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Determination of the DNA Sequences of Acetylcholinesterase and Butyrylcholinesterase from Cat and Demonstration of the Existence of Both in Cat Plasma

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ABSTRACT. Cat serum contains 0.5 mg/L of butyrylcholinesterase (BChE, EC 3.1.1.8) and 0.3 mg/L of acetylcholinesterase (AChE, EC 3.1.1.7); this can be compared with 5 mg/mL and < 0.01 mg/L, respectively, in human serum. Cat BChE differed from human BChE in the steady-state turnover of butyrylthiocholine, having a 3-fold higher k_{cat} and 2-fold higher K_m and K_{ss} values. Sequencing of the cat *BCHE* cDNA revealed 70 amino acid differences between cat and human BChE, three of which could account for these kinetic differences. These amino acids, which were located in the region of the active site, were Phe398Ile, Pro285Leu, and Ala277Leu (where the first amino acid was found in human and the second in cat). Sequencing genomic DNA for cat and human *ACHE* demonstrated that there were 33 amino acid differences between the cat and human AChE enzymes, but that there were no differences in the active site region. In addition, a polymorphism in intron 3 of the human *ACHE* gene was detected, as well as a silent polymorphism at Y116 of the cat *ACHE* gene. BIOCHEM PHARMACOL **60**;4:479–487, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. feline; *Felis*; cholinesterase; acetylcholinesterase

Both acetylcholinesterase (*ACHE*† for gene; AChE for protein, EC 3.1.1.7) and butyrylcholinesterase (*BCHE* for gene; BChE for protein, pseudocholinesterase, EC 3.1.1.8) are present in vertebrates; however, tissue locations of the enzymes, specific activities, and levels of activities vary from species to species. AChE is vital in nerve impulse transmission, serving to break down acetylcholine that travels across nerve synapses during the nerve signal relay. When this AChE is inhibited, death may result. The natural role of butyrylcholinesterase is not certain. It has been suggested that BChE may detoxify ingested plant toxins [1–3], and it has been shown to regulate cell proliferation of the embryonic chicken neural crest [4].

It is expected that the Feline Genome Project will complete its first genetic map this year, consisting of about 950 markers spread throughout the cat genome. Although there are no plans to sequence the entire feline genome, the cat was selected for gene mapping because it shares many inherited diseases with humans. In addition to using cat sequences to look for human disease genes, evolutionary comparisons of AChE and BChE are helpful in understanding the possible functions of the enzymes, and can indicate the importance of specific residues by their conservation. Although the major function of AChE is known, AChE on erythrocytes and in plasma may serve another function.

We show here the DNA sequences representing the protein coding regions of BChE and AChE in the domestic cat, and BChE in the Bengal tiger. The *BCHE* cDNA and the *ACHE* gene with intervening introns were sequenced. Cat plasma was found to contain 40% AChE and 60% BChE; Bengal tiger plasma contained 23% AChE and 77% BChE. Purified cat BChE hydrolyzed butyrylthiocholine but not benzoylcholine, three times faster than human BChE.

MATERIALS AND METHODS

Samples and Enzyme Preparation

Blood from 4–5 cats (pooled) was purchased from Pel-Freez Biologicals. Cat pituitary and additional blood were donated by the UNMC Comparative Medicine Department. The Henry Doorly Zoo of Omaha provided Bengal tiger blood and pituitary gland, Sumatran tiger blood, and Siberian tiger serum. Human blood was obtained by venipuncture. The anticoagulant in human, cat, and tiger whole blood samples was liquid citrate. Cat BChE and human

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† *Abbreviations*: AChE, acetylcholinesterase enzyme; *ACHE*, acetylcholinesterase gene; BChE, butyrylcholinesterase enzyme; *BCHE*, butyrylcholinesterase gene; iso-OMPA, tetraisopropyl pyrophosphoramide; cDNA, complementary DNA; and RBC, red blood cell.

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BChE were purified from plasma by ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography on procainamide-Sepharose [5, 6].

