We cloned and expressed a cDNA encoding acetylcholinesterase (AChE) of type T from Electrophorus electricus organs. When expressed in COS, HEK, and Chinese hamster ovary cells, the AChE<sub>T</sub> subunits generated dimers and tetramers. The cells produced more activity at 27 than at 37 °C. The kinetic parameters of a recombinant enzyme, produced in the yeast Pichia pastoris, were close to those of the natural AChE. Analysis of genomic clones showed that the coding sequence is interrupted by an intron that does not exist in Torpedo and differs in its location from that observed in the mouse. This intron is preceded by a sequence encoding a non-conserved 29-amino acid peptide, which does not exist in Torpedo or mammalian AChEs. According to a three-dimensional model, this non-conserved peptide is located at the surface of the protein, opposite from the entry of the catalytic gorge; its deletion did not modify the catalytic parameters. Sequence analyses and expression of various constructs showed that the gene does not contain any H exon. We also found that splicing of transcripts in mammalian cells reveals cryptic donor sites in exons and acceptor sites in introns, which do not appear to be used in vivo.

The molecular structure of acetylcholinesterase (AChE, EC 3.1.1.7) attracts considerable interest, because of its fundamental role in cholinergic synapses. It appeared useful to clone AChE from Electrophorus, since this enzyme has been used as a model in numerous biochemical studies (1, 2), due to its exceptional abundance in electric organs. The catalytic mechanism of AChE has been explored by site-directed mutagenesis, using mostly Torpedo and mammalian AChEs (3, 4). The comparison of AChEs from different species has also been extremely informative, particularly for the structure of the peripheral site in enzymes that show little or no inhibition by peripheral site inhibitors, such as Bungarus AChE (5) or chicken AChE (6).

It was also interesting to analyze the polymorphism of catalytic subunits, generated by alternative splicing, in a Teleost fish. In Torpedo and in mammals, the catalytic domain of AChE may be associated with different C-terminal peptides that determine the quaternary associations and anchoring of the enzyme in membranes or extracellular matrices (7) as follows: the H and T peptides, encoded by alternative exons, characterize, respectively, AChE<sub>H</sub> subunits, producing glycoporphatidylinositol (GPI)-anchored dimers, and AChE<sub>T</sub> subunits, producing an array of homo- and hetero-oligomers, including collagen-tailed molecules. This splicing pattern is, however, variable, since Drosophila possesses only AChE<sub>H</sub> subunits, and chicken possesses only AChE<sub>T</sub> subunits. To understand this variability, it is necessary to explore the structure of AChE genes in the various vertebrate lineages.

**EXPERIMENTAL PROCEDURES**

**Materials**

Reagents for biochemistry were purchased from Prolabo (Paris, France) or from Sigma. Products, enzymes, and kits for molecular biology were from Ambion, Biobas, Bioprobe Systems, Life Technologies, Inc., Invitrogen, Machery-Nagel, Pharmacon Biotech Inc., Promega, and U. S. Biochemical Corp. Oligonucleotides were synthesized by Genset. Radioactive a-<sup>32</sup>P-labeled and a-<sup>35</sup>S-labeled nucleotides were from Amersham Corp. Phosphatidylinositol-phospholipase C from Bacillus thuringiensis was obtained from Immunotech (Marseille, France). The M2 monoclonal antibody directed against the “flag” epitope was from Eastman Kodak. Live Electrophorus were obtained from Worldwide Scientific Animals (Apopka, FL); they were rapidly sacrificed, and their tissues were stored in liquid nitrogen.

**Determination of a cDNA Sequence Encoding Electrophorus AChE**

Purification of Electrophorus AChE and Determination of Peptide Sequences—Collagen-tailed AChE was extracted from Electrophorus electric organs in a high salt buffer and purified by a combination of affinity chromatography, low ionic strength aggregation, and isopycnic centrifugation in CsCl (8, 9). Tryptic fragments of the catalytic subunit were sequenced by Prof. Joel Vandekerckhove (Rijksuniversiteit Gent, Belgium), as described (10).

