Bovine acetylcholinesterase: cloning, expression and characterization

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The bovine acetylcholinesterase (BoAChE) gene was cloned from genomic DNA and its structure was determined. Five exons coding for the AChE T-subunit and the alternative H-subunit were identified and their organization suggests high conservation of structure in mammalian AChE genes. The deduced amino acid sequence of the bovine T-subunit is highly similar to the human sequence, showing differences at 34 positions only. However, the cloned BoAChE sequence differs from the published amino acid sequence of AChE isolated from fetal bovine serum (FBS) by: (1) 13 amino acids, 12 of which are conserved between BoAChE and human AChE, and (2) the presence of four rather than five potential N-glycosylation sites. The full coding sequence of the mature BoAChE T-subunit was expressed in human embryonal kidney 293 cells (HEK-293). The catalytic properties of recombinant BoAChE and its reactivity towards various inhibitors were similar to those of the native bovine

enzyme. Soluble recombinant BoAChE is composed of monomers, dimers and tetramers, yet in contrast to FBS-AChE, tetramer formation is not efficient. Comparative SDS/PAGE analysis reveals that all four potential N-glycosylation sites identified by DNA sequencing appear to be utilized, and that recombinant BoAChE comigrates with FBS-AChE. A major difference between the recombinant enzyme and the native enzyme was observed when clearance from circulation was examined. The HEK-293-derived enzyme was cleared from the circulation at a much faster rate than FBS-AChE. This difference in behaviour, together with previous studies on the effect of post-translation modification on human AChE clearance [Kronman, Velan, Marcus, Ordentlich, Reuveny and Shafferman (1995) *Biochem. J.* 311, 959–967] suggests that cell-dependent glycosylation plays a key role in AChE circulatory residence.

INTRODUCTION

Acetylcholinesterases (AChEs; EC 3.1.1.7) play a key role in termination of transmission at synapses, and are the major target for organophosphorus intoxication. AChEs have also been identified in a variety of non-cholinergic loci, where their function is not yet fully understood (reviewed in [1]). In vertebrates, AChE is encoded by a single gene, yet the enzyme occurs in various tissues in multiple molecular forms. Polymorphism is achieved through alternative splicing, generating two catalytic subunits with different C-termini (H-subunits and T-subunits) and through formation of homo-oligomeric or hetero-oligomeric structures associated with non-catalytic subunits (20 kDa subunit collagen tail).

The architecture of AChEs was originally investigated through analysis of molecular forms isolated from different tissues of fish and mammalian origin [2–10]. In recent years, further insight into AChE structure was gained by cloning AChEs from various species [11–14] and expressing functional enzyme subunits in tissue-culture systems. Expression of the human [15,16], mouse [17,18] and rat [19] AChEs in recombinant systems allowed for a thorough analysis of the architecture as well as the catalytic function of the enzymes. Furthermore, the ability to generate large quantities of human recombinant enzyme in human embryonal kidney 293 cells (HEK-293) [20], together with mutagenesis studies dissecting the interactions of this enzyme with its various ligands [21–26], has set a basis for the use of human AChE (HuAChE) as a therapeutic agent in organophosphorus poisoning. However, the use of recombinant HuAChE (rHuAChE) as a biological scavenger is complicated by its rapid clearance from the circulation [27].

One of the more intriguing forms of naturally occurring AChE is the soluble T-subunit tetramer found in fetal bovine serum

(FBS) [7]. The existence of such a form in the circulation of the fetus raises questions related to its biological function, the site and regulation of its synthesis, and its interrelation with butyrylcholinesterase, which appears in the serum of adult mammals in a very similar molecular form (reviewed in [28]). Most importantly, the structural features which allow its long-term residence in the bloodstream remain to be elucidated.

The amino acid sequences of the bovine acetylcholinesterase (BoAChE) T-subunit isolated from fetal serum [29], as well as those of a few peptides derived from the bovine brain enzyme [30], have already been determined. Nevertheless, further elucidation of the unique features of BoAChE requires cloning and establishment of appropriate expression systems.

To this end, we isolated the bovine AChE gene, determined its coding sequences and expressed the corresponding T-subunit in HEK-293 cells. This provided a recombinant mammalian AChE for which a native soluble counterpart is readily available, and allowed for comparison of the enzymic, structural and pharmacokinetic features of recombinant BoAChE (rBoAChE) with those of FBS-AChE. We found that the recombinant enzyme, like the native enzyme, forms soluble oligomers, carries four glycosylation sites and displays the expected kinetic properties, yet, in contrast with the native FBS-derived enzyme, the recombinant enzyme is cleared rapidly from the circulation.

EXPERIMENTAL

Cloning of the BoAChE gene

The coding sequence of the BoAChE gene was determined through PCR amplification of bovine genomic adult male kidney DNA and based on two independent clone isolates for each

Abbreviations used: AChE, acetylcholinesterase gene; AChE, acetylcholinesterase; BoAChE, bovine acetylcholinesterase; CMV, cytomegalovirus; FBS-AChE, fetal bovine serum acetylcholinesterase; HEK-293, human embryonal kidney 293 cells; HuAChE, human acetylcholinesterase; ChE, cholinesterase; rBoAChE, recombinant bovine acetylcholinesterase; rHuAChE, recombinant human acetylcholinesterase; WT, wild-type.