BChE and AChE in Blood

Specific inhibitors were used to determine the proportions of AChE and BChE in cat and tiger plasma. For controls, fetal bovine serum, which contains AChE but no BChE, and human plasma, which contains BChE and <1% AChE, were tested at the same inhibitor concentrations used with the cat and tiger plasma. The inhibition was followed by measuring the hydrolysis of 0.5 mM acetylthiocholine by 10 μL plasma [7] in 3 mL of 0.1 M potassium phosphate buffer, pH 8.0, at 25°, in the presence of (A) the general cholinesterase inhibitor eserine (physostigmine; Sigma; [8]) at 10^{-5} M, (B) the AChE-specific inhibitors BW 284C51 {1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide; Sigma A-9013; [9]} at 10^{-7} M, and edrophonium (3-hydroxy-*N*,*N*-dimethyl-*N*-ethylanilinium chloride; Sigma) at 10^{-4} M, and (C) the BChE-specific inhibitors ethopropazine (2-diethylamino-1-propyl-*N*-dibenzoparathiazine hydrochloride; Sigma) at 10^{-5} M, bambuterol {dimethylcarbamic acid 5- [2-[(1,1-dimethylethyl]amino]-1-hydroxyethyl]-1,3-phenylene ester; Astra Draco AB; [10]} at 10^{-5} and 10^{-7} M, iso-OMPA (Sigma T-1505) at 10^{-4} M, dibucaine {2-butoxy-*N*-[2-(diethylamino)ethyl]-4-quinolinecarboxamide hydrochloride; Sigma; [11]} at 10^{-4} M, and Ro 2–0683 {the dimethyl carbamate of (2-hydroxy-5-phenyl-benzyl) trimethylammonium bromide created by Hoffmann-La Roche; [12, 13]} at 10^{-7} M. For the BW 284C51 inhibitions, the buffer was 0.01 M instead of 0.1 M potassium phosphate, since this peripheral site ligand has a lower dissociation constant in the lower ionic strength buffer [14]. Samples were preincubated with inhibitors for 30 min at 25° before being assayed for activity. An uninhibited plasma control was prepared with every reaction, and all reactions were performed in triplicate.

To determine the relative levels of cholinesterase activity, cat, tiger, and human plasma samples (10 μL in 3-mL reactions) were assayed for activity with 1 mM butyrylthiocholine or 0.5 mM acetylthiocholine [7] in 0.1 M potassium phosphate buffer, pH 8.0, at 25°, in triplicate. Plasma samples were also assayed using 50 μM benzoylcholine as substrate [15] in 0.067 M sodium/potassium phosphate buffer, pH 7.4, at 25°, in triplicate. Benzoylcholine is a specific substrate for BChE but not for AChE. Acetylthiocholine is a substrate for both AChE and BChE, whereas butyrylthiocholine is more specific for BChE but is hydrolyzed weakly by AChE.

RBC membranes from cat and human were assayed for relative activity of AChE, with 0.5 mM acetylthiocholine [7] in 0.1 M potassium phosphate buffer, pH 8.0, at 25°, in triplicate. Membranes were prepared from the packed RBCs [16], and the protein concentration was determined using the Micro BCA Protein Assay Reagent Kit (Pierce). Plasma samples were also assayed for inhibition with 50 μM sodium fluoride [17].

Nondenaturing Gel Electrophoresis

Polyacrylamide gradient gels (4–30%), 0.75 mm thick, with 4% stacking gels, were electrophoresed at 125 V for 24 hr at 4°. The gels were stained for cholinesterase activity [18] with 1.7 mM acetylthiocholine iodide or 2 mM butyrylthiocholine. To identify AChE bands, one gel was preincubated in 30 μM BW 284C51, a specific AChE inhibitor, for 30 min before the addition of acetylthiocholine. To identify BChE bands, another gel was preincubated in 10 μM iso-OMPA, a specific BChE inhibitor, for 30 min before the addition of butyrylthiocholine [19].

Western Blot

Highly purified cat and human BChE were reduced, denatured, and applied in 5-ng quantities to an SDS–polyacrylamide 4–30% gradient gel. After electrophoresis for 24 hr at 125 V, the proteins were transferred to a polyvinyl difluoride membrane (Immobilon-P, Millipore Corp.). The blot was incubated with a polyclonal anti-human BChE antibody in rabbit serum, using 10 μL antibody in 10 mL blocking buffer. This antibody was custom made by Hazleton Research Products (now Covance Research Products Inc.) in rabbits by injecting purified human BChE that had been boiled in 0.1% SDS. The blot was hybridized with a second antibody, goat anti-rabbit IgG conjugated to horseradish peroxidase (Dako P448). Electrochemical luminescence was detected with LumiGLO reagents [54-61-00] from Kirkegaard & Perry. A good signal was obtained after 30 sec of exposure to x-ray film.

DNA Amplification and Sequencing

Genomic DNA was isolated from the white blood cell layer [20]. Cat and tiger cDNA was prepared from anterior pituitary tissue of single animals, using the 3′RACE System from Gibco-BRL. PCR amplification was performed on cat and tiger cDNA templates and human and cat genomic DNA, using a panel of oligonucleotide primers directed against human *BCHE* [21, 22] and human *ACHE* [23, 24] sequences. After obtaining partial cat cDNA sequences, new primers were made for cat and tiger DNA amplifications.