Construction of a cDNA Library and Amplification of Partial Coding Fragments—mRNAs from Electrophorus electric organs were reverse-transcribed using oligonucleotide hexamers, for construction of a AZAP-II library, which contained 160,000 independent recombinants. The cloning strategy is schematically indicated in Fig. 1. To obtain a non-degenerate probe, we first amplified a fragment of the coding region by PCR, using degenerate oligonucleotide primers. The forward primer F<sub>1</sub>, was deduced from the sequence of a tryptic peptide, LLDQR (170–174, Torpedo numbering). The reverse primer R<sub>1</sub> was based on the peptide sequence WPEWGM (452–457), which is identically conserved in all known sequences of vertebrate and invertebrate cholinesterases. We thus amplified a fragment of 910 bp, called E<sub>1</sub>. This PCR product was ligated in the PCRII plasmid (Invitrogen) and sequenced by the method of Sanger, with the Sequenase 2.0 kit (U. S. Biochemical Corp.). We amplified an overlapping fragment of 320 bp (E<sub>2</sub>), extending further upstream, using a forward degenerate primer, F<sub>2</sub>, based on another conserved region (EMWINP, 82–97) and a reverse primer R<sub>2</sub>.

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‡ Recipient of a doctoral fellowship from the French Ministry of Research.

1 The abbreviations used are: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; GPl, glycoporphatidylinositol; PCR, polymerase chain reaction; RT-PCR, reverse-transcription and PCR; RPA, ribonuclease protection assay; RACE-3', rapid amplification of cDNA 3' ends; S, Svedberg units; EU, Ellman units; CHO, Chinese hamster ovary; bp, base pairs.

2 A. Anselmet, unpublished results.
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duced from the sequence of EL. The cloned EL and EL fragments were used to synthesize radioactive α-32P probes to screen the cDNA library. We thus obtained clones C1, C2, C3 and C4, as shown in Fig. 1.

Amplification and Cloning of the 3′ Part of AChE Transcripts (RACE-3′)—Total RNA was extracted from electric organs (Bioprobe Systems). Approximately 1 µg of total RNA was reverse transcribed from the poly(A) tail, using the oligonucleotide (dT)17-Ri-Ro (11) and 5 units of avian myeloblastosis virus reverse transcriptase (Promega) in 20 µl. The products were diluted to 100 µl with water, and 10 µl of this dilution were used for amplification by PCR, using the forward primer F2 in the C3 sequence and Ro as the reverse primer (Fig. 1). For a second amplification, we used a specific downstream (nested) forward primer F4 and the Ri reverse primer, with 5 µl of the 100-fold diluted product of the first reaction. The RACE-3′ product, obtained after two amplifications, was cloned in the PCR-II vector.

Construction of a Complete Coding Sequence: Subcloning in the pcDNA3 Mammalian Expression Vector—A complete coding region was constructed from the C3 cDNA fragment and the RACE-3′ product, using a unique HgoI restriction site and ligated into pcDNA3 (Invitrogen), after digestion by HindIII and EcoRI. The resulting construct was called cDNAAC.

Modeling of the Three-dimensional Structure

A model of Electrophorus electricus AChE, obtained by the first approach protocol of Swiss-Model, using the structure of Torpedo californica AChE as template, was displayed with the RASMOL program.

Expression of AChE in COS, HEK, and CHO Cells—Plasmidic DNA was prepared with the Nucleobond plasmid purification kit (Macherey-Nagel). COS7, HEK293, and CHO cells were transfected with 5 µg of DNA per 10-cm dish containing about 106 cells, using the DEAE-dextran method for COS cells, as described previously (17), or the calcium phosphate method for HEK and CHO cells (18). Conversely, P. pastoris, CHO cells do not possess the SV40 T antigen and thus do not allow the replication of the pcDNA3 vector. Transiently transfected cells were generally grown at 37 °C for 36–48 h after transfection and then transferred to 27 °C for 48–72 h.

