Materials and Methods. (B) HIS extracts of transfected COS-7 cells were incubated with inhibitors for 20 min prior to being assayed for activity with ATCh as described in Materials and Methods. The inhibitors used were physostigmine (●), BW284c51(▽), Ethopropazine (▼), and iso-OMPA (○).

Fig. 5.

Molecular forms of Dv-ACE-1 produced *in vitro*. Extracts and media from COS-7 cells transfected with cDNA for the catalytic subunit for Dv-ACE-1 were subjected to velocity sedimentation on sucrose gradients in the presence and absence of the non-ionic detergent Brij 97 and assayed for AChE activity as described in Materials and Methods. (A) HIS extracts, (B) media; presence of Brij 97 (●), absence of Brij 97 (○). In A, the grey peaks correspond to G_1^a / G_2^a and the arrows indicate their shifts to lower sedimentation coefficients in the presence of detergent. In B, the grey peaks correspond to G_2^a and the arrow indicates its shift to lower sedimentation coefficient in the presence of the detergent.

Fig. 6.

Molecular forms of Dv-ACE-1 produced *in vitro* when expressed with PRiMA and ColQ. Extracts of COS-7 cells transfected with cDNA for the catalytic subunit for DV-ACE-1 and (A) PRiMA or (B) ColQ were analyzed by velocity sedimentation on sucrose gradients as described in Materials and Methods. (A) Absence of PRiMA ($\circ$), presence of PRiMA ($\bullet$); the grey peak corresponds to G_4^a (G_4 -PRiMA). (B) Presence of ColQ ($\bullet$), presence of ColQ and collagenase ($\circ$); the grey peak corresponds to the A_{12} form of the enzyme, three catalytic tetramers covalently associated with a triple helical ColQ tail.

Fig. 7.

Molecular forms of Dv-ACE-1 produced *in vitro* in the presence of poly-L-proline. (A, B) Cells were transfected for AChE and treated with poly-L-proline for 48 hours. Extracts (A) were applied to gradients: control ($\circ$), 2×10^{-7} M poly-L-proline ($\bullet$), 2×10^{-5} M poly-L-proline ($\blacktriangledown$). Alternatively, cells were transfected for AChE, and after 48 hours extracts (B) were harvested and incubated without ($\circ$) or with 2×10^{-7} M poly-L-proline ($\bullet$) or 2×10^{-5} M poly-L-proline ($\blacktriangledown$) for 4 hours at 37°C before being analyzed by velocity sedimentation on sucrose gradients. For A and B, the shades of grey peaks correspond to G_4^{na} in the absence (light grey) and presence of 2×10^{-7} M (dark grey) and 2×10^{-5} M (medium grey) poly-L-proline.

Table 1

Aromatic amino acids in the catalytic gorge of AChEs from *D. viviparus* and *T. californica*^a

Subsite	Dv-ACE1	Dv-ACE2	Dv-sACE1	Dv-sACE2	T. californica
Peripheral	Ser68	Thr72	Thr105	Thr105	Tyr70
	Tyr121	Tyr121	Tyr156	Tyr156	Tyr121
	Trp283	Tyr281	Ser315	Gly315	Trp279
Choline binding and hydrophobic	Trp82	Trp86	Trp119	Trp119	Trp84
	Tyr130	Tyr130	Tyr165	Tyr165	Tyr130
	Tyr332	Tyr335	Phe367	Phe367	Phe330
	Phe333	Trp336	Trp368	Trp368	Phe331
Acyl pocket	Ala290	Met291	Met324	Met324	Phe288
	Phe292	Phe293	Phe326	Phe326	Phe290
	Phe411	Phe419	Phe453	Phe453	Val400
	Trp114	Trp114	Trp149	Trp149	Trp114
Wall of gorge	Trp232	Trp233	Trp268	Trp268	Trp233
	Tyr336	Tyr339	Tyr371	Tyr371	Tyr334
	Trp443	Trp452	Trp486	Trp486	Trp432
	Tyr453	Tyr462	Tyr496	Tyr496	Tyr442

^aNumbering is based on amino acid sequences after actual or putative signal peptides have been removed. Residues in *T. californica* are used as a reference value. Conserved aromatic residues are shown in bold type.

Table 2Kinetics of AChE from *D. viviparus*.

Substrate	K_m (M)	K_{ss} (M)	b	V_{max} Substrate/ V_{max} ATCh
ATCh	$7.51 \pm 0.21 \times 10^{-4}$	$2.36 \pm 0.10 \times 10^{-1}$	0	1.00
PTCh	$1.59 \pm 0.03 \times 10^{-3}$	$3.51 \pm 0.19 \times 10^{-1}$	0	0.78
BTCh	$4.32 \pm 1.61 \times 10^{-5}$	$3.90 \pm 0.33 \times 10^{-3}$	3.69	0.05

Data are the mean $\pm$ SD of 3 determinations.

Table 3Pharmacology of AChE from *D. viviparus*.

Inhibitor	IC ₅₀ (M)
Physostigmine	$1.26 \pm 0.18 \times 10^{-7}$
Ethopropazine	$1.37 \pm 0.02 \times 10^{-4}$
BW284c51	$3.03 \pm 0.95 \times 10^{-7}$
Iso-OMPA	$>3 \times 10^{-3}$

Data are the mean $\pm$ SD of 3 determinations.