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Figure 1 Strategy for cloning and expression of bovine *AChE*

(a) Map of the bovine *AChE* gene as deduced from DNA sequencing. (b) DNA fragments used for cloning of the bovine *AChE* gene (see text for details). (c) Plasmid pBo2346 carrying the four exons coding for BoAChE T-subunit. (d) The tripartite expression vector pBoAChE-nc carrying the bovine *AChE*, *neo* and *cat* genes.

amplified DNA fragment. Primers designed to isolate the various exons were based on the similarity in mammalian *AChEs*. Amplifications were done using the Expand High Fidelity PCR system (Boehringer Mannheim) in the presence of 1 mM $MgCl_2$. This system utilizes both Taq and Pwo polymerases and is designed to give PCR products with high fidelity. Preliminary denaturation was carried out at 95 °C for 3 min followed by 35 cycles consisting of a 45 s denaturation period at 95 °C, a 45 s annealing period at 55 °C and a 3 min extension period at 68 °C. The reaction was terminated by a 7 min extension period at 68 °C.

Six amplified DNA fragments (Figure 1b) were used to determine the bovine AChE sequences: DNA fragments that contain the C-terminal part of exon 2 (fragment I), as well as the entire exons 3 (fragment II) and 6 (fragment III) were each amplified using degenerate primers derived from the published FBS-AChE amino acid sequence and subcloned separately into pGEM-T (Promega). To determine the sequences at boundaries of exons 2 and 3, two additional fragments were amplified and cloned: the N-terminal part of exon 2 (fragment IV) and intron 2 (fragment V). In order to clone exons 4 and 5 and to establish the boundaries of exons 3 and 6, a ~ 2.6 kb fragment (fragment

VI) that spans from the end of exon 3 to the beginning of exon 6 was also amplified.

Construction of expression vector

The contiguous sequence coding for subunit-T of BoAChE was generated by joining exons 2, 3, 4 and 6, through the use of appropriate synthetic DNA linkers. To construct the expression vector for BoAChE the coding sequences of mature bovine enzyme were excised from the pBo2346 plasmid by *BspEI*–*SalI*, linked to a synthetic DNA duplex coding the signal peptide of human AChE and ligated into *EcoRV*–*SalI* of the pHuAChE-nc plasmid [31]. This resulted in the formation of pBoAChE-nc (Figure 1d), which drives BoAChE transcription by the cytomegalovirus (CMV) promoter and carries the *neo* and *cat* genes as markers.

Sequencing

DNA sequences were determined with the ABI prism rhodamine terminator reaction kit using the ABI310 Genetic Analyzer (Applied Biosystems). Each of the individual exons was sequenced on both strands after subcloning. The final sequence

of BoAChE T-subunit was verified again using expression vector pBoAChE-nc. Sequencing was performed on two independent isolates for each of the subclones, as well as for the final construct. Sequences of exons 2, 3, 4 and 6 were deposited in the GenBank database under accession numbers AF061813, AF061814, AF061815 and AF061816 respectively.

Expression, production and purification of BoAChE and HuAChE mutants

HEK-293 cells were transfected by the pBoAChE-nc plasmid using a modification [21] of the calcium phosphate procedure [32]. Stably transfected cell pools were generated by G418 selection [16], and cultivated in medium containing 10% (v/v) FBS from which endogenous AChE was depleted, as described previously [15]. BoAChE secretion was monitored by measuring enzymic activity (see below) in cell medium. Enzyme was harvested by collecting medium every 24 h for 3 days.

BoAChE was purified on procainamide columns as previously described [27]. The same purification procedure was used to produce native FBS-AChE derived from calf serum (Biological Industries, Bet Haemek, Israel), recombinant HuAChE and the glycosylation mutants [27] HuAChE D61N and HuAChE D61N/S541N.

AChE activity

AChE activity was measured according to the method of Ellman et al. [33]. Standard assays were performed in the presence of 0.5 mM acetylthiocholine, 50 mM sodium phosphate buffer, pH 8.0, 0.1 mg/ml BSA and 0.3 mM 5',5'-dithiobis-(2-nitrobenzoic acid). The assay was carried out at 27 °C and monitored using a Thermomax microplate reader (Molecular Devices).

The concentration of enzyme subunits was determined by active-site titration. Various amounts of the potent phosphonate inhibitor, soman, were added to enzyme solutions in the presence of 0.1 mg/ml BSA in 50 mM sodium phosphate buffer, pH 8.0. Inhibition was allowed to proceed to completion and residual activity was plotted against concentration of inhibitor.

K_m values of BoAChE for acetylthiocholine were obtained from Lineweaver–Burk plots and k_{cat} calculations were based on active-site titration. Interactions of BoAChE with the AChE-specific inhibitors edrophonium, propidium and BW284C51 were analysed as described previously [23].