Polymerase chain reaction with *Taq* polymerase was conducted as described by Bartels *et al.* [24]. The exception was cat *ACHE* intron 2, which could be amplified with 1 mM but not with 0.5 or 1.5 mM $MgCl_2$. The cloned amplicon of this DNA was sequenced at 1 mM $MgCl_2$, since it could not be sequenced at the standard 2 mM $MgCl_2$ sequencing reaction concentration.

DNA cycle sequencing was performed using the ABI 373-stretch analyzer with dye-terminator chemistry. All human sequences were amplified and sequenced several times. The polymorphic sites of human *ACHE* were examined in 17 individuals. The cat *ACHE* and *BCHE* and tiger *BCHE* exon sequences were amplified and sequenced at

least twice, to identify potential errors caused by *Taq* polymerase.

Enzyme Kinetics (k_{cat} and K_m)

With purified cat and human BChE, hydrolysis of butyrylthiocholine iodide (Sigma) was tested at 18 concentrations ranging from 0.008 to 25 mM, in 0.1 M potassium phosphate buffer, pH 7.0, at 25° to obtain the K_m and V_{max} values. The K_m and V_{max} values for benzoylcholine (Sigma) were determined from 10 concentrations ranging from 12.5 to 140 μM. To convert V_{max} into k_{cat}, the active sites of purified cat BChE and purified human BChE were titrated with diisopropyl fluorophosphate (Sigma) as described by Leuzinger [25].

RESULTS

Gels

Cat plasma was characterized by comparison with purified human BChE, human serum, fetal bovine serum, and purified cat BChE using nondenaturing polyacrylamide gel electrophoresis (Fig. 1). The gel in Fig. 1A was stained for activity with acetylthiocholine, which is active with both AChE and BChE. Cat plasma showed two bands of AChE activity (marked) and three bands of BChE activity (unmarked). Bengal tiger plasma samples gave bands about the same as those of cat plasma (data not shown). AChE bands could be identified because they were inhibited by the specific AChE inhibitor BW 284C51 (30 μM) but not by the specific BChE inhibitor iso-OMPA (10 μM, data not shown). The major AChE band in cat migrated at about the same level as the strong fetal bovine AChE band. This fetal bovine band has been identified as a tetramer [26]. Fetal bovine serum contains only AChE. Thus, the majority of AChE present in cat plasma is probably tetrameric. The primary BChE band in the human serum and purified human BChE were tetrameric (Fig. 1B). Purified cat BChE migrated at approximately the same position as human BChE and thus appeared to be tetrameric. Human serum showed a small AChE band. Human plasma is known to contain almost no AChE [27]. The single human AChE band contributes 0.2% of the cholinesterase activity in human plasma. Cat plasma, however, was surprisingly rich in AChE, which amounted to 40% of the total cholinesterase activity. The basis for this number will be developed in the next section.

When gels were stained for activity using butyrylthiocholine as the substrate (Fig. 1B), AChE activity was depressed severely, whereas BChE activity was enhanced. Fetal bovine serum AChE stained weakly on the butyrylthiocholine gel. Human serum BChE showed tetramer, trimer, dimer, and monomer bands as well as a band labeled C2. The C2 band is a conjugate between a BChE monomer and albumin [28]. It was visible on both the acetylthiocholine and the butyrylthiocholine gels. Bands located above the primary tetrameric band were due to protein aggregation. The cat plasma showed bands that migrated to positions similar to those of human BChE, including what appeared to be a cat BChE C2 form. Purified cat and human BChE samples showed tetramer plus faint dimer and trimer bands.

