Identification of a Novel Type of Alternatively Spliced Exon from the Acetylcholinesterase Gene of Bungarus fasciatus

MOLECULAR FORMS OF ACETYLCHOLINESTERASE IN THE SNAKE LIVER AND MUSCLE*

The venom of the snake Bungarus fasciatus contains a hydrophilic, monomeric species of acetylcholinesterase (AChE), characterized by a C-terminal region that does not resemble the alternative T- or H-peptides. Here, we show that the snake contains a single gene for AChE, possessing a novel alternative exon (S) that encodes the C-terminal region of the venom enzyme, located downstream of the T exon. Alternative splicing generates S mRNA in the venom gland and S and T mRNAs in muscle and liver. We found no evidence for the presence of an H exon between the last common “catalytic” exon and the T exon, where H exons are located in Torpedo and in mammals. Moreover, COS cells that were transfected with AChE expression vectors containing the T exon with or without the preceding genomic region produced exclusively AChET subunits. In the snake tissues, we could not detect any glycosylphosphatidylinositol-anchored AChE form that would have derived from H subunits. In the liver, the cholinesterase activity comprises both AChE and butryrylcholinesterase components; butryarylcholinesterase corresponds essentially to nonamphiphilic tetramers and AChE to nonamphiphilic monomers (G1ns). In muscle, AChE is largely predominant: it consists of globular forms (G1s and G1m) and trace amounts of asymmetric forms (A1 and A2s), which derive from AChET subunits. Thus, the Bungarus AChE gene possesses alternatively spliced T and S exons but no H exon; the absence of an H exon may be a common feature of AChE genes in reptiles and birds.

Acetylcholinesterase (AChE)3 (EC 3.1.1.7) is an essential component of cholinergic synapses, in the nervous tissues and muscles of vertebrates (1). This enzyme is also found in nonsynaptic contexts, where its function is unclear. In the blood of mammals, AChE exists in the form of soluble tetramers (G4ns), probably originating from the liver, and of membrane-bound dimers (Gn2ns), anchored by a glycosylphosphatidylinositol (GPI) to the surface of erythrocytes and lymphocytes (2); these enzymes could serve as a safeguard against any diffusion of acetylcholine from synapses into the circulation. The venoms of various Elapidae from the genera Bungarus, Hemachatus, Naja, and Ophiophagus represent a particularly rich source of nonsynaptic AChE (3, 4).

The presence of AChE in snake venoms is mysterious because it is nontoxic by itself and does not enhance the toxicity of other venom components. This enzyme has been characterized as a true AChE, possessing the characteristic catalytic activity of AChEs from cholinergic tissues of other species: it hydrolyses acetylcholine faster than propionylcholine or butyrylcholine and it is inhibited by eserine (5). Moreover, the primary sequences of Naja and Bungarus venom AChEs present a strong homology to those of other AChEs, as shown by analysis of partial peptidic sequences (5, 6) and by analysis of the complete sequence of Bungarus AChE deduced from cDNA clones (7).

The cloning of AChE from Bungarus venom revealed, however, that this homology is limited to the catalytic domain and that the C-terminal sequence is entirely different from both C-terminal H- and T-peptides, which are encoded by alternatively spliced exons in the single AChE gene and characterize AChETsubunits of other vertebrates (review in Ref. 1). These C-terminal peptides determine the mode of post-translational processing and quaternary associations of AChE catalytic subunits. Thus, AChET subunits are modified by cleavage and addition of a GPI anchor, as well as by the formation of an intersubunit disulfide bond, generating GPI-anchored dimers (8). The AChET subunits produce monomers and a variety of disulfide-linked oligomeric forms, including homo-oligomers (dimers or tetramers) and hetero-oligomers, which incorporate structural collagen subunits (collagen-tailed forms) or hydrophobic subunits (hydrophobic-tailed tetramers). These hetero-oligomeric forms are tethered to extracellular matrices at neuromuscular synapses (9) or attached to cellular membranes, particularly in the brain (10). The T-peptide may adopt an amphiphilic α helical structure, thus explaining the observation that monomers and dimers of AChET subunits can interact with detergent micelles and membranes phospholipids (1). In contrast with all molecular forms that are normally produced by AChET subunits, the venom AChE consists of soluble, hydrophilic monomers.