Sucrose-density-gradient centrifugation and gel electrophoresis

Analytical sucrose-density-gradient centrifugation was performed on 5–25% sucrose gradients containing 0.1 M NaCl/50 mM sodium phosphate buffer, pH 8.0. Centrifugation was carried out in an SW41 Ti rotor (Beckman) for 26 h at 160 000 g. Fractions of 0.3 ml were collected and assayed for AChE activity. Alkaline phosphatase was used as a sedimentation marker.

Purified enzyme was subjected to 0.1% (v/v) SDS/7.5% polyacrylamide gel analysis [34]. Where noted, samples were digested before loading with 0.6 units of *N*-glycanase (Genzyme, Cambridge, MA, U.S.A.) at 37 °C, as reported previously [27]. Bands were visualized by Coomassie Blue staining or by Western blotting with antibodies to rHuAChE, as previously described [35].

In vivo clearance experiments

Clearance experiments in mice were performed as previously described [27]. Purified BoAChE (20 µg) or HuAChE (20 µg) was injected into the tail vein of each mouse (male, ICR strain,

25–30 g). Samples of blood (5 µl) were removed at various intervals, diluted and monitored for AChE activity. All values were corrected for background activity. Blood AChE values 1 min after injection were referred to as input activity. The clearance patterns of the enzyme in the circulation were fitted to a bi-exponential elimination pharmacokinetics model [27].

RESULTS

Cloning of the BoAChE gene

Bovine kidney genomic DNA was used to clone the BoAChE gene. Access into the various exons was gained by primers directed towards regions which display high conservation in amino acid sequence between FBS-AChE and HuAChE, as well as high conservation in DNA sequence between human and mouse *ACHE* genes. These primers allowed sequencing and identification of authentic bovine *ACHE* sequences. Based on the derived sequences, new primers were designed to confirm genomic sequences of regions spanning the primers used for the initial cloning.

Altogether, six DNA fragments from the bovine genome were amplified and cloned (Figure 1b), and for each PCR-amplified fragment two independent clones were isolated. These fragments were used to determine the putative amino acid sequence of the catalytic subunits of BoAChE (see below) and to delineate the organization of the bovine gene (Figure 1a). A strong resemblance in the organization of the BoAChE gene to its human and mouse counterparts [36] was revealed. The structure and the size of all exons and introns (0.35, 1.2, 0.13 and 0.67 kb for introns [37] 2, 3, 4 and 5 respectively) appear to be similar to those of other mammalian AChE genes.

Amino acid sequence of the BoAChE T-subunit

The significant similarity in gene structure and amino acid sequence of mammalian AChE, together with the identification of typical splicing signals on the bovine gene (not shown), allowed us to deduce the putative amino acid sequence of the mature T-subunit of the bovine AChE (Figure 2a). The sequence for each exon is based on 2–4 independent PCR-derived clones. Comparison of this sequence with the published amino acid sequence of FBS-AChE [29] reveals differences at 13 positions. At two of these divergent positions, Arg-16 and Ser-541, the BoAChE sequence is in agreement with the sequence determined from selected peptides of bovine brain AChE [30]. Arg-16 was also identified in erythrocyte bovine AChE [30] and Ser-541 was confirmed by sequencing of DNA coding for the bovine H-subunit C-terminus [38]. Four putative glycosylation sites are deduced from the rBoAChE T-subunit sequence (Figure 2a). Side-chains could be attached to asparagines at positions 61, 265, 350 and 464. It should be noted that in the published FBS-AChE sequence, position 541 is also occupied by an asparagine residue, and thereby should contribute to the formation of a fifth glycosylation sequon (Asn-Ala-Thr). As noted above, no evidence for the presence of asparagine at this position is provided by the rBoAChE sequence.

The signal peptide of BoAChE (Figure 2b) resembles in sequence its murine and human counterparts and displays the characteristic features of mammalian signal peptides, namely the presence of a hydrophobic core and of alanine residues at positions –1 and –3 ([16] and references cited therein).

The sequence between exons 4 and 6 also allowed the identification of the putative coding sequence for the alternative exon 5. This sequence (Figure 2c) is in full agreement with the