Preparation of Cellular Extracts from Transfected Cells and from Electrophorus Tissues—The transfected cells were scraped in 2 ml of cold TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl) and centrifuged 5 min at 1,500 × g at 4 °C. The cellular pellet was resuspended in 200 µl of extraction buffer (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2; 0.1% SDS, 1% Triton X-100) and homogenized in a Potter glass-teflon homogenizer. The supernatant obtained after centrifugation at 1,500 × g at 4 °C, and the supernatant was used as the cellular extract. Extracts from various Electrophorus tissues were prepared by homogenizing approximately 0.5 g of tissue in 7 volumes of extraction buffer without detergent with an Ultraturrax T25 homogenizer (Janke and Kunkel, IKA Labotechnik). The supernatant obtained after centrifugation at 30,000 rpm in a Beckman SW41 rotor for 20 min, at 7 °C, was the “soluble” fraction. The pellet was re-homogenized in 7 volumes of extraction buffer containing 2% Triton X-100 and centrifuged at 40,000 rpm for 30 min, at 7 °C, yielding the “detergent-soluble” fraction.

Assay of AChE Activity: Catalytic Parameters—The AChE activity of tissue extracts, cellular extracts, and culture media was determined by the colorimetric method of Ellman et al. (19). Enzyme samples were added to 0.2 ml of the assay medium (1 mM acetylthiocholine, in 50 mM phosphate buffer, pH 7.4, 0.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid), 0.1 mg/ml bovine serum albumin), and the reaction was monitored at 414 nm at 30-s intervals, over a period of 5 min, using a Multiskan RC microplate reader (Labsystems). One Ellman unit (EU) corresponds to an increase in optical density of 1 per min, with a path length of 1 cm. For the determination of kinetic parameters, we used crude extracts from electric organs and from transfected cells, as well as secreted AChE from P. pastoris. The extracts were diluted to 0.5 EU/ml in 10 mM Tris, pH 7, 0.1% bovine serum albumin; in some experiments, we added 1 M NaCl, as indicated. The values of Km and Vmax, as defined by the Haldane equation, were fitted with the Kaleidagraph software, as described previously (5). Catalytic turnover numbers (kcat) were determined by titrating the active sites of AChE with the irreversible inhibitor O-ethyl-S-[2-diisopropylamino]-ethyl)methylphosphonothioate (20). These experiments were performed at the Centre d’Etudes du Bouchet.

Analysis of AChE by Sedimentation and Electrophoresis—For sedimentation analyses, samples of tissue extract, cell extracts, or culture medium containing about 100 mEU of AChE activity were centrifuged in 5–20% sucrose gradients, in 20 mM Tris-HCl, pH 7.0, 8 mM MgCl2, 0.08 mg/ml bacitracin, either without detergent or in the presence of 1% Triton X-100 or Brij-96, as described previously (5, 21, 22). The samples were mixed with Escherichia coli β-galactosidase (16 S) and alkaline phosphatase (6.1 S) as internal standards of sedimentation coefficients. 
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RESULTS

Structure of Electrophorus AChE

Construction of a Complete cDNA Encoding Electrophorus AChE—To clone AChE from Electrophorus, we used a partial sequence obtained from purified Electrophorus AChE, LLDQR, corresponding to residues 170–174 of Torpedo AChE (note: the number refers to the corresponding Torpedo residue that is considered as a reference (24, 25)) together with totally conserved peptide motifs EMWNPN at positions 82–87 and WPEWMG at positions 432–437. As shown in Fig. 1, oligonucleotide primers allowed us to amplify successive fragments of the coding sequence by PCR, from cDNA obtained by reverse transcription of electric organ poly(A)+ RNA.

The EI fragment clearly encodes AChE, including the characteristic peptide motif that surrounds the active site serine, FGESAG. An R1 primer, derived from EI, was combined with F2, corresponding to the EMWNPN peptide sequence, allowing us to amplify an overlapping upstream fragment, EI. Using radioactive probes derived from EI and EI fragments, we obtained several cDNA clones from a random-primed cDNA library, constructed from electric organ mRNA. The clone that contained the longest insert, C3, was totally sequenced; it extended 117 bp upstream of the initiation codon of the AChE open reading frame but is incomplete at the 3’ end. Using the RACE-3’ method, we obtained the 3’ end of the coding sequence and a 3’-untranslated sequence of approximately 770 bp that precedes the poly(A) tail. A complete coding sequence was constructed from these elements (Fig. 1) and used for expression of Electrophorus AChE. Partial genomic and other constructs are described in Fig. 2.