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§The abbreviations used are: AChE, acetylcholinesterase; A8 and A12, asymmetric forms composed of two or three AChE tetramers, associated with a triple helical collagen tail; AChE, AChE, and AChET, AChE subunits of type H, S and T, generated from transcripts terminating with the H, S, and T exons; AChET, construction containing the intron that precedes exon T; AChET truncated AChE subunit limited to the catalytic domain; BChE, butyrylcholinesterase; G1s, G1m, and G1n, amphiphilic globular monomer, dimer, and tetramer, respectively; G2ns, nonamphiphilic globular monomer and tetramer; GPI, glycosylphosphatidylinositol; iso-OMPA, tetraisopropyl pyrophosphoroamide; PI-PLC, phosphatidylinositol phospholipase C; PCR, polymerase chain reaction; bp, base pair(s).
lated to the fact that the venom AChE possesses a specific C-terminal peptide, which we called SARA. It defines AChE subunits, which produce only soluble monomers when expressed in COS cells (7).

The presence of the SARA sequence, replacing the H or T sequences, which are encoded by alternative exons in Torpedo and mammalian AChE genes, raises the problem of the relationship between the venom enzyme and the AChE molecules that occur in cholinergic synapses of the snake. Several hypotheses may be considered to explain the production of this unusual type of AChE in venom glands. First, although previously studied vertebrates possess a single AChE gene (11–14), the snake might possess two distinct AChE genes, expressed in cholinergic tissues and the venom glands, respectively. Such a duplication would be similar to the duplication of cholinesterase genes, which generate the twin enzymes AChE and butyrylcholinesterase (BChE) (EC 3.1.1.8) in vertebrates (15). Second, the venom enzyme may derive from the same gene as AChE in other tissues. In this case, the SARA sequence could be encoded by a novel type of alternative exon or by “readthrough” transcripts. Readthrough transcripts, in which the genomic sequence following the common catalytic exons is maintained, have been characterized in Torpedo electric organs (16), in mouse MEL cells (13) and embryonic diaphragm (17), and in rat embryonic liver (18). Readthrough transcripts are expected to produce nonamphiphilic, monomeric AChE, but the corresponding proteins have never been characterized in vivo.

In the present report, we show that Bungarus possesses a single AChE gene containing a novel alternative exon, S, localized downstream of the T exon. We identified the alternative splicing of the AChE transcripts in the venom glands, the liver, and the muscles, and we characterized the resulting molecular forms in vivo, as well as in COS cells expressing various constructs.

MATERIALS AND METHODS

RNA Purification—Bungarus fasciatus snakes were kindly provided by Prof. Xiong Yu-Liang and Dr. Zhang Yun (Kunning Institute of Zoology, Academia Sinica, Kunming, Yunnan, China). They were sacrificed in China, and the tissues (venom gland, muscle and liver) were immediately frozen and transported in dry ice. Total RNA was extracted using RNeasy (Qiagen), according to the method of Chomczynski and Sacchi (19).

Reverse Transcription and PCR Experiments—For reverse transcription-PCR experiments, 1 μg of total RNA was reverse transcribed using 200 units of Superscript-Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) with 10 pmol of oligo-dT, 2 pmol of specific primer, or 25 pmol of hexanucleotides, as described in the legend to Fig. 4. PCR was performed essentially as described previously (7) with Taq polymerase from Promega in a PTC150 thermocycler (M. J. Research).