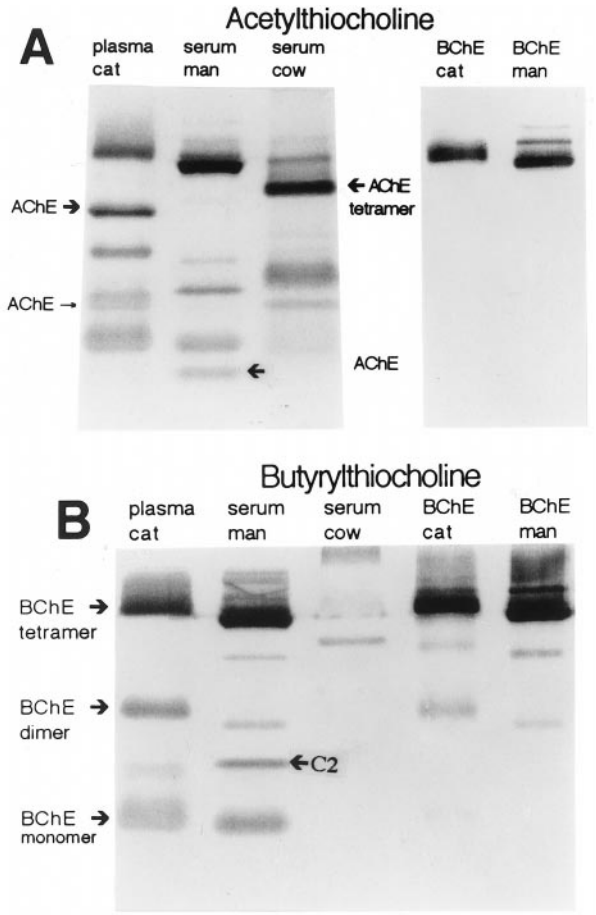

FIG. 1. Nondenaturing gels stained for activity. Lanes contained 5 μL plasma or serum, or 0.015 U (assayed with butyrylthiocholine) of purified BChE. (A) This gel was stained for activity with acetylthiocholine. The unlabeled bands in the cat and human samples are BChE. (B) This gel was stained for activity with butyrylthiocholine. The band labeled C2 consists of a BChE monomer bound to albumin.

On the SDS reducing gel (Fig. 2), purified cat BChE migrated at exactly the same position as the 85-kDa human BChE monomer. The rabbit polyclonal anti-human BChE, prepared against purified and denatured human BChE, reacted strongly against cat BChE.

Relative Abundance of AChE and BChE in Sera

Table 1 presents the proportions of AChE and BChE activity in cat and Bengal tiger plasma. Specific AChE-inhibiting or BChE-inhibiting compounds showed that the cholinesterase activity in cat plasma was 40% AChE and 60% BChE, whereas the cholinesterase activity in Bengal tiger plasma was 23% AChE and 77% BChE. The various inhibitors gave the same proportions of cat AChE and BChE consistently. The general cholinesterase inhibitor

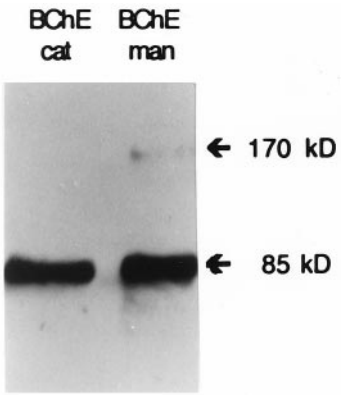

FIG. 2. Western blot. Purified cat and human BChE samples containing 5 ng protein were reduced with mercaptoethanol and denatured in 2% SDS before loading onto an SDS–polyacrylamide gel. The protein was visualized by treating with a polyclonal antibody against purified, denatured human BChE.

eserine inhibited almost all of the cholinesterase present in cat and Bengal tiger plasma. The human plasma (BChE) and fetal bovine serum (AChE) results indicated the relative efficiency and selectivity of each of the inhibitors. The presence of AChE in cat plasma has not been reported previously.

Total cholinesterase activity in cat and tiger plasma was lower than in human plasma (Table 2). The AChE activity of RBC membranes was also lower for cat than for human (Table 2). AChE, but not BChE, is found on RBC membranes. Cat AChE activity was 1.6% that of human. This agrees with the 2% determined by Callahan and Kruckenberg [29] and the 1.3% determined by Zajicek and Datta [30]. For purposes of comparison, we have converted the AChE and BChE activity values into concentrations (mg/L) and presented them in Table 3. The value we obtained for BChE in human serum, 5 mg/L, is equivalent to 60 nM, which is essentially identical to the value of 80 nM reported for human blood [31]. The value we obtained for AChE in packed human RBC membranes, 0.5 mg/L, is equivalent to 7 nM. Considering that packed RBCs take up 40% of the volume of blood, that would make the concentration of AChE equal to 3 nM in whole human blood. This is identical to the value of 3 nM AChE reported for human blood [31].

Enzyme Activity

The K_m and k_{cat} values of purified cat BChE with benzoylcholine were approximately the same as those of human BChE (Table 4). However, cat BChE showed a K_m with butyrylthiocholine that was twice that of human BChE, and a k_{cat} that was 3-fold higher than the human enzyme. Both human and cat BChE were activated by high concentrations of butyrylthiocholine, resulting in a 2- to 3-fold increase in k_{cat}. This substrate activation is measured by the b value and the K_{ss} value. The Bengal tiger BChE was examined to test whether the results from the domestic cat were characteristic of felines in general. The K_m, K_{ss}, and b values of Bengal tiger BChE (plasma), measured with butyrylthiocholine, were the same as those of the purified cat BChE.

The cat and tiger *BCHE* DNA sequences indicated that these enzymes might be resistant to sodium fluoride. When tested, cat and tiger cholinesterase activity in plasma showed no inhibition by 5×10^{-5} M sodium fluoride, a concentration that inhibits wild-type human plasma BChE by approximately 55%, confirming the prediction from the sequence.