Primary Structure of Electrophorus AChE: a Catalytic Subunit of Type T—The open reading frame encodes a protein of 633 amino acids, beginning with a putative signal peptide of 23 amino acids. The mature protein contains the peptides that were labeled and sequenced by Salih et al. (26). (These sequences were published after our cloning of Electrophorus AChE.) Fig. 3 shows an alignment of the deduced primary sequence of Electrophorus AChE with that of Torpedo AChE (type T). The catalytic domains of the two proteins present 68% identity, without taking into account a 29-amino acid insertion that exists only in Electrophorus AChE, between residues 415 and 416 of Torpedo AChE. The additional peptide is highly hydrophilic, containing 7 glycines and 7 serines. A three-dimensional model of the catalytic domain of Electrophorus AChE, deduced from the structure of Torpedo AChE, indicates that this peptide is located at the surface of the molecule and may partially adopt an α-helical structure (Fig. 4).

As expected, Electrophorus AChE possesses the 6 cysteines that form three intracatenary disulfide bonds, the three amino acids of the catalytic triad (Ser-200, Glu-327, and His-440), the aromatic residues Phe-288 and Phe-295, which define an acetylcholine-specific acyl pocket, the tryptophan residue Trp-84, which interacts with the choline moiety in the active site, as well as the 14 aromatic residues lining the walls of the gorge; 13 of these residues are identical, but Phe-330 of Torpedo AChE is replaced by Tyr in Electrophorus AChE, as well as in Drosophila, Bungarus, mouse, and human AChEs (3). Together with Trp-84, this residue constitutes the choline-binding subsite of the catalytic site; both the Electrophorus Tyr-330 and Torpedo Phe-330 residues were found to react with the photoactivable ligand p-(N,N-dimethylamino)-benzenediazonium fluoroborate (27, 28).

The peptide sequence contains 6 putative sites for N-glycosylation, one of which (Asn-161, corresponding to His-159 in Torpedo) has not been observed previously in other ChEs; it seems to be localized at the surface of the protein, in the loop joining the secondary structure elements β6 and αC.

The C-terminal sequence (underlined in Fig. 3) clearly corresponds to a T peptide; it contains all the aromatic residues that are conserved in T peptides of vertebrate cholinesterases and a comparison with the Torpedo C-terminal 40 residues revealed only 9 differences (4, 29). The cloned cDNA therefore encodes a catalytic subunit of type T, AChE, in agreement with the fact that Electrophorus electric organs only contain collagen-tailed forms of AChE (7).

Expression of Electrophorus AChE in COS, HEK, and CHO Cells

Effect of Temperature on the Production of Active AChE—COS, HEK, and CHO cells were transiently transfected with a
pCDNA3 vector encoding the *Electrophorus* AChET subunit. The production of active AChE was considerably increased when the cells were incubated at 27 °C, rather than 37 °C, after transfection, as reported previously in the case of *Torpedo* AChE (17). After 72 h, 40% of the total activity was released in the culture medium, as shown in Table I, indicating that secretion of this enzyme is somewhat less efficient than that of AChET from rat, but more than that of *Torpedo*, at 27 °C (30).

**Molecular Forms of AChE—** Sedimentation analyses showed that extracts from transfected cells contained mostly amphiphilic dimers, G₂ₐ, sedimenting at 5.9 S in the presence of Brij-96 and 7.1 S in the presence of Triton X-100, together with smaller proportions of amphiphilic tetramers, G₄ₐ, sedimenting at 10.3 S in Brij-96 and 11 S in Triton X-100, and of nonamphiphilic tetramers, G₄ₐ, sedimenting at 12 S in the presence of both detergents (Fig. 5A). As shown in Fig. 5B, the culture medium also contained a majority of amphiphilic dimers, G₂ₐ, but the proportion of tetramers, G₄ₐ (12 S) and G₄ₐ, was higher than in the cell extract. In addition, the secreted amphiphilic AChE forms (G₂ₐ and G₄ₐ) sedimented slightly but reproducibly faster than the cellular forms, 6.3 S in Brij-96 and 7.3 S in Triton X-100 for the secreted G₂ₐ form and 11 S in Brij-96 for the secreted G₄ₐ form.