a. Mature T - subunit

FBS AChE	EGPEDPELLV MVRGGRLRGL RLMAPRGVPS AFLGIPFAEP FVGPRRFLPP	50
BoAChE	EGPEDPELLV MVRGGRLRGL RLMAPRGVPS AFLGIPFAEP FVGPRRFLPP	
HuAChE	EGPEDPELLV MVRGGRLRGL RLMAPRGVPS AFLGIPFAEP FVGPRRFLPP	
FBS AChE	EPKRFWPGVL NATAFQSVCY QYVDLYPGF EGTEMWNPNR ELSIEDCLYLN	100
BoAChE	EPKRFWPGVL NATAFQSVCY QYVDLYPGF EGTEMWNPNR ELSIEDCLYLN	
HuAChE	EPKRFWPGVL NATAFQSVCY QYVDLYPGF EGTEMWNPNR ELSIEDCLYLN	
FBS AChE	VMTFYPSPSS PTPVLVMIYG GGFYSGASSL DVIDGRFLTQ AEGTVLVSMN	150
BoAChE	VMTFYPSPSS PTPVLVMIYG GGFYSGASSL DVIDGRFLTQ AEGTVLVSMN	
HuAChE	VMTFYPSPSS PTPVLVMIYG GGFYSGASSL DVIDGRFLTQ AEGTVLVSMN	
FBS AChE	YRVGAFGFLA LPGSREAPGN VGLLDQRLAL QWQENVAAF GGDPTSVTLF	200
BoAChE	YRVGAFGFLA LPGSREAPGN VGLLDQRLAL QWQENVAAF GGDPTSVTLF	
HuAChE	YRVGAFGFLA LPGSREAPGN VGLLDQRLAL QWQENVAAF GGDPTSVTLF	
FBS AChE	GESAGAAVSG MHLSPSPSRG LFHRAVLQSG APNGPWATVG VGEARRRATL	250
BoAChE	GESAGAAVSG MHLSPSPSRG LFHRAVLQSG APNGPWATVG VGEARRRATL	
HuAChE	GESAGAAVSG MHLSPSPSRG LFHRAVLQSG APNGPWATVG VGEARRRATL	
FBS AChE	LARLVGCPPG GAGNDTBLV ACLRARPAQD LVDHEWRVLP QESVFRFSFV	300
BoAChE	LARLVGCPPG GAGNDTBLV ACLRARPAQD LVDHEWRVLP QESVFRFSFV	
HuAChE	LARLVGCPPG GAGNDTBLV ACLRARPAQD LVDHEWRVLP QESVFRFSFV	
FBS AChE	PVVDGDFLSD TPEALINAGD FHGLQVLGV VKDEGSYFLV YGAPGFSKDN	350
BoAChE	PVVDGDFLSD TPEALINAGD FHGLQVLGV VKDEGSYFLV YGAPGFSKDN	
HuAChE	PVVDGDFLSD TPEALINAGD FHGLQVLGV VKDEGSYFLV YGAPGFSKDN	
FBS AChE	ESLISRAQFL AGVRVGVPAQ SDLAEEAVLV HYTDWLHPED PARLREALSD	400
BoAChE	ESLISRAQFL AGVRVGVPAQ SDLAEEAVLV HYTDWLHPED PARLREALSD	
HuAChE	ESLISRAQFL AGVRVGVPAQ SDLAEEAVLV HYTDWLHPED PARLREALSD	
FBS AChE	VVGDHNVVCP VAQLAGRLAA QGARVYAYIF EHRASTLSWP LMMGVPHGYE	450
BoAChE	VVGDHNVVCP VAQLAGRLAA QGARVYAYIF EHRASTLSWP LMMGVPHGYE	
HuAChE	VVGDHNVVCP VAQLAGRLAA QGARVYAYIF EHRASTLSWP LMMGVPHGYE	
FBS AChE	IEFIFGLPLE PSNLNTIEER TFAQLRMRYW ANFARTGDPN DPRDPKAPQW	500
BoAChE	IEFIFGLPLE PSNLNTIEER TFAQLRMRYW ANFARTGDPN DPRDPKAPQW	
HuAChE	IEFIFGLPLE PSNLNTIEER TFAQLRMRYW ANFARTGDPN DPRDPKAPQW	
FBS AChE	PPYTAGAQYQ VSLNLRPLEV RRGRLAQACA FWNRFPLKLL SATDTLDEAE	550
BoAChE	PPYTAGAQYQ VSLNLRPLEV RRGRLAQACA FWNRFPLKLL SATDTLDEAE	
HuAChE	PPYTAGAQYQ VSLNLRPLEV RRGRLAQACA FWNRFPLKLL SATDTLDEAE	
FBS AChE	RQWKAEPFRW SSMVMHWKQV FDHYSKQDRC SDL*	583
BoAChE	RQWKAEPFRW SSMVMHWKQV FDHYSKQDRC SDL*	
HuAChE	RQWKAEPFRW SSMVMHWKQV FDHYSKQDRC SDL*	

b. Signal peptide

BoAChE	MRPPWCPLHTPSLTPTPLLLLL FLLGGGAAE
HuAChE	MRPPQCLHTPSLTASPLLLLLWLLGGGVGA
MoAChE	MRPPWYPLHTPSLTAPPLLLLLSLGGGARA

c. Exon 5 - C-terminus of the H-subunit

BoAChE	ASEAPCTCSGPAHGEAAPPRLPGLPLPILLLLFLLL SRIILRL*
HuAChE	ASEAPSTCPGFTGHEAARRP GLPLPILLHQLLLFLSHLRRL*
MoAChE	ATEAPCTCPSPAHGEAAPPRL GPDLAISLLFLLFLH SGLPWL*

Figure 2 Amino acid sequence of BoAChE: comparison with other AChEs

The mature T-subunit sequence (a) was compared with the FBS amino acid sequence [29] and with the human enzyme sequence [12]. The signal peptide (b) and the C-terminus of the H-subunit (c) were compared with the corresponding regions of the mouse and human enzymes. Gaps were introduced to allow maximal alignment. An asterisk denotes a stop codon.

previously published sequence of this exon [38]. Comparison with the mouse and bovine counterparts suggests that the selective pressure for conservation of this exon in mammals is not as strong as for the other exons (60–70 % identity for exon 5 versus close to 95 % for the other exons)