RNase Protection Assay—For RNase protection assays, we introduced exon 4 and the T or S exon, under control of T7 promoter to produce antisense probes. 10 μg of these constructs (described in Fig. 5) were digested overnight with 20 units of EcoRI in a final volume of 30 μl. The DNA was extracted with phenol and chloroform and then precipitated by ethanol in the presence of ammonium acetate. DNA was resuspended in 20 μl of RNase-free water and quantified using a GeneQuant spectrophotometer (Amersham Pharmacia Biotech). For probe synthesis, 3 μg of DNA were incubated with 100 μCi of [32P]dUTP (800 Ci/mmol; Amersham Pharmacia Biotech) and 150 units of T7 RNA polymerase (Promega) during 1 h at 37 °C and then digested using 2 units of RNase-free RNase (Ambion). The probe was purified by denaturing polyacrylamide gel electrophoresis and eluted for 2 h at 37 °C in a solution containing 0.5 m ammonium acetate, 0.2% SDS, and 1 μM EDTA. Total RNA (10 μg) was then co-precipitated overnight with 100,000 cpm of probe (200,000 cpm in the case of venom gland RNA, in which the AChE mRNA is more abundant) with 0.5 m ammonium acetate. The pellet was then resuspended in Hybdeasy buffer (Ambion).

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Sedimentation and Electrophoretic Analyses—AChE and BChE were analyzed by sedimentation in 5–20% sucrose gradients containing 10 mM Tris-HCl, pH 7.0, and 5 mM MgCl2, either without detergent or in the presence of 0.2% Triton X-100 or 1% Brij 96. AChE and BChE activities were assayed in the presence of specific inhibitors as indicated below. For analysis of asymmetric forms, the gradients contained 0.4 M NaCl and 1% Triton X-100. E. coli alkaline phosphatase (6.1 S) and E. coli β-galactosidase (16 S) were included as internal sedimentation standards.

Collagenase and PI-PLC Treatment—Collagenase form III (27) was purchased from Advance Biofactures Co. (Lynnbrook, NJ). A high salt extract containing AChE was incubated with 40 units of collagenase in a buffer containing 50 mM Tris-HCl, pH 8.0, and 5 mM MgCl2, either without detergent or in the presence of 0.5 M NaCl and 1% Triton X-100. E. coli alkaline phosphatase (6.1 S) and E. coli β-galactosidase (16 S) were included as internal sedimentation standards.

Assays of AChE and BChE Activity—AChE and BChE were assayed by the colorimetric method of Ellman et al. (29). Acetylthiocholine was used as a substrate for both enzymes. AChE was assayed in the presence of the specific anti-BChE inhibitor iso-OMPA (10−5 M), and BChE was assayed in the presence of the specific anti-AChE inhibitor BW284C51 (1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide) (10−3 M).
Genomic DNA was extracted and isolated by a salting-out protocol (30): liver was crushed in liquid nitrogen and transferred in 10 volumes of extraction buffer (10 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 20 mg/ml pancreatic RNase, 0.5% SDS, and 0.5 M NaCl) and incubated at 50 °C for 30 min. Proteinase K was added at a final concentration of 100 μg/ml, and incubation was performed overnight at 50 °C. Saturated NaCl (1/4 volume) was then added (1/4 volume) and agitated. After centrifugation (15 min at 5000 rpm), 2 volumes of cold ethanol were added to the supernatant, which was then kept on ice for 10 min. After centrifugation (15 min at 5000 rpm), the DNA pellet was redissolved in Tris-EDTA buffer.

PCR was performed with primer oligonucleotides corresponding to sequences of exons 3, 4, T, and S. To search for the presence of a putative exon H between exons 4 and T, we made several constructs by inserting various 3’ sequences, using a unique BglII site, located in exon 4, as shown in Fig. 7.

Transfection of COS Cells—COS cells were transfected by the DEAE-dextran method, as reported previously (31), using 5 μg of DNA encoding the catalytic subunit AChE T with or without DNA encoding the QN/HC binding protein (31, 32), as specified. The cells were maintained at 37 °C and extracted 2–4 days after transfection. The culture medium (7 ml/10-cm dish containing about 5 × 10^6 cells) was collected after variable periods of time, as indicated, for analysis of released AChE activity. The extracts and culture media were stored at −80 °C.

