Solubilization, molecular forms, purification and substrate specificity of two acetylcholinesterases in the medicinal leech (*Hirudo medicinalis*)

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Two acetylcholinesterases (AChE) differing in substrate and inhibitor specificities have been characterized in the medicinal leech (*Hirudo medicinalis*). A 'spontaneously-soluble' portion of AChE activity (SS-AChE) was recovered from haemolymph and from tissues dilacerated in low-salt buffer. A second portion of AChE activity was obtained after extraction of tissues in low-salt buffer alone or containing 1% Triton X-100 [detergent-soluble (DS-) AChE]. Both enzymes were purified to homogeneity by affinity chromatography on edrophonium- and concanavalin A-Sepharose columns. Denaturing SDS/PAGE under reducing conditions gave one band at 30 kDa for purified SS-AChE and 66 kDa for DS-AChE. Sephadex G-200 chromatography indicated a molecular mass of 66 kDa for native SS-AChE and of 130 kDa for DS-AChE. SS-AChE showed a single peak sedimenting at 5.0 S in sucrose gradients with or without Triton X-

100, suggesting that it was a hydrophylic monomer (G1). DS-AChE sedimented as a single 6.1–6.5 S peak in the presence of Triton X-100 and aggregated in the absence of detergent. A treatment with phosphatidylinositol-specific phospholipase C suppressed aggregation and gave a 7 S peak. DS-AChE was thus an amphiphilic glycolipid-anchored dimer. Substrate specificities were studied using *p*-nitrophenyl esters (acetate, propionate and butyrate) and corresponding thiocholine esters as substrates. SS-AChE displayed only limited variations in K_m values with charged and uncharged substrates, suggesting a reduced influence of electrostatic interactions in the enzyme substrate affinity. By contrast, DS-AChE displayed higher K_m values with uncharged than with charged substrates. SS-AChE was more sensitive to eserine and di-isopropyl fluorophosphate (IC_{50} 5×10^{-8} and 10^{-8} M respectively) than DS-AChE (5×10^{-7} and 5×10^{-5} M).

INTRODUCTION

Among protostomian invertebrates, acetylcholinesterase (AChE, EC 3.1.1.7) has been characterized in insects [1], nematodes [2], molluscs and annelida [3–5]. It was shown genetically that AChE was encoded by a single gene, *ace*, in *Drosophila* (as in vertebrates), but by three distinct genes, *ace-1*, *ace-2* and *ace-3*, in the nematode *Caenorhabditis elegans*. Genes encoding AChE in the fruitfly *Drosophila* and in the mosquito *Anopheles* have been cloned and sequenced [6,7]. Recently one of the three *ace* genes in *C. elegans* (*ace-1*) was also characterized [8]. As ACh is used essentially in the sensory system in insects and as a major neurotransmitter in the motor system of nematodes [9], it is not clear whether the expression of three genes of AChE in nematodes is a primitive trend or if it is correlated with the existence of a cholinergic motor system. We therefore decided to study AChE in other invertebrates in which motor systems are cholinergic and which are considered to be more highly evolved than nematodes. The medicinal leech (*Hirudo medicinalis*; Annelida) appears as an excellent example of the presence of AChE in the motor system of an invertebrate. *Hirudo* dorsal muscle has been for years a valuable bioassay for acetylcholine; for this assay the muscle needed to be treated with eserine, proof of the efficiency of endogenous cholinesterase [3].

We report here biochemical data obtained on acetylcholinesterase from the *H. medicinalis*. Sequential extractions were used for characterization of molecular forms, and catalytic properties were studied after purification of differently solubilized fractions.

MATERIALS AND METHODS

Animals

They were provided by a leech breeder in Pesaro, Italy, and kept alive in natural fresh water.

Composition of buffers used for extractions

Low-salt (LS) buffer contained 20 mM Tris/HCl, pH 7.5, 5 mM *N*-ethylmaleimide (Sigma), 1 mM benzamidine (Sigma), 5 mM EDTA, 0.1 mg/ml bacitracin (Sigma) and aprotinin from bovine lung (Sigma) (proteinase inhibitors) at a concentration of 7.5×10^{-3} trypsin-inhibitory units/ml. Low-salt Triton (LST) buffer contained LS buffer with the proteinase inhibitors described above plus 1% Triton X-100. HS and HST buffers were LS and LST buffers with the proteinase inhibitors described above plus 1M NaCl.