Expression of BoAChE T-subunit in HEK-293 cells

Exons 2, 3, 4 and 6 were assembled to generate a continuous open reading frame coding for the BoAChE T-subunit (Figure

1c), as described in the Experimental section. This sequence, which lacks the original BoAChE signal peptide sequence, was linked to the signal sequence of HuAChE [12] to allow for enzyme secretion from cells. The hybrid sequence was then introduced downstream from the CMV promoter to generate a tripartite expression vector (pBoAChE-nc; Figure 1d), resembling vectors used by us previously for efficient expression of HuAChE [15,16,22]. After transient transfections of HEK-293 cells by this vector, AChE activity could be recovered in the cell medium after 24 h. The amount of enzyme retained in the cell was negligible, indicating that, like rHuAChE [39], rBoAChE T-subunits are produced in HEK-293 cells in a soluble and secreted form. Treatment of the transfected cells with G418 yielded a stable cell pool expressing about 15 mg·24 h⁻¹·l⁻¹ rBoAChE. These high levels of expression are comparable with those reached for rHuAChE production in this cell line and are probably achieved through the unique features of the tripartite vector used in these systems [16].

Enzymic characterization of rBoAChE

Quantification of rBoAChE produced by transfected HEK-293 cells was carried out by measurements of enzyme activity and active-site titration. Interestingly, in spite of the strong similarity in sequences, antibodies to the human enzyme failed to detect rBoAChE in a capture ELISA, even though these same antibodies interacted with the enzyme on a Western blot. One should note that the interaction of the denatured BoAChE band with anti-human AChE antibodies was as efficient as the interaction of these antibodies with the homologous human AChE (see Figure 4b). Kinetic constants determined for rBoAChE are displayed in Table 1. The K_m and the k_{cat} values of the recombinant bovine enzyme are indistinguishable, within experimental error, from those of the native FBS-AChE, as well as from those of the recombinant human enzyme. Likewise, inhibition constants (K_i) for the active-site inhibitor edrophonium, the peripheral anionic-site inhibitor propidium, and the bis-quaternary inhibitor BW284C51 are very similar for the three enzymes (Table 1).

Biochemical characterization of soluble rBoAChE

The molecular forms detected by sucrose-density-gradient centrifugation of rBoAChE correspond to tetramers, dimers and monomers (Figure 3a). Analysis of several preparations indicates that only 2–7 % of the enzyme preparations consists of tetramers. On the other hand, native FBS-AChE, submitted to the same analysis, sediments as a distinct tetramer (Figure 3b), as shown previously [7]. The sedimentation profile of rBoAChE actually resembles that of rHuAChE produced in the same cells [15,40]. This observation, together with studies on other recombinant cholinesterases [41–44], suggests that cell lines cannot duplicate the efficient tetramerization of native enzymes (see Discussion).

Table 1 Comparison of reactivities of rBoAChE, FBS-AChE and rHuAChE towards substrate and inhibitors

Type of AChE	K_m (mM)	$10^5 \times k_{cat}$ (min ⁻¹)	$10^8 \times K_{app}$ (M ⁻¹ ·min ⁻¹)	K_i	$10^6 \times$ Edrophonium (M)	$10^6 \times$ Propidium (M)	$10^9 \times$ BW284C51 (M)
rBoAChE	0.14	4.0	29	0.8	1.9	18	
FBS-AChE	0.13	3.8	30	0.5	1.4	19	
rHuAChE	0.14	4.2	30	0.7	1.4	16

Figure 3 Sucrose-density-gradient centrifugation profiles of rBoAChE and native FBS-AChE

rBoAChE (A) and FBS-AChE (B) in similar amounts (~ 50 ng, 0.3 units) and at a concentration of ~ 500 ng/ml, were loaded on sucrose gradients. After centrifugation, 0.2 ml fractions were collected and examined for enzymic activity. An arrow denotes sedimentation of alkaline phosphatase. AChE molecular forms are marked as G4 (tetramers), G2 (dimers) and G1 (monomers).

Analysis of rBoAChE by SDS/PAGE reveals a ladder (Figure 4a) composed of three major bands of about 72, 70 and 68 kDa. Native FBS-AChE migrates as a 72 kDa band, accompanied by a faint 70 kDa band. When these two preparations were treated with *N*-glycanase to remove N-linked oligosaccharide side-chains, both migrated as single bands and had the same size, ~ 64 kDa (Figure 4a). All these bands are authentic AChE derivatives, as indicated by the fact that they can be visualized by antibodies directed against rHuAChE in a Western blot (Figure 4b). These observations suggest that the recombinant BoAChE T-subunit resembles the native subunit in the net size of the polypeptide chain and that a significant fraction of the molecules in the recombinant enzyme preparation carry the same number of sugar side-chains as the native enzyme. It should be noted, however, that heterogeneity in glycosylation is higher in the recombinant bovine enzyme and probably results from partial utilization of some of the glycosylation sites, as previously suggested for the human enzyme [31].