RESULTS

Molecular Forms of Cholinesterases in Bungarus Liver and Muscles—Extracts from snake liver were found to hydrolyze butyrylthiocholine, as well as acetylthiocholine, indicating the presence of both AChE and BChE. As shown in Fig. 1, an AChE component sedimented at 4.5 S (about 80% of the total cholinesterase activity), and a BChE component sedimented at 10.9 S (about 20% of the total activity); only the latter component hydrolyzed butyrylthiocholine (not shown). The sedimentation patterns were not modified by incubation with PI-PLC (not shown) and were identical in the presence of Triton X-100 (Fig. 1), in the presence of Brij-96, or without detergent (not shown), indicating that both components were nonamphiphilic, corresponding to a monomeric form of AChE (G1 na) and a tetrameric form of BChE (G4 na). The residual activity observed around 11 S in the presence of iso-OMPA, a specific inhibitor of BChE, and the fact that BW284C51, a specific inhibitor of AChE, reduced the cholinesterase activity of the same fractions suggests the presence of a small contribution of tetrameric AChE (G4 na). The absence of amphiphilic forms and the fact that PI-PLC had no effect on these profiles indicated that the snake liver did not produce any GPI-anchored form of AChE.

Fig. 2 shows that detergent-soluble extracts from Bungarus muscles contained a much smaller proportion of BChE, repre-
senting less than 2% of the total cholinesterase activity. The sedimentation patterns, obtained in the presence of Triton X-100 (Fig. 2A) or of Brij-96 (Fig. 2B), showed three AChE components (a major one, corresponding to amphiphilic tetramers (G4a) sedimenting at 9.5 S in Brij-96 and 10.9 S in Triton X-100, and two minor ones, corresponding to amphiphilic monomers (G1a) sedimenting at 3.5 S in Brij-96 and 4.5 S in Triton X-100) and nonamphiphilic AChE monomers (G1na) sedimenting at 4.5 S in both conditions. The same muscle extract contained a small proportion of BChE, corresponding to amphiphilic tetramers (G4a) sedimenting at 9.8 S in Brij-96 and 11 S in Triton X-100. The AChE and BChE G4a peaks presented a small but definite difference in their sedimentation when assayed in the same gradient fractions, showing that they really correspond to distinct molecules. There was no indication of the presence of a dimeric AChE form, and treatment with PI-PLC did not modify the sedimentation profiles.

To examine whether snake muscles also contained collagen-tailed AChE forms, which aggregate at low ionic strength, the pellet obtained after two successive extractions in the low salt detergent buffer was re-extracted in a high salt detergent buffer. The AChE activity that was solubilized under these conditions was analyzed by sedimentation in a gradient containing 0.4 M NaCl and 0.2% Triton X-100, with or without prior digestion by collagenase. Fig. 3 shows that in addition to major G1a and G4a components, the high salt extract contained a small proportion of asymmetric A8 and A12 forms, which were modified by collagenase in a characteristic manner: their sedimentation coefficients were increased from 13.4 to 15.1 S and from 17.3 to 18.5 S, respectively. Taken together, these two molecular forms represent less than 3‰ of the total AChE activity in the muscle extracts.

The presence of G1a, G4a, A8, and A12 forms clearly demonstrates that AChE1ₜ₏ subunits are not produced in Bungarus liver or muscles. Therefore, it appears that only AChE₁ₜ and AChE₉ₙ are produced in these tissues. To confirm this, we analyzed the structure of AChE transcripts and of the AChE gene.

**AChE Transcripts Are Generated by a Single Gene in Bungarus Muscles, Liver and Venom Glands**—A Southern blot of digested Bungarus genomic DNA was hybridized with a probe
corresponding to nucleotides 1610–1727 of the AChE cDNA from venom glands, within the coding region of the catalytic domain. We obtained a single labeled band after digestion by EcoRI and BamHI, suggesting that *Bungarus* possesses a single gene for AChE (not shown).