Sequential extractions of *Hirudo* AChE

The spontaneously-soluble (SS) fraction of AChE activity was recovered after dilaceration of tissues (1 g in 10 ml of LS buffer) and centrifugation for 1 h at 100 000 *g* (SS1 in Table 1). The operation was repeated, giving the SS2 and SS3 fractions. The pellet recovered at the end of the third round was homogenized in LS buffer (at 1:10, w/v) in a glass-glass Potter–Elvehjem homogenizer at 4 °C. The 100 000 *g* supernatant gave the low-salt-soluble 1 (LSS1) fraction. The pellet was submitted to another cycle of homogenization in LS buffer and centrifugation

Abbreviations used: AChE, acetylcholinesterase; AcSCho, BrSCho and PpSCho, acetyl-, butyryl- and propionyl-thiocholine; DFP, di-isopropyl fluorophosphate; DS, detergent-soluble fraction [in low-salt/Triton (LST) medium]; HSS, high-salt-soluble fraction [in high-salt (HS) medium]; LSS, low-salt-soluble fraction [in low-salt (LS) medium]; ND-PAGE, non-denaturing PAGE (SDS/PAGE is denaturing); PI-PLC, phosphatidylinositol-specific phospholipase C; SS-AChE, spontaneously soluble acetylcholinesterase.

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(giving the LSS2 fraction). Two additional cycles performed with LST buffer (1:5, w/v) resulted in detergent-soluble 1 (DS1) and DS2 extracts. Two cycles using HS buffer (1:5, w/v) gave high-salt-soluble 1 (HSS1) and HSS2 fractions.

Sucrose-gradient centrifugation

Sedimentation analyses were performed in 11 ml, 5–20%-(w/v)-sucrose gradients in HS or HST buffers. Samples (150–250 ml loaded) were centrifuged for 16 h at 36000 rev./min at 4 °C in a SW41 rotor (200000 g). A total of 40 fractions was collected from the bottom of each gradient and assayed for AChE activity at pH 7. Alkaline phosphatase from calf intestine (Boehringer; $s_{20,w}$ 6.1 S) and *Escherichia coli* β -galactosidase (Boehringer; $s_{20,w}$ 16 S) were used as internal markers of migration. In some experiments samples were centrifuged on gradients containing 0.5% Brij 96 (10-oleyl ether, Sigma) as a detergent. In these cases, samples were preincubated with 0.5% Brij 96 for 30 min before loading.

AChE activity and protein determination

AChE activity of extracts and fractions of sucrose gradients was assayed at 25 °C according to the spectrophotometric method of Ellman et al. [10] with 1 mM acetylthiocholine iodide (AcSCho, Sigma) or propionylthiocholine (PpSCho, Sigma) as substrate in the presence of 0.2 mM 2,2'-dinitro-5,5'-dithiodibenzoic acid (Boehringer) in 0.1 M phosphate buffer, pH 7.2. One enzyme unit corresponds to the amount of enzyme which hydrolyses 1 μ mol of substrate/min. Protein content of samples was determined by the bicinchoninic acid method following the manufacturer's (Pierce, Rockford, IL, U.S.A.) instructions.

Non-denaturing (ND-) PAGE

Electrophoretic runs were performed as described previously [11] in 8 cm $\times$ 7.3 cm $\times$ 0.1 cm polyacrylamide gels in a vertical slab-gel apparatus (Bio-Rad). Gels and running buffers contained 50 mM Tris/Glycine, pH 8.9, and either 0.5% Triton X-100 or no detergent. Gels were rinsed in distilled water and stained for AChE activity by the method of Karnovsky and Roots [12] with PpSCho as substrate. Detection of non-specific esterases was achieved using indoxyl acetate and Nitro Blue Tetrazolium.