To further evaluate the extent of rBoAChE glycosylation we have used rHuAChE mutants carrying varying numbers of N-linked sugar side-chains engineered by us previously [27]. Comparative SDS/PAGE analysis (Figure 4c) of rBoAChE and rHuAChE molecules carrying three [wild-type (WT)], four (Asp-61 $\rightarrow$ Asn) and five (Asp-61 $\rightarrow$ Asn/Ser-541 $\rightarrow$ Asn) N-linked glycans reveals that rBoAChE bands migrate faster than the pentaglycosylated rHuAChE, slower than the WT triglycosylated enzyme and very close to the tetraglycosylated rHuAChE molecule. This indicates that the bovine enzyme does in fact carry four functional N-glycosylation sites, as suggested by the updated sequence (Figure 2a).

Figure 4 SDS/PAGE analysis of rBoAChE, FBS-AChE and rHuAChE derivatives

(a) rBoAChE and FBS-AChE before or after treatment with *N*-glycanase (Coomassie Blue staining). (b) rBoAChE, FBS-AChE and rHuAChE visualized by Western blotting with antibodies to rHuAChE. (c) rBoAChE as well as tri-glycosylated (rHuAChE-WT), tetra-glycosylated (rHuAChE-Asp-61 $\rightarrow$ Asn) and penta-glycosylated (rHuAChE-Asp-61 $\rightarrow$ Asn/Ser-541 $\rightarrow$ Asn) HuAChE derivatives (Coomassie Blue staining; one-letter symbol used for amino acids). Migration of prestained (New England Biolabs, Inc.) molecular-mass markers (kDa) is denoted by arrows.

Figure 5 Time course of rBoAChE clearance from the circulation: comparison with FBS-AChE and tetra-glycosylated rHuAChE

Purified enzyme preparations were administered to mice (20 µg/mouse in 0.2 ml). AChE activity values in sample removed immediately after injection were assigned a value of 100% and used for calculation of residual activity. Enzymes used in this experiment were rBoAChE (○), tetraglycosylated rHuAChE (●) and FBS-AChE (▲). Background cholinesterase levels (serum pseudocholinesterase and erythrocyte acetylcholinesterase) in blood of untreated mice is less than 2 units/ml. Exogenous AChE was introduced at levels that are at least 30-fold higher than the background level.

Circulatory residence of soluble rBoAChE

To evaluate the pharmacokinetics behaviour of rBoAChE, purified enzyme was administered to mice and enzyme levels in the bloodstream were determined at different time intervals. The clearance profile can be described by a typical biphasic curve (Figure 5), as has been found previously for other cholinesterases [27,45,46]. In contrast to its native counterpart, the recombinant enzyme produced in HEK-293 cells was cleared rapidly from the circulation (Figure 5). The half-life for the rapid phase ($t_{1/2\alpha}$) was 4 min and that for the slow phase ($t_{1/2\beta}$) was 29 min. The corresponding values for native FBS-AChE were 40 min and 990 min. Treatment of native FBS-AChE as well as rBoAChE with sialidase resulted, as expected [27,53], in a very rapid clearance (a few minutes) of the two preparations (results not shown). These observations indicate that the ability of native FBS-AChE to be retained in the circulation over long periods of time does not stem from unique features within its primary sequence, but rather from post-translational modifications.

In fact, HEK-293-derived rBoAChE was cleared from the circulation at rates similar to those of a recombinant HuAChE engineered to carry an additional N-glycosylation site. This mutant, rHuAChE Asp-61 → Asn, mimics BoAChE in its glycosylation-site profile (Asn-61, Asn-265, Asn-350, Asn-464) and is cleared very rapidly (Figure 5) from the circulation ($t_{1/2\alpha}$ = 4.5 min; $t_{1/2\beta}$ = 40 min).

Taken together, these observations indicate that the differences in primary sequence between the human and bovine enzymes do not contribute to the circulatory longevity of the bovine-derived enzyme, yet the composition of N-linked glycans plays an essential role in the circulatory retention of cholinesterases.

DISCUSSION

Native mammalian AChEs from different tissues differ in their quaternary structure [1–10]. Muscle-derived AChE is an asymmetric hetero-oligomer composed of multiple catalytic and collagen-tailed subunits, erythrocyte AChE is an H-subunit dimer anchored onto the membrane by a glycoinositol phospholipid,

brain AChE is a T-subunit tetramer bound to the cell membrane with a small peptide anchor, and serum AChE is a soluble homooligomer consisting solely of catalytic subunits. While the first three forms were identified in various species, the serum-derived form was only characterized in FBS [7]. Whether such forms exist in other species at the embryonic stage remains to be investigated. Homo-oligomeric forms of human, mouse and rat AChE produced in recombinant expression systems have been studied extensively and examined for structure/activity relationship. Nevertheless, the absence of a native homologous counterpart hampered our understanding of the relevance of certain structural features to AChE function. The study presented here enables comparison of recombinant and native soluble AChEs of the same species, in terms of quaternary architecture, glycosylation, enzymic activity and residence in circulation, and provides the tools to understand the inter-relationship between these various features.

Structure of the bovine *ACHE* gene

To express BoAChE we have cloned the gene from genomic bovine DNA. This enabled characterization of the bovine gene and its comparison with other *ACHE* genes.