Butyrylcholinesterase Sequence

The higher k_{cat} (with butyrylthiocholine) and the faster spontaneous reactivation rate found with cat BChE prompted us to sequence the cat cDNA to determine if there were any amino acid differences near the active site that might have affected the kinetics. There was 88% amino acid identity (504 out of 574) and 90% nucleotide identity (1546 out of 1722) between DNA sequences representing cat and human mature BChE proteins.* There were exactly the same number of amino acids in both the cat and human sequences, so the numbering of the residues for the two proteins is identical. Human BChE has nine asparagine-linked carbohydrates at residue positions 17 (19),† 57 (59), 106 (108), 241 (243), 256 (258), 341 (343), 455 (457), 481 (483), and 486 (488) [34]. The cat DNA sequence showed asparagines at eight of these positions, all with the Asn-X-Thr/Ser sequence required for *N*-linked carbohydrate chain attachment. The missing carbohydrate in cat BChE was at Asn 17, where the n + 2 residue was Pro instead of Thr or Ser. The intrachain and interchain disulfide bond cysteines were all conserved in the cat [C65 (67)–C92 (94), C252 (254)–C263 (265), C400 (402)–C519 (521), and C571 (573)]. The catalytic triad, S198 (200), E325 (327), and H438 (440), was also conserved. Of the 36 amino acids that line the active site gorge of human BChE ([35, 36]; Bartels CF, unpublished observation), 33 were conserved. The three differences were at positions 277, 285, and 398, where the cat BChE had L277, L285, and I398 in place of human A277, P285, and F398. The cat sequence differed from the human sequence in 67 other locations. Fifty of the differences were located on the surface, and as such were not likely to affect the activity at the active site. Thirteen residues were buried in the protein structure, well away from the active site gorge at positions 110 (112), 162 (164), 202 (204), 227 (229), 234 (236), 293 (295), 357 (359), 368 (370), 374 (376), 390 (392), 393

* Butyrylcholinesterase sequence data have been deposited with the GenBank Data Library under Accession Numbers AF053483 (cat *BCHE* cDNA), and AF053484 (Bengal tiger *BCHE* cDNA).

† By agreement at the 3rd and 4th International Conferences on Cholinesterases, all AChE and BChE amino acids generally are defined by the numbering of the *Torpedo californica* (electric fish) AChE sequence. The *T. californica* numbering is given in italics following the specific enzyme amino acid number [33].

TABLE 1. Determination of proportions of AChE and BChE activity in domestic cat and Bengal tiger plasma by use of specific inhibitors

Domestic cat

Inhibitor	% inhibition of cat plasma ChE	% inhibition of fetal bovine serum AChE	% inhibition of human plasma BChE	% BChE in cat plasma	% AChE in cat plasma
General cholinesterase inhibitors					
10^{-5} M eserine	100.0 ± 0	96.0 ± 1.01	99.8 ± 0.4	—	—
Acetylcholinesterase inhibitors					
10^{-7} M BW284C51	38.1 ± 2.7	93.2 ± 0.3	0 ± 1.6	59.6	40.4
10^{-4} M edrophonium	39.5 ± 1.5	95.0 ± 0.5	0.5 ± 6.2	58.7	41.3
Butyrylcholinesterase inhibitors					
10^{-5} M ethopropazine	58.0 ± 2.7	0 ± 3.3	95.9 ± 0.4	60.5	39.5
10^{-7} M bambuterol	61.3 ± 1.9	2.9 ± 2.4	95.5 ± 0.5	63.1	36.9
10^{-5} M bambuterol	62.8 ± 1.2	6.4 ± 0.3	97.7 ± 0.1	61.8	38.2
10^{-4} M iso-OMPA	62.1 ± 2.9	3.4 ± 1.1	98.2 ± 0.4	61.9	38.1
10^{-4} M dibucaine	60.1 ± 1.9	10.0 ± 3.2	95.5 ± 0.1	58.6	41.4
10^{-7} M Ro 2-0683	62.6 ± 2.7	24.4 ± 3.1	97.0 ± 0.2	52.6	47.4