Experiments involving rapid amplification of cDNA ends were unsuccessful to identify the 3' region of the coding sequence of AChE cDNA in muscle. To obtain the complete coding sequence, we amplified a cDNA fragment encoding the C-terminal region of muscle AChE, by reverse transcription-PCR.

Reverse transcription of mRNA was performed with random hexanucleotides associated with Ri and Ro sequences for PCR priming (20) (Fig. 4A). PCR was then performed with Ri and Ro reverse primers and a forward primer corresponding to a fragment from the AChE cDNA previously cloned from venom gland (7). We thus amplified a fragment of about 200 bp, which was subcloned and sequenced (Fig. 4B). According to this sequence, the end of the catalytic domain is identical to that of the venom cDNA clone, but it is associated with a different C-terminal region. In agreement with our analysis of AChE forms in muscles, this region corresponds to a T-peptide, as shown by its alignment with sequences from *Caenorhabditis*, *Torpedo*, avian, and mammalian AChEs (Fig. 4C). The strict conservation of eight aromatic residues and of a cysteine residue at position 242 of the C terminus is particularly noticeable. Thus, the AChE gene that produces the venom enzyme also generates T transcripts in muscles.

Reverse transcription-PCR experiments showed that both T and S transcripts are produced in *Bungarus* muscle. The 3' genomic region of the gene of AChE from *Bungarus* was cloned by PCR amplification. We sequenced the region containing introns 3–4 and 4–T (exons are numbered by analogy with the mammalian AChE gene), as well as the noncoding region situated between the T and S coding sequences. The S sequence is encoded by a new alternative exon, called S. Putative polyadenylation sites are underlined. Constitutive introns are shown in lowercase letters, but the alternatively spliced 3' regions are in uppercase letters. The BglII site used for constructions (see Fig. 7) is indicated by a dotted line. Note that the coding sequence of the S-peptide is preceded by a classical consensus splicing acceptor site and that the preceding untranslated region contains GC-rich domains. The end of the presented sequence corresponds to the beginning of the poly(A) tail of S transcripts (7).
and T transcripts exist in venom glands, liver, and muscle (not shown). RNase protection assays indicated that the venom glands contain mostly S transcripts, with only a small fraction (<5%) T transcripts, whereas the other tissues approximate two-thirds S transcripts and one-third T transcripts (Fig. 5).

Genomic Structure of the 3' Region of the Bungarus AChE Gene — We explored the structure of the Bungarus AChE gene by PCR amplification of genomic DNA, using primers corresponding to various exonic sequences. The results are shown in Fig. 6. The sequence encoding the catalytic domain is interrupted by an intron (about 1.3 kb) at the level of amino acid 475 (according to the Torpedo numbering), as in other vertebrates. The last common exon (encoding 35 residues) is followed by a genomic region of 1741 base pairs, preceding the T exon. The region encoding the C-terminal part of the venom AChE (SARA, or S) is located about 300 nucleotides downstream of the stop codon of the T exon. Therefore, the SARA region does not derive from a readthrough sequence but from a novel type of exon, which we call S (Fig. 7). It is interesting to note that the 300-nucleotide-long sequence located between the sequence encoding the T-peptide and exon S contains GC-rich domains.

In Torpedo and mammalian AChE genes, the H exon is located between the last common catalytic exon and exon T (1). The 1741 bp-long corresponding region in Bungarus AChE gene was fully sequenced: its analysis did not reveal the presence of any H-like open reading frame that might encode a GPI-addition C-terminal signal peptide (33). Therefore, the Bungarus AChE gene does not appear to contain an H exon.