BoAChE displays the structural characteristics of human *ACHE* and mouse *ACHE* genes [36], suggesting that *ACHE* gene organization in mammals is strongly conserved. The coding sequences are organized in five different exons intersected by introns. The intron–exon boundaries are located at the same sites as in other mammals [36] and the size of exons and introns is conserved. Exons 2, 3, 4 and 6 code for a viable BoAChE T-subunit, as indicated by the fact that assembly of these exons and expression of the fused sequence yields an active recombinant enzyme displaying the same catalytic properties as native BoAChE (Table 1).

The product of *ACHE* alternative splicing was not expressed in this study, yet the presence of a typical exon 5 within the sequence, between exon 4 and 6 (Figure 1 and [38]), strongly suggests that the bovine H-subunit is generated through the same mechanism as in other vertebrates [47,48].

Existence of a third *ACHE* gene product (the R-subunit), generated by read-through downstream from exon 4, has been suggested in torpedo and mouse [13,47]. Sequencing downstream from exon 4 in bovine *ACHE* (I. Mendelson, C. Kronman, N. Ariel, A. Shafferman and B. Velan, unpublished work, and [38]) reveals an open reading frame of 21 amino acids that could form a third alternative bovine splicing form. It may be interesting to note that this putative addendum is very different in sequence from the C-terminus of the mouse R-subunit.

Comparison of the amino acid sequence of BoAChE with other mammalian AChEs

The central role of AChE in signal transmission is manifested by a rather strong evolutionary conservation in amino acid sequences [1]. The DNA-derived BoAChE sequence presented here differs from the published amino acid sequence of AChE isolated from FBS by 13 amino acids. This could be the result of either an extensive polymorphism within the bovine species, or ambiguities related to the amino acid sequencing technology. We believe that the latter explanation is more plausible for the following reasons. (1) In contrast to butyrylcholinesterase, reports on polymorphism in *ACHEs* within the same species are very limited. (2) The fidelity of the DNA sequencing used here is rather high, since both strands were sequenced using two independent bacterial clones for each PCR-amplified fragment. For some regions, four independent sequences were determined

Figure 6 Mapping of divergent amino acids between BoAChE and HuAChE onto the three-dimensional model of HuAChE

The protein backbone is depicted as a line ribbon and the C- α atoms of the divergent residue are shown as full circles. Note that all divergent residues map to the enzyme surface. The enzyme is presented in two orientations: with the gorge entrance (marked by an arrow) parallel to the plane of the picture (left panel) and a rotated view (right panel, rotated 120° around the y -axis). Labels have been added to representative residues as indicators of relative orientation.

due to overlap of fragments (see Figure 1). The possibility that the differences in sequence result from errors introduced by PCR amplification are highly unlikely since each of the two clones represents an independent PCR event. (3) Two of the mismatches that we have found here were also observed by sequencing selected brain BoAChE peptides or DNA stretches within the bovine enzyme [30,38]. (4) Out of the 13 amino acids where divergence was observed between rBoAChE and FBS-AChE, 12 are identical with those of HuAChE and 10 are identical with mouse AChE.

Alignment of the updated BoAChE and HuAChE sequences reveals a higher identity than previously believed: only 34 out of the 583 amino acid of the mature T-subunits are different. All residues implicated in folding, such as the seven cysteines involved in intra- and inter-chain bonding, as well as residues Asp-175 and Asp-404 involved in salt-bridge formation [21], are conserved in the updated bovine *ACHE* sequence. In addition, the charged and aromatic residues implicated in the functional subsites of HuAChE [17,21–24] are also conserved. These include Phe-295 and Phe-297 which form the acyl-binding pocket, Tyr-337 and Phe-338 which constitute part of the hydrophobic subsite together with Trp-86 the choline-binding subsite, as well as Tyr-133, Glu-202 and Glu-450 which participate in the hydrogen-bond network. Furthermore, all residues implicated in the array of peripheral anionic binding sites of HuAChE, namely Tyr-72, Asp-74, Tyr-124, Trp-286 and Tyr-341 [26], are also present in BoAChE.

The conservation of all these functional residues probably explains the essentially identical kinetic behaviour of BoAChE and HuAChE with respect to hydrolysis and to interactions with the three distinct inhibitors, edrophonium, propidium and BW284C51 (Table 1).

The high resemblance in sequence between the human and the bovine enzymes appears to be in contrast to the differences in antigenicity between the two AChEs, as manifested by ELISA (see above). To examine this, we have mapped onto the three-dimensional model of HuAChE all positions where divergence between BoAChE and HuAChE was observed (Figure 6). All 34 divergent amino acids are located on the molecule surface and at least two distinct topological clusters can be identified. These surface-directed divergences could explain why antibodies against

HuAChE interact with denatured BoAChE molecules in the context of a Western blot (Figure 4b) and yet fail to interact with the same enzyme in a capture-ELISA where the molecule is presented in its native conformation. In addition to their effect on antigenicity, differences on the AChE surface could lead to functional manifestations related to the non-cholinergic activities of AChE. BoAChE and HuAChE could also differ in their interactions with other proteins, and this, in turn, could affect the pharmacokinetic behaviour of AChE, as discussed below.