Digestion by phosphatidylinositol-specific phospholipase C (PI-PLC)

PI-PLC (EC 3.1.4.10) from *Bacillus thuringiensis* was purchased from Boehringer. AChE in solution was digested for 1 h at 37 °C with stock PI-PLC diluted to 5 units/ml.

Disulphide-bond reduction

Reduction of purified SS- and DS-AChEs was conducted at pH 8.0 at room temperature for 1 h. Dithiothreitol (Sigma, final concn. 10^{-2} or 10^{-4} M) was used in the presence of 0.2 mg/ml edrophonium chloride (Sigma) for protection of the active site. *N*-Ethylmaleimide (Sigma; 5×10^{-2} or 5×10^{-4} M) was added after 1 h for 15 min. Reduced samples were dialysed against LST buffer before ultracentrifugation.

Purification procedure

The same procedure was followed for the purification of AChE in SS and DS fractions. Edrophonium chloride (Sigma) was coupled to Sepharose (Pharmacia) as described by Hodgson and Chubb [13]. Crude preparations (SS- and DS-AChEs; total

activity 29 and 16 units respectively) were applied to 3 ml edrophonium-Sepharose columns that had been pre-equilibrated with 10 vol. of LS or LST buffers (only 0.1% Triton X-100 in LST buffer in this case). Columns were then washed with 100 ml of LS and LST (0.1% Triton X-100) buffers containing 30 mM NaCl and then with 20 ml of the same buffer with 10 mM NaCl. SS- and DS-AChEs were eluted with 20 mM edrophonium chloride (Sigma) in LS or LST buffers. Fractions 0.5 ml were collected, and the more active were pooled and loaded on to 0.5 ml concanavalin A-Sepharose columns (Sigma). Washing of columns was achieved first with 20 ml of LS or LST buffer containing 0.5 M NaCl then with LS or LST buffer only. The enzyme activity was specifically eluted with 0.3 M α -methyl glucamannopyranoside (Sigma) in LS or LST buffer. Fractions collected were dialysed against LS or LST buffer.

Denaturing electrophoresis and silver staining

SDS/PAGE of purified enzymes was performed in 12% acrylamide gels as described by Laemmli [14], and gels were stained with AgNO₃ by the method of Blum et al. [15].

Gel-filtration chromatography

Gel filtration of purified extracts (0.5 ml), in HS and HST buffers, was performed on Sephadex G-200 columns (1.5 cm $\times$ 70 cm). Stokes radii (a) were determined by comparison to standards: apoferritin, catalase, alkaline phosphatase, alcohol dehydrogenase and cytochrome *c* (Sigma). Estimated molecular masses (M) were calculated from $s_{20,w}$ (s) and a values according to the equation:

$$M = 413sa$$

derived from Svedberg's equation:

$$M = 6\pi\eta aNs / (1 - (\rho/\rho_m))$$

where η is the viscosity of water, N is Avogadro's number and ρ and ρ_m are the densities of water and enzyme respectively.

Substrate and inhibitor specificities

K_m and V_{max} were determined by computer analysis of the Lineweaver-Burk double-reciprocal plots of experimental data obtained with increasing concentrations of substrates (AcSCho, PpSCho and BrSCho) ranging from 3 to 100 μ M, in 100 mM phosphate buffer, pH 7.2, in the presence of 0.2 mM 2,2'-dinitro-5,5'-dithiodibenzoic acid at 25 °C ('Ellman medium'). Inhibition constants (K_i) for the reversible inhibitors edrophonium and procainamide were determined using the equation:

$$K_i = I / (K_m^{-1} / K_m^0 - 1)$$

K_i for the reversible ligand of the peripheral-site propidium (Sigma) was calculated according to the equation:

$$K_i = I / (V_{max}^0 / V_{max}^{-1} - 1)$$

K_m^0 , V_{max}^0 , K_m^{-1} and V_{max}^{-1} (K_m and V_{max} values in the absence, 0 , or presence, $^{-1}$, of inhibitor) were determined from Lineweaver-Burk plots in which straight lines were fitted to experimental data by linear regression. Three concentrations of inhibitor were used: 0.1, 0.2 and 1 μ M for edrophonium, 1.5 and 10 μ M for procainamide and 1, 2 and 5 μ M for propidium with 10 μ l of purified enzyme in the presence of 3, 5, 10, 30, 50 and 100 μ M PpSCho.