Expression of Bungarus AChE in Transfected COS Cells: Existence of an H Exon? — We transfected COS cells with expression vectors containing either the cDNA sequence encoding the AChE T subunit or a partial genomic construct (AChEgT), which included the 1741-bp intron preceding exon T, where a putative H exon would be expected to be localized (see Fig. 7). The total AChE activity and the proportion that was secreted in the culture medium were the same in both cases: 30–45% of the activity was recovered in the medium, 2 days after transfection. As illustrated in the case of the AChE T construct (Fig. 8, A and B), sedimentation analysis showed that the cells and the culture medium contained G1a, G2a, and G4a forms, as well as heavy polydisperse aggregates. These aggregates, which have not been observed in other AChE species, accounted for as much as 80% of the total activity in cell extracts. The culture medium contained the same type of molecules, including aggregates, with a higher proportion of G4a than in the cells. We obtained the same results with the AChEgT construct (not shown). We could not detect any PI-PLC-sensitive AChE, showing that only AChEH subunits were produced. This demonstrates the absence of any functional exon H, that would generate AChEgH subunits.

In co-transfection with the binding protein QN/Hc (31, 32),

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**Fig. 7. Genomic structure of Bungarus AChE.** A, the Bungarus AChE gene contains two exons encoding the catalytic domain and two alternatively spliced exons, T and S. Constitutive splicing is indicated by solid lines and alternative splicing by dashed lines. The genomic region located between the 3' end of the second catalytic exon and the T exon does not contain an H exon. The noncoding regions of exons are indicated by gray boxes (assuming that T transcripts terminate at a polyadenylation site located upstream of exon S; see Fig. 6). B, constructs used in transfection experiments. The arrowheads indicate the BglIII site used for constructions.

**Fig. 8. Molecular forms of AChE produced in COS cells expressing Bungarus AChE, alone or with QN/Hc.** A and B, AChE alone; C and D, AChE with QN/Hc. The cell extracts (A and C) and the culture medium (B and D) were analyzed by sedimentation in sucrose gradients containing Brij-96 (C) or Triton X-100 (D). In the case of co-transfection with QN/Hc, a sample of the cell extract (C) was treated with PI-PLC (D). The presence of aggregates sedimenting above 15 S was clearly visible in all gradients, but their proportion was markedly decreased by QN/Hc, which induced the association of monomers and dimers into GPI-anchored tetramers (G4a) and the subsequent production of lytic nonamphiphilic tetramers (G4na) and monomers (G4na) (31). A small proportion of G4a was recovered in the culture medium (D), possibly attached to membrane fragments.
the proportion of monomers, dimers, and aggregates was markedly decreased; AChE subunits were largely assembled into GPI-anchored tetramers characterized by their sensitivity to PI-PLC, both in the case of the cDNA (Fig. 8, C and D) and in the case of the AChET construct (not shown). These results were confirmed by electrophoretic analyses in nondenaturing polyacrylamide gels (Fig. 9). The GPI-anchored G₃α form was converted by PI-PLC to a hydrophilic derivative, which migrated faster in nondenaturing electrophoresis (Fig. 9). The production of such heteromeric molecules is a further confirmation that Bungarus AChEt subunits can form the same types of quaternary associations as those of other species.

When we varied the quantity of vector DNA used for transfection, the production of secreted AChE activity increased at low doses, and reached a plateau for about 5 μg of DNA/dish. Although the saturating dose was approximately the same, the yields obtained varied widely with the different constructions: the secreted AChE activity was about 10-fold lower in the case of AChEs than of AChET or of AChEgT. In the case of AChEs or AChET, the cells only produced monomers (G₁α), and the activity was at least 85% secreted (7). We also compared the production of AChE activity with corresponding rat AChEα and AChEγ. As shown previously (7), the yield of secreted AChE was about 30-fold higher for Bungarus AChEs or AChEγ than for rat AChEα. Similarly, the yield was higher for Bungarus AChEγ than for rat AChEγ, but in this case the ratio was only 2-fold.

**DISCUSSION**

*Bungarus Possesses a Single AChE Gene, with a Novel Type of Alternative Exon—*Analyses of genomic DNA and of AChE cDNA from muscle showed that Bungarus, like other vertebrates, possesses a single AChE gene, and that AChEs and AChEγ subunits are produced by alternative splicing. The S-peptide is not encoded by a readthrough sequence but by a bona fide alternative exon, called S, which is located 3' of the T exon.