The nature and the proportions of AChE forms produced in transfected HEK, COS, and CHO cells appeared identical, according to sedimentation analyses (not shown). However, in nondenaturing electrophoresis, the migration of AChE forms produced in CHO cells appeared slightly different from that of the corresponding molecules produced in COS cells (Fig. 6) and in HEK cells (not shown); this was probably due to differences.
in glycosylation.

Cleavage of a C-terminal Flag Peptide upon Secretion—A flag epitope, when inserted at the end of the AChET protein, did not alter the production of active AChE, or the proportions of the different oligomeric forms, but increased secretion by more than 50%. Although the cellular enzyme was retarded by the anti-flag M2 monoclonal antibody in nondenaturing electrophoresis, most of the secreted enzyme was not affected (Fig. 7, lanes 1–6), indicating that proteolytic removal of a peptide fragment may occur upon release, as previously observed in the case of rat AChE<sub>r</sub> (30).

Co-expression of Electrophorus AChE<sub>r</sub> and QN/Stop—When Electrophorus AChET subunits were expressed in COS cells together with the Q N/stop protein, which contains the AChE binding domain of the N-terminal region of the collagen tail (17, 30), they produced soluble tetramers that were mainly recovered in the medium (data not shown).

Expression of Electrophorus AChE in P. pastoris: Catalytic Parameters

Expression of a Truncated Electrophorus AChE in P. pastoris—Because the transfected mammalian cells secreted very little Electrophorus AChE<sub>r</sub> in their culture medium, we attempted to produce this enzyme in the methylotrophic yeast P. pastoris. This yeast was found to express rat AChE in an active form but secreted it better when the C-terminal T peptide was at least partly deleted (16). We therefore introduced a stop codon immediately after the catalytic domain, replacing the

| Total Activity (cells and medium) | Cellular activity |
|---------------------------------|------------------|
| %                               | %                |
| T<sup>a</sup>                   | 100              | T<sup>a</sup>      | 61 ± 3            |
| iT<sup>a</sup>                  | 280 ± 20         | iT<sup>a</sup>     | 62 ± 3            |
| iT<sup>b</sup>                  | 250 ± 10         | iT<sup>b</sup>     | 29 ± 3            |

<sup>a</sup> >10 different transfections.
<sup>b</sup> 6–10 transfections.
<sup>c</sup> Three transfections.

Electrophorus AChE<sub>r</sub> subunits were expressed in COS cells together with the Q<sub>N</sub>/stop protein, which contains the AChE binding domain of the N-terminal region of the collagen tail (17, 30), they produced soluble tetramers that were mainly recovered in the medium (data not shown).

Expression of Electrophorus AChE<sub>r</sub> in P. pastoris:

**Table I**

| Production of recombinant Electrophorus AChE activity in transfected COS cells |
|---------------------------------|------------------|
| Total activity (cellular and secreted) obtained after 72 h at 27 °C (see "Experimental Procedures") with the iT and iT constructs is given as the proportion of that obtained in parallel transfections with a cDNA construct encoding AChE<sub>r</sub> (T), defined as 100%. The cellular activity represents the proportion of total activity that was recovered in a detergent cell extract. |

**Fig. 4.** Three-dimensional structure of Electrophorus and Torpedo AChEs. Left panels, Torpedo AChE; right panels, Electrophorus AChE. The ribbon structures of the two enzymes are shown in similar orientations, with the opening of the catalytic gorge (indicated by an arrow) on the right side (top) and on the left side (bottom). Selected residues are shown in space filling view; the active site serine (Ser-200, according to Torpedo numbering) is shown in red; in the peripheral site, tyrosine 279 is shown in green; the Ω loop (Cys-67–Cys-94) is shown in blue, with the two cysteines as well as the tyrosine 84 in the active site in dark blue. The non-conserved peptide of Electrophorus AChE and the two residues corresponding to its extremities in Torpedo AChE (415–416) are shown in yellow. Part of this peptide is predicted to assume an α-helical conformation.