Series of eserine and di-isopropyl fluorophosphate (DFP; Sigma) dilutions (10^{-10} – 10^{-2} M) were prepared in Ellman medium without substrate. An aliquot of the sample (10 μ l) was incubated in each solution for 30 min at room temperature and the substrate was then added at a final concentration of 1 mM.

RESULTS

Sequential extraction of AChE from *Hirudo*

A significant amount of AChE activity was recovered from

Table 1 AChE recovery in sequential extractions

Three successive dilacerations of 1 g of tissue in 10 ml of LS buffer gave SS1–SS3 fractions. Homogenization in LS, LST and HS buffers gave LSS, DS and HSS fractions.

Fraction	Activity (units/ml)	Total activity (units)	Recovery (%) [*]	Specific activity (units/mg of protein)
SS1	0.078	0.78	28	0.241
SS2	0.026			0.074
SS3	0.011			0.065
LSS1	0.100	1.00	35	0.287
LSS2	0.046			0.332
DS1	0.253	1.14	38	0.899
DS2	0.071			0.864
HSS1	0.019	0.09	2	0.062

^{*} Percentage of total activity recovered in, from top to bottom as grouped, SS, LSS, DS and HSS fractions respectively.

Hirudo after dilaceration of tissues with no need of homogenization. This fraction of activity was called 'spontaneously-soluble' (SS-) AChE. It corresponded to soluble enzyme present in haemolymph and in a free form within tissues. SS-AChE was exhausted after three dilacerations (Table 1): it accounts for approx. 30 % of total activity. The LSS fraction was recovered after homogenization in a glass–glass Potter–Elvehjem homogenizer in LS buffer; 35 % of the total AChE activity was recovered in two successive LSS fractions. DS-AChE was solubilized in LST buffer; 35 % of the total activity was recovered in the DS1 and DS2 fractions. Only 2 % was released by HS buffer (HSS1), and no activity was detectable in the HSS2 fraction, indicating that AChE extraction was completed (Table 1).

Sucrose-gradient analysis of AChE from each solubilization fraction

Figure 1 shows the sedimentation analysis of AChE recovered in each fraction. SS-AChE showed a single peak sedimenting at 5.0S in the absence of detergent in the gradient or in the presence of Triton X-100 (Figure 1a). This behaviour suggests that it contains only hydrophilic monomer (G1h form). AChE in LSS and DS fractions sedimented as a single peak at 6.1 S in the presence of Triton X-100 was shifted to 4.5 S in the presence of Brij 96 and aggregated in the absence of detergent. PI-PLC treatment suppressed the aggregation (major 7 S peak with a

Figure 1 Sedimentation analysis of AChE activity recovered in different fractions

(a) SS-AChE; (b), (c) and (d), successive extractions in LS, LST and HS buffers respectively (LSS, DS and HSS fractions). ●, Sucrose gradient containing 1 % Triton X-100. ○, Gradients with no detergent; □ in (b) and (c), samples treated by PI-PLC and run on gradients with no detergent; ■ in (c), gradient containing 0.5 % Brij 96. All gradients contained 1 M NaCl. Centrifugation was for 16 h at 200 000 *g* at 4 °C. Arrowheads show the position of internal markers of migration: β -galactosidase (16 S, left) and alkaline phosphatase (6.1 S, right). Assay of fractions was performed as described by Ellman et al. [10] with 1 mM PpSCo as substrate.

Figure 2 Non-denaturing electrophoresis of SS-AChE (a) and DS-AChE (b and c)

SS-AChE (lane a) gave a faint band of high mobility (open arrow). DS-AChE was treated by PI-PLC in lane c, converting amphiphilic dimers (G2a in lane b) into hydrophilic dimers (G2h in lane c). Staining was performed as described by Karnovsky and Roots [12] with 1 mM PpScho as substrate.