Assembly of rBoAChE subunits

A significant difference between the native serum-derived and recombinant BoAChEs is manifested in subunit assembly. Circulating native enzyme is almost exclusively tetrameric, whereas the enzyme derived from HEK-293 cells is predominantly dimeric (assuming that monomers are breakdown products of dimers as suggested for other recombinant cholinesterases [40,42]). We have shown previously, with HuAChE, that the recombinant system is very efficient in assembling disulphide-bound AChE dimers [35], yet fails to assemble tetramers at comparable efficiencies. Inefficient tetramer formation is not unique to HEK-293 cells nor to the human and bovine enzymes. Large populations of dimers, relative to tetramers, were also found in the torpedo [49], rat [14] and chicken [41] AChEs and in the related enzyme butyrylcholinesterase [42]. These observations were made using a variety of cells, such as COS, CHO and RBL cells, or even non-vertebrate cells, such as yeast [43]. The high proportion of tetramers found in the circulation of the bovine fetus could be explained either by physiological selection mechanisms eliminating all other forms, or by the existence of an effective tetramerization machinery in the cells which secrete these molecules. More effective tetramerization can possibly be achieved by a tighter control between rates of subunit synthesis and enzyme secretion from the cell, or alternatively by the presence of cellular assembly cofactors [50].

Glycosylation

All vertebrate as well as insect cholinesterases characterized to date are glycoproteins carrying variable numbers of N-linked side-chains [1]. Glycans are not required for enzymic activity, at

least in the human [27,31] and *Drosophila* [51] enzymes, yet could play a role in other functions of cholinesterases. The involvement of sugar side-chains in circulatory residence of proteins is a well-documented phenomenon (reviewed in [52]) and has also been implicated in cholinesterase clearance [27,53]. Interestingly, the number of N-linked sugar side-chains in the two best-characterized serum cholinesterases, human serum butyrylcholinesterase and FBS-AChE, appeared to be higher than in other cholinesterases. While human, mouse, rat and torpedo enzymes carry 3–4 putative glycosylation sites [11–13], human butyrylcholinesterase carries nine side-chains [28] and the FBS was claimed to carry five such chains [29]. The controversy concerning the number of sugar side-chains on the bovine enzyme is reflected in failure to confirm a glycosylation site at position 541 of the bovine sequence [30,38], and in the fact that the glycan content of native purified FBS-AChE was found to be lower [46] than expected from an enzyme carrying five glycosylation sites. Results presented here indicate that the number of sugar side-chains carried by bovine AChE is indeed four. The updated sequence confirms this fact, and the SDS/PAGE analysis suggests that rBoAChE, as well as native FBS-AChE, resembles in size rHuAChE engineered to carry four sugar side-chains (Figure 4c). It should be mentioned that three of the four BoAChE glycosylation sites, Asn-265, Asn-350 and Asn-464, are located at the same positions as the glycosylation sites of HuAChE.

Circulatory residence

Native BoAChE from fetal serum, unlike HuAChE, is characterized by a prolonged circulatory residence [27]. Despite the extensive amino acid similarity between the two enzymes, one cannot exclude the possibility that the difference in clearance resides in the divergent, surface-located amino acids. Alternatively, the difference between the clearance patterns of these two enzymes can be explained by differences in their post-translational modifications. The ability to produce BoAChE and HuAChE in the same cell line now allows us to evaluate the possible contributions of primary sequence and cell-dependent processing on the pharmacokinetics of cholinesterases. In this study we have demonstrated that BoAChE produced in HEK-93 cells loses its prolonged circulatory residence, indicating on one hand, that cell-dependent protein modification has an over-riding effect on circulatory clearance, and that on the other hand, the BoAChE-specific amino acid residues do not confer any apparent pharmacological advantage to BoAChE as compared with the human-derived enzyme.

The effect of cell-dependent post-translational modification on clearance can be attributed to differences in assembly or to differences in glycosylation. In a previous study on HuAChE [27] we demonstrated that the degree of subunit oligomerization has a minor effect on circulatory residence, whereas the terminal-glycosylation profile is a major factor in AChE pharmacokinetics. We believe that the same is true for BoAChE. In fact, rBoAChE, which contains four sites for N-glycosylation, is cleared from the circulation in a manner very similar to that of a mutated rHuAChE which also contains four utilized N-glycan sequons. This is in accordance with our previous observations that an increase in the number of sugar side-chains of recombinant cholinesterases could lead to inefficient terminal glycosylation [27].

Taken together, these findings suggest that the major decisive factor determining circulatory retention of AChEs is the oligosaccharide content and composition and not the amino acid sequence of the enzyme. Therefore, further improvement in the therapeutic value of cholinesterases as organophosphate scavengers

should rely on modification of the sugar composition of the enzyme in a manner which will allow long-term protein residence in the bloodstream. Alternatively, chemical modification of recombinant AChEs, such as polyethylene glycol addition, could be used to override the adverse effects of inappropriate glycosylation and thereby generate molecules with advantageous pharmacokinetic profiles.

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