Bengal tiger

Inhibitor	% inhibition of tiger plasma ChE	% inhibition of fetal bovine serum AChE	% inhibition of human plasma BChE	% BChE in tiger plasma	% AChE in tiger plasma
General cholinesterase inhibitors					
10^{-5} M eserine	98.9 ± 1.9	96.0 ± 1.01	99.8 ± 0.4	—	—
Acetylcholinesterase inhibitors					
10^{-7} M BW284C51	17.8 ± 1.9	93.2 ± 0.3	0 ± 1.6	81.4	18.6
10^{-4} M edrophonium	27.8 ± 5.3	95.0 ± 0.5	0.5 ± 6.2	71.1	28.9
Butyrylcholinesterase inhibitors					
10^{-5} M ethopropazine	76.7 ± 2.7	0 ± 3.3	95.9 ± 0.4	80.8	20.0
10^{-7} M bambuterol	73.8 ± 4.3	2.9 ± 2.4	95.5 ± 0.5	77.3	22.7
10^{-5} M bambuterol	76.2 ± 2.3	6.4 ± 0.3	97.7 ± 0.1	78.0	22.0
10^{-4} M iso-OMPA	80.0 ± 1.6	3.4 ± 1.1	98.2 ± 0.4	80.8	19.2
10^{-4} M dibucaine	72.0 ± 0.4	10.0 ± 3.2	95.5 ± 0.1	72.5	27.5
10^{-7} M Ro 2-0683	79.3 ± 1.7	24.4 ± 3.1	97.0 ± 0.2	75.6	24.4

Inhibitors were preincubated with plasma or serum for 30 min before the addition of acetylthiochline to a final concentration of 0.5 mM.

The cholinesterase present in human plasma is almost 100% BChE.

The cholinesterase in fetal bovine serum is 100% AChE.

Example calculation for % of total activity that is BChE in cat plasma after inhibition with iso-OMPA:

$$\frac{62.1-3.4}{98.2-3.4} \times 100 = 61.9\%$$

The error limits refer to standard deviation; assays were run in triplicate.

(395), 468 (470), and 470 (472) The four remaining residues are not included in the model.

One of the buried positions, 390 (392), is worthy of special note. The cat had an Asp at this location in place of the Gly normally found for human BChE. In the naturally occurring fluoride variant of human BChE, Gly390 is changed to Val, resulting in resistance to inhibition by sodium fluoride [37]. This suggested that cat BChE, with Asp at this position, might be resistant to inhibition by sodium fluoride also. Kinetic determination of the sensitivity of cat BChE to fluoride confirmed this suggestion (see the section entitled "Enzyme Activity").

Bengal tiger *BCHE* was also sequenced* to determine if the differences found in cat were a general property of feline *BCHE*. Tiger *BCHE* differed from cat at only 14 nucleotides and three amino acids [Y61H (63), I99S (101), and V162I (164), where the first residue refers to cat and the second to tiger]. This very high degree of identity indicates that there is a general feline *BCHE* genotype. These and other cholinesterase sequences are available through the ESTHER server at *http://meleze.ensam.inra.fr/cholinesterase/*.

* Butyrylcholinesterase sequence data have been deposited with the GenBank Data Library under Accession Numbers AF053483 (cat *BCHE* cDNA), and AF053484 (Bengal tiger *BCHE* cDNA).

TABLE 2. Cholinesterase activity in plasma and red blood cells*

Substrate	Human	Cat	Bengal tiger	Sumatran tiger	Siberian tiger
	Total activity in plasma (U/mL†)				
Butyrylthiocholine	4.41	0.96	0.90	1.07	0.88
Benzoylcholine	0.89	0.09	0.13	0.10	0.10
Acetylthiocholine	2.19	0.69	0.51	0.50	0.53
	Activity in red blood cells (U/mg protein)				
Acetylthiocholine	0.58	0.0088	0.0097	NM‡	NM‡

Standard deviation was 10% or less in each case.

*Assays were performed in triplicate, as described in Materials and Methods.

†A unit of activity is defined as the formation of 1 μmol product per minute, under the selected conditions.

‡NM stands for not measured.

Acetylcholinesterase Sequence

The nucleotide sequence of cat AChE was determined and compared to that of humans. We found a 94% amino acid identity (550 out of 583) and 90% nucleotide identity (1572 out of 1749) between human and cat AChE (tetrameric form) sequences. The three glycosylated asparagines at 265 (258), 350 (343), and 464 (457) were conserved. There were 33 amino acid differences between cat and human AChE. Twenty-six were on the surface and 7 were buried in the protein structure. The 36 amino acids lining the active site gorge, including the catalytic triad, were all conserved, as were the six intrachain and one interchain cysteines.

We sequenced > 4 kb of the cat and human *ACHE* genes, each as a continuous sequence. The sequence began in intron 1, ended in exon 6, and contained the entire protein coding region including both alternatively spliced 3′ exons. The cat *ACHE* sequence* was missing nine nucleotides that would correspond to human amino acids 3, 4, and 5. Because of this difference, and an additional Glu residue in the cat sequence, we are uncertain where the cat AChE signal peptide ends and the mature protein begins. Amino acid alignment with the human sequence showed two possible Glu +1 residues. Asp is a more common +2 residue than Ala; Ala is a more common −3 residue than Gly [38], so the second of the two possible Glu residues is most likely the +1 residue.