Figure 3 Effect of dithiothreitol (10^{-2} M) on DS-AChE

In sucrose gradients containing 1% Triton X-100 the peak at 6.5 S (G2a, ○) was shifted to 4.0 S (G1a, ●) after S–S bond reduction. Samples were dialysed extensively before gradient sedimentation. The shoulder at about 6 S in the reduced sample indicated that reduction was not totally complete. Conditions of sedimentation and assay were as in Figure 1. The inset shows ND-PAGE of the control DS-AChE (G2a in lane a) and after DTT reduction (G1a in lane c). In lane b, G2h after PI-PLC treatment of DS-AChE is shown for comparison of migration. 'o' shows the origin of migration.

shoulder near 5 S in the absence of detergent in the gradient; Figures 1b and 1c). These properties suggest that the major molecular form in LSS and DS fractions is an amphiphilic dimer possessing a glycosyl-phosphatidylinositol anchor. Hereafter this form is termed 'G2a' (for G2 amphiphilic). The small amount of activity in HSS fraction (Table 1) suggested that there was no specific HSS form of AChE in *Hirudo* and, indeed, sedimentation analysis showed that it contained a few amphiphilic dimers

Table 2 Yields of recovery during purification of SS- and DS-AChEs

(a) SS-AChE

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
100 000 g supernatant	28	29	1.04	100	1
Edrophonium + concanavalin A	0.28	9	32.14	31	31

(b) DS-AChE

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification factor
100 000 g supernatant	41.6	16	0.38	100	1
Edrophonium + concanavalin A	0.3	9	32	56	78

(Figure 1d) sensitive to PI-PLC (results not shown). DS-AChE was thus taken to represent all LSS, DS and HSS fractions.

Structure of SS- and DS-AChEs

Susceptibility of the G2a form to PI-PLC in DS-AChE was also demonstrated in ND-PAGE (Figure 2). The slow-migrating G2a was converted into faster-migrating G2h after digestion by PI-PLC. The migration of the latter was far slower than that of hydrophilic G1 from SS-AChE.

Dithiothreitol (10^{-2} M) was shown to convert the G2a form into a monomeric form (peak at 6.5 S shifted to 4.0 S) with no loss of enzymic activity (Figure 3), indicating that G2a was a disulphide-bonded dimer. In ND-PAGE (inset to Figure 3), only a small increase in migration was observed after reduction, indicating that the monomer still bound detergent and was thus amphiphilic. This amphiphilic monomer was thus termed 'G1a' as for *Drosophila* AChE [16]. Dithiothreitol and PI-PLC treatments had no effect on SS-AChE (results not shown).

Purification of SS- and DS-AChEs

SS and DS fractions were purified by affinity chromatography using successively edrophonium and concanavalin A as ligands. Table 2 summarizes yields of recovery. SDS/PAGE under reducing conditions was used to check the purity of the preparations obtained (Figure 4a). Only one band at 30 kDa was found for SS-AChE and one band at 66 kDa for DS-AChE. In gel chromatography on Sephadex G-200 columns, purified SS-AChE gave a single peak (α 3.2 nm) when HS or HST buffers were used for elution. Purified DS-AChE showed a peak at 5.8 nm in the presence of Triton X-100, but only at 4.8 nm after treatment by PI-PLC in the absence of detergent (Figure 4b). Combining α and $s_{20,w}$ values found respectively in gel filtration and ultracentrifugation in sucrose gradients (see the Materials and methods section), molecular masses were estimated at 66 kDa for SS- and 130 kDa for DS-AChE treated with PI-PLC. The molecular mass for DS-AChE was estimated at 146–160 kDa. This confirmed that DS-AChE was an amphiphilic glycolipid-

Figure 4 Analysis of purified SS- and DS-AChEs

(a) SDS/PAGE; 7.5% polyacrylamide gels were run under reducing conditions and silver-stained. Comparison with markers (BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa) indicated a molecular mass (M) of 30 kDa for SS-AChE and 66 kDa for DS-AChE. (b) Gel-filtration chromatography on Sephadex G-200. SS-AChE was run in HS buffer ($\square$). DS-AChE was run in HST ($\bullet$) buffer or after treatment by PI-PLC in HS buffer. Stokes radii (a) were determined by comparison with markers (vertical arrows from left to right): apoferritin (6.1 nm), catalase (5.2 nm), alcohol dehydrogenase (4.6 nm), alkaline phosphatase (3.3 nm) and cytochrome c (1.7 nm). The void volume (determined by Dextran Blue) is shown by an arrowhead. Molecular masses of 66 kDa for SS-AChE, of 130 kDa for DS-AChE after PI-PLC and of 146–160 kDa for DS-AChE in the presence of Triton X-100 were calculated (see the Materials and methods section).