The peptideic sequence encoded by the T exon is highly homologous to the C-terminal T-peptides of other AChEs and BChEs. Among the conserved residues, it is interesting to note the presence of a cysteine residue near the C terminus, involved in intersubunit disulfide bridges, as well as of several aromatic residues, probably involved in the hydrophobic character of an amphiphilic α helix, in the N-terminal part of the peptide (1). The capacity of Bungarus AChEs to form heteromeric quaternary structures with a proline-rich attachment domain (PRAD) (31, 34) is demonstrated by the presence of collagen-tailed AChE forms in Bungarus muscle and the formation of GPI-anchored tetramers with the QN/HCh chimera in transfected COS cells.

The 3' untranslated region of the muscle transcripts encoding AChEγ subunits contains GC-rich domains, so that it was not possible to define its extremity by the rapid amplification of cDNA ends 3' method. Three putative polyadenylation sites are located 190 bases 3' of the T exon stop codon. The T transcripts may terminate at such sites or include the S coding sequence, in the same manner as the T sequence is included in the 3' region of H transcripts of mouse AChE (14).

In the AChE genes of Torpedo and mammals, the H exon is located upstream of the T exon. Several lines of evidence indicate that the Bungarus AChE gene does not possess an H exon: (a) we could not find any GPI-anchored AChE dimers, which would be generated from AChEsγ subunits, in Bungarus tissues (this is particularly significant in the liver, because this organ is rich in GPI-anchored AChE in rat) (18, 35, 36); (b) the genomic region separating the last common exon from the T exon does not contain any sequence that might encode an H-peptide; and (c) COS cells transfected with a construct, AChEγT, in which this region was included produced only AChEsγ subunits, showing that it does not contain any alternative exon. In the case of human AChE, a similar construction led to the production of both H and T subunits (37).

It is interesting that H exons have not been found in the genes of mammalian BChE, quail AChE (1), and Electrophorus AChE (38). In fact, among vertebrates, only Torpedo and mammals have been shown to possess a GPI-anchored AChE form.

*Expression in Transfected Cells—*It has been shown that the C-terminal peptides of AChE subunits determine the fate of the enzyme in a tissue-specific manner, and in particular its metabolic stability: thus, the rat RBL cells express rat AChEγ subunits and expose them at their surface much more efficiently than AChEγ subunits, despite the fact that the two proteins seem to be synthesized at equivalent levels (39). We found that AChE activities were systematically higher in...
transfected COS cells expressing *Bungarus* AChE than in cells expressing rat AChE. This was true for AChE_T subunits and was even more marked in the absence of the T-peptide (AChE_S or AChE_M). In all cases, we observed a similar influence of the amount of vector DNA used for transfection, with saturation at approximately the same dose (5 µg/dish), so that the difference could not be ascribed to transcription; translation is also very unlikely to differ between constructions such as AChE_T and AChE_M, which differ only by the presence or absence of the C-terminal 40-amino acids T-peptide. Comparisons of AChE activities obtained with the different constructions clearly showed that the presence of a C-terminal T-peptide reduced the yield of active enzyme and that the catalytic domains of *Bungarus* and rat AChEs present intrinsic differences. The snake enzyme may be able to fold more efficiently into its active conformation, as suggested by its capacity to renature after exposure to guanidinium hydrochloride.²

Whereas the truncated *Bungarus* or rat AChE_M subunits remained exclusively monomeric, the AChE_S subunits of both species generated monomers, dimers, and tetramers, as expected (31). However, the major part of active species generated monomers, dimers, and tetramers, as expected approximately the same dose (5 rem).

In addition, AChE does not appear to contribute to the toxicity of the venom (7). It will therefore be interesting to examine whether the presence of an S exon is correlated with expression of AChE in the venom, in particular in *Dendroaspis* snakes (mambas), which do not contain AChE in their venom (4), and whether S exons also exist in other reptiles. The evolutionary significance of the absence of H exons and the presence of S exons in *Elapidae* snakes clearly deserves more detailed studies.

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