Vertebrate ACHE mRNA is alternatively spliced at the 3′ end, encoding either a glycophospholipid-anchored form of the enzyme (exon 4 spliced to exon 5), or a tetrameric form (exon 4 spliced to exon 6). The glycophospholipid-anchored AChE is found on red blood cells. The tetrameric form of AChE is hydrophobically anchored on brain plasma membrane [39], is collagen-bound within neuromuscular synapses, and is also the form found in plasma. There is also the possibility of a "read-through" where intron 4 is not excised but remains in mature mRNA transcripts [40, 41]. The importance of this last transcript is not known. We were able to amplify and sequence both alternatively spliced ACHE transcripts, as well as the intron 4 read-through transcript, from pituitary cDNA. The transcript encoding the tetrameric form of the enzyme was the one most strongly amplified, as expected, since this is the most abundant form of AChE.

Cat *ACHE* exons and introns were approximately the same sizes as their human *ACHE* counterparts, except for intron 3 where a 300-bp *Alu* repeat in the human sequence made human *ACHE* intron 3 longer than cat *ACHE* intron 3. Our sequencing showed a polymorphism (g/a) 316 nucleotides downstream from the human *ACHE* exon 3/intron 3 junction. Within a 17-person sample, the frequency of the allele with guanidine was 0.62, and with adenosine was 0.38. There was a silent polymorphism at cat Y116 (TAT/TAC) in exon 2.

TABLE 3. Amount of AChE and BChE in human and cat blood

	Human plasma, mg/L	Human RBC, mg/L packed membranes	Cat plasma, mg/L	Cat RBC, mg/L packed membranes
AChE	< 0.01	0.5	0.3	0.008
BChe	5	0	0.5	0

BChE concentration in plasma was calculated from the measured benzoycholine activity (U/mL) and the specific activity of pure BChE with benzoylcholine (200 U/mg BChE, with 50 μM benzoylcholine). The benzoylcholine activity was used since benzoylcholine is specific for BChE. The concentration of AChE in cat plasma was calculated from the concentration of BChE and the fact that 40% of the total cholinesterase activity in cat plasma is attributable to AChE. The AChE concentration in RBC membranes was calculated from the measured acetylthiocholine activity (U/μg RBC membrane protein), the concentration of protein in packed RBC membranes (5 mg/mL), and the specific activity of pure AChE with acetylthiocholine (5800 U/mg AChE, with 0.5 mM acetylthiocholine). Assays were performed in triplicate, as described in Materials and Methods. The standard deviation was 10% or less in each case.

DISCUSSION

We have determined the nucleotide sequences of both *ACHE* and *BCHE* for domestic cat, and we have determined the nucleotide sequence of *BCHE* for Bengal tiger. With this information, we are in a position to address questions regarding the structure and activity of cat and tiger AChE and BChE on an amino acid level. Furthermore, by comparing this information with similar data from human BChE and AChE we can obtain a better insight into the mechanisms that control the activity of both enzymes.

* Acetylcholinesterase sequence data have been deposited with the GenBank Data Library under Accession Numbers AF053485 (>4 kb of continuous cat genomic DNA containing all of the AChE protein coding sequence in exons 2–6), and L42812 (human *ACHE* genomic DNA, including exons 2–6).

TABLE 4. Kinetic values of cat, human, and tiger BChE

	Cat purified	Human purified	Tiger plasma
Butyrylthiocholine			
K_m	44 ± 2 μM	23 ± 3 μM	44 ± 4.9 μM
K_{ss}	3.0 ± 0.3 mM	1.4 ± 0.2 mM	2.6 ± 0.4 mM
b	2.1 ± 0.04	2.5 ± 0.2	2.3 ± 0.09
k_{cat}	110,000 ± 3000 min^{-1}	34,000 ± 2000 min^{-1}	—
$b \cdot k_{cat}$	230,000 ± 6000 min^{-1}	88,000 ± 5000 min^{-1}	—
Benzoylcholine			
K_m	8.4 ± 0.4 μM	7.7 ± 0.4 μM	—
k_{cat}	13,600 ± 100 min^{-1}	18,000 ± 700 min^{-1}	—

K_m, K_{ss} and b values were calculated by nonlinear computer fitting of the following equation [32] using Sigma Plot (Jandel Scientific)

$$\upsilon = \frac{k_{cat}\, e_0\, (1 + b[S] / K_{ss})}{(1 + K_m/[S])\,(1 + [S]/K_{ss})}, \text{ where}$$

υ is the measured velocity of the enzyme reaction,

K_m is the substrate concentration that gives half maximal velocity (= the Michaelis constant),

K_{ss} is the binding constant for the binding of a second substrate molecule to an enzyme–substrate complex,

k_{cat} is the catalytic constant ($= V'_{max}/e_0$) where V_{max} is the maximal velocity determined from low substrate concentrations and e_0 is the number of enzyme active sites,

b is the value for the efficiency of product formation by the ternary substrate–enzyme–substrate complex in the presence of excess substrate; $b < 1$ indicates substrate inhibition, $b > 1$, substrate activation, and

$b \cdot k_{cat}$ is the maximum catalytic activity.