anchored dimer. Detergent bound to DS-AChE is the explanation for its a being higher than that of the PI-PLC-treated enzyme (the detergent is bound to the glycolipid). Regarding SS-AChE, the single band in SDS-containing gel observed at 30 kDa may be the result of a cleavage of a single catalytic subunit into two subfragments (in some other experiments we sometimes obtained a doublet in the 30 kDa region for purified SS-AChE). Thus we suggest that both catalytic subunits of SS-AChE and DS-AChE could have similar sizes in native state (about 60–66 kDa) and that SS-AChE is a hydrophilic monomer.

Substrate and inhibitor specificity of purified SS- and DS-AChEs

SS- and DS-AChEs showed distinct substrate specificity: for both PpSCho was the best substrate, but they showed different activities towards BrSCho and AcSCho (the Pp-/Br-/Ac-SCho $V_{\max}$ ratio was approx. 10:7:3 for SS-AChE and 10:1:7 for DS-AChE; see Table 3). For SS-AChE only limited changes were

Table 3 Kinetic parameters of purified SS- and DS-AChEs

Three charged substrates (AcSCho, PpSCho and BrSCho) and their neutral counterparts [nitrophenyl acetate, nitrophenyl propionate and nitrophenyl butyrate (NpAc, NpPp and NpBr respectively)] were used as substrates. The $V_{\max}$ ratios are indicated for thiocholine esters (100% for PpSCho). Concentrations of enzyme in purified samples were determined assuming a molecular mass of 66 kDa for SS-AChE and 130 kDa for DS-AChE.

(a) SS-AChE

Substrate	K_m (M)	$V_{\max}$ (units/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Charged				
AcSCho	7.0×10^{-5}	9.50 (30%)	11.7	1.67×10^5
PpSCho	12.3×10^{-5}	33.70 (100%)	41.6	3.38×10^5
BrSCho	7.8×10^{-5}	23.60 (70%)	29.1	3.37×10^5
Neutral				
NpAc	56×10^{-5}	0.75 (23%)	0.93	1.66×10^3
NpPp	38×10^{-5}	3.29 (100%)	4.06	1.05×10^4
NpBr	16×10^{-5}	1.69 (52%)	2.09	1.31×10^4

(b) DS-AChE

Substrate	K_m (M)	$V_{\max}$ (units/mg)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \cdot \text{s}^{-1}$)
Charged				
AcSCho	2.5×10^{-5}	20.30 (67%)	29.73	1.19×10^6
PpSCho	3.2×10^{-5}	30.40 (100%)	44.52	1.39×10^6
BrSCho	3.5×10^{-5}	2.70 (9%)	3.95	1.13×10^5
Neutral				
NpAc	2.0×10^{-3}	0.16 (15%)	0.23	0.11×10^3
NpPp	2.5×10^{-3}	1.09 (100%)	1.59	0.64×10^3
NpBr	0.4×10^{-3}	0.02 (1.8%)	0.03	0.07×10^3

Figure 5 Inhibitor-sensitivity of SS- and DS-AChEs

Eserine and DFP were used in (a) and (b) respectively. $\bullet$, SS-AChE; $\circ$, DS-AChE. The IC_{50} values for SS-AChE were 5×10^{-8} M (eserine) and 10^{-8} M (DFP). For DS-AChE, the values were 5×10^{-7} M (eserine) and 5×10^{-5} M (DFP). Error bars represent the S.D. calculated for four different determinations for each inhibitor concentration.