Error limits refer to standard deviation; assays were run in triplicate.

BChE Sequence and Mechanism

Between cat and tiger BChE there were only three amino acid differences, none of which was in the active site gorge of the enzymes. These enzymes, therefore, can be considered to be essentially identical. This identity is reflected in the identical butyrylthiocholine steady-state turnover kinetics found for these two enzymes (Table 4).

There are, however, notable differences between cat BChE and human BChE. These differences can be found in the steady-state kinetics of butyrylthiocholine turnover, i.e. cat BChE turned over butyrylthiocholine 3 times faster than human BChE, with 2-fold higher K_m and K_{ss} values (Table 4). These kinetic differences are necessarily the consequence of amino acid differences between cat and human BChE. The amino acid sequence of cat BChE differed from that of human BChE at 70 positions. Three of those differences were located in the active site gorge. Though an amino acid change anywhere in the BChE structure could, in principle, lead to a conformational change that might affect the kinetics, for example, the effect of the residue at position 390 on the sensitivity of BChE to fluoride inhibition, amino acid changes in the active site gorge are the most likely candidates. Two of the active site gorge mutations were located near the mouth of the gorge: Ala 277 (279), which is Leu in cat BChE, and Pro 285 (287), which is Leu in cat BChE. Position 277 is an important component of the peripheral anionic site in *Torpedo* and human AChE, where it is a Trp. The third mutation, Phe 398 (400), was located near the bottom of the gorge and is Ile in cat BChE. We suggest that the kinetic differences may all be attributed to the amino acid changes at these three positions. Mouse BChE also differs from human BChE at positions 277, 285, and 398, where mouse BChE has Arg 277, Ile 285, and Ile 398 [42].

Another interesting situation occurs at position 390 in the BChE sequence. Normal human plasma BChE is inhibited 55% by 5×10^{-5} M sodium fluoride, but the naturally occurring G390V variant of human BChE is inhibited only 36% by this concentration of fluoride [37, 43]. This indicates that sensitivity to fluoride inhibition is dependent upon the residue in position 390. We found that neither cat nor tiger BChE was inhibited at all by 5×10^{-5} M sodium fluoride. Both the cat and tiger BChE contained an Asp at position 390. Therefore, it is reasonable to propose that D390 (392) is the source of resistance to fluoride inhibition in cat and tiger BChE. Of the completed mammalian BChE sequences (mouse [42], rabbit [44], horse [45], cat, tiger, and human [21]), only the human sequence contains Gly at position 390. All others contain Asp. Our results predict that mouse, rabbit, and horse BChE will not be inhibited by fluoride. Horse BChE (along with monkey, guinea pig, and dog) has been shown to be resistant to fluoride inhibition [46], confirming the prediction. Conversely, one would predict that the BChE of monkey, guinea pig, and dog will contain Asp in position 390, since they are resistant to fluoride inhibition. Furthermore, the finding that human BChE appears to be unique in containing Gly at position 390 bears on the practice of adding sodium fluoride to human blood samples to inhibit cocaine hydrolysis during storage. It suggests that this method of inhibition may be feasible only with human blood.

For the most part, the sequences of human and cat BChE

were identical (88%). This suggests that the overall structure of the two enzymes is the same. This expectation is consistent with the gel electrophoresis results (Figs. 1 and 2). The distribution of forms that cat BChE can adopt in serum was essentially identical to those of human BChE. Slight differences in migration between cat and human BChE on nondenaturing gel electrophoresis can be attributed to differences in glycosylation, since cat BChE has one less glycosylation site.

AChE in Cat Plasma

Another significant difference between blood cholinesterase of cat and human was the presence of a substantial proportion of AChE activity in cat serum. Since the human case is often taken as the norm, it is important to emphasize the occurrence of AChE in the serum whenever it constitutes a significant fraction of the total.

Polymorphism

In addition to the differences in the primary sequences of cat and human AChE, we found a polymorphism in intron 3 of human *ACHE* and a silent polymorphism at Y116 in cat AChE. Reports of polymorphisms in AChE are rare. Only five other polymorphisms have been found. All five are in human *ACHE*. Three are in exons: H322N (315), P446P (439), and P561R *(h)* [24]. Two are in retained intron 5, a 3-bp deletion near the midpoint of the intron, and a c/t polymorphism 10 bp before the junction with exon 6 [47]. At all five sites, the cat sequence was identical to the more frequent human sequence, H322, P446 (CCC), and P561 of the exons, and the undeleted three bases and c at the −10 position of the human retained intron.

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