Table 4 Inhibition of SS- and DS-AChEs by edrophonium, procainamide and propidium

Edrophonium and procainamide inhibited both enzymes competitively: they bind to the active site. Propidium inhibited SS- and DS-AChEs non-competitively; this inhibitor binds to a peripheral site distinct from the catalytic site [18].

Inhibitor	Form	Inhibition	K_i (μ M)
Edrophonium	SS	Competitive	0.12
	DS	Competitive	0.13
Procainamide	SS	Competitive	1.00
	DS	Competitive	5.50
Propidium	SS	Non-competitive	0.32
	DS	Non-competitive	2.75

observed in K_m values between choline esters and corresponding neutral esters (2–8-fold increase), whereas under the same conditions a 10–100-fold increase was observed in the K_m for DS-AChE (Table 3). These distinct properties of both enzymes against neutral substrates are particularly apparent when k_{cat}/K_m values are compared (Table 3). The above observations again indicated that SS-AChE and DS-AChEs were two distinct enzymes. This was confirmed by their sensitivity to eserine and DFP (Figures 5a and 5b). SS-AChE appeared more sensitive than DS-AChE to both inhibitors (IC_{50} for eserine and DFP respectively were 5×10^{-8} M and 10^{-8} M for SS-AChE and 5×10^{-7} M and 5×10^{-5} M for DS-AChE). Both enzymes were inhibited by edrophonium and procainamide in a competitive manner and by propidium in a non-competitive manner (Table 4), two classical properties of AChEs (see, for example, [17]). Inhibition by propidium suggested the existence of a peripheral site of substrate binding [18] that is also involved in excess-substrate inhibition. SS-AChE and DS-AChE both exhibited inhibition by excess AcSCho above 5×10^{-4} M and 5×10^{-3} M respectively (results not shown).

DISCUSSION

Two enzymes hydrolysing choline esters were identified as AChEs (EC 3.1.1.7) in *H. medicinalis*. They both hydrolysed PpSCho optimally. This substrate preference might permit one to call them 'propionylcholinesterases', a term previously used for the enzymes in other annelids (*Allolobophora* [5]) and gastropods (the land snail *Helix* [19] and the sea snail *Murex* [4]). However, we used 'AChEs' for both enzymes to underline the fact that they fulfilled conditions required to define AChE (EC 3.1.1.7): (i) they hydrolysed AcSCho significantly; (ii) they were inhibited by eserine, edrophonium and DFP at concentrations usual for AChEs; (iii) they were inhibited by excess AcSCho.

The present demonstration of two AChEs contrasts with the previous report of only one enzyme species in *Hirudo* [20]. This is explained by the fact that the latter study was restricted to the soluble enzyme, with no extraction of tissues, and was thus limited to the present 'SS'-AChE. Kinetic parameters reported by Principato et al. [20] are in good agreement with those we

obtained for SS-AChE. The second AChE (DS-AChE), which required extraction in detergent-containing buffer and possessed a glycolipid anchor, was probably associated with cell membranes *in situ*. It likely corresponds to the AChE activity found associated, by histochemical staining, with nerve trunks, synapses and muscle fibres and less frequently with nerve cell bodies [3].

Kinetic parameters for SS-AChE indicate that it does not discriminate between substrates on the basis of increasing steric hindrance and hydrophobic interaction. The similarity between K_m values obtained with charged and uncharged substrates suggests a reduced influence of electrostatic interactions in the enzyme–substrate affinity. DS-AChE, by contrast, resembles vertebrate AChEs, with strong ionic-interaction effects on the formation of DS AChE–substrate complex.

SS-AChE was found as a presumptive hydrophilic monomer with high electrophoretic mobility in non-denaturing gels, whereas DS-AChE was a glycolipid-anchored dimeric form. The differences between pharmacological properties of SS- and DS-AChEs in *Hirudo* rule out the possibility that SS enzyme could represent a degradation product of the membrane-bound form (progressive conversion of G2a into G2h and G1h, as observed with *Drosophila* AChE [16]), but rather suggest the existence of two distinct species encoded by two different genes. To support this hypothesis we are trying to amplify fragments of AChE gene(s) from *H. medicinalis* with degenerated oligonucleotides deduced from conserved sequences in the AChE family. This has not been successful so far.

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