**Fig. 5.** Sedimentation analysis of AChE forms produced by expressing Electrophorus AChE<sub>r</sub> in COS cells. A, cell extract; B, culture medium. The AChE activity is plotted on an arbitrary scale, as a function of S values, determined from the positions of sedimentation standards. The sedimentation was performed in sucrose gradient containing no detergent (■—■), Triton X-100 (●—●), or Brij-96 (○—○).
first residue of the T peptide and thus creating a truncated protein, which was limited to the catalytic domain, AChE
(Fig. 2). In another construction, AChE_CAT, we replaced the non-conserved 29-amino acid peptide of Electrophorus AChE by the corresponding 3 residues of Torpedo AChE, NGT, thus adding a potential N-glycosylation site. These mutated con-
structions were inserted in the Pichia expression vector pHILD2 and integrated in yeasts in the AOX gene (HIS/"Muts")
or in the HIS4 gene (HIS/"Mut+"). In both cases, the recombinant yeasts secreted active AChE, at similar levels.

Catalytic Parameters—We compared the catalytic parameters of natural AChE, purified from Electrophorus electric or-
gans, and of recombinant enzymes (Table II). The expression vectors contained cDNAs encoding AChE_P, AChE_T, or AChE_CAT
or partial genomic constructs including 3’ introns, with or without exon T (Fig. 2). We found no significant difference
between the K_m values of natural AChE and of recombinant enzymes obtained in COS cells and in Pichia, either without
salt or in the presence of 1 M NaCl. The low salt values are very similar to those obtained previously for AChE from Electroph-
orus electric organ (26, 31). The increase observed at high ionic strength values illustrates the fact that electrostatic interac-
tions participate in the binding of ligands to AChE. The higher values of K_m observed for AChE produced in Pichia, compared
with natural enzyme and that obtained in mammalian cells, is possibly related to a difference in glycosylation. The K_m values
were very similar for the recombinant enzyme secreted by Pichia and the natural enzyme from electric organs.

Structure of the Electrophorus AChE Gene

Position of Introns in the Sequence Encoding the Catalytic Domain—As indicated in Fig. 2, we amplified fragments of
genomic DNA by PCR to detect the presence of introns and to define their positions. The gene contains at least one 5’ non-
coding exon, which has not been determined (named exon 1 by analogy with that of Torpedo). The first coding exon of the
Torpedo AChE gene, which encodes most of the catalytic do-
main, corresponds to two exons in Electrophorus (exon 2 and exon 3, see Fig. 8), as in the mouse gene, but the additional
intron is located at a different position. It is noteworthy that the sequence encoding the non-conserved peptide is located at
the 3’ end of exon 2. The position of exon 4, which encodes the
end of the catalytic domain, is conserved in all three species.

Existence of an H Exon?—GPI-anchored dimers, generated from AChE/Eq subunits, have not been reported previously in Electrophorus electric organs or other tissues (24). In the present study, we failed to detect any phosphatidylinositol phospholipase C-sensitive AChE form in the liver, spleen, and electric organs (not shown). Exon 4 is separated from exon T, which encodes the C-terminal T peptide, by an intronic region of 1349 nucleotides. In the Torpedo and mammalian AChE genes, this region contains an alternative exon H, encoding a C-terminal region that would encode the C-terminal T peptide, by an intronic region of 1349 nucleotides. An analysis of this region did not reveal any open reading frame that would encode the GPI-addition signal (32, 33). An analysis of this region did not reveal any open reading frame that would encode the GPI-addition signal (32, 33). An analysis of this region did not reveal any open reading frame that would encode the GPI-addition signal (32, 33). An analysis of this region did not reveal any open reading frame that would encode the GPI-addition signal (32, 33).

To verify that transcripts AChE/VLECC or AChE/ECC generated the non-amphiphilic dimers produced from the iAT construction or a mutated imAT construction, respectively (in which the acceptor VLECC site was mutated), we inserted the coding sequence of the flag epitope immediately before the stop codon. We found that the resulting cellular and secreted enzymes did carry this epitope, as shown by retardation with the M2 monoclonal antibody in non-denaturing gel electrophoresis (Fig. 7, lanes 7–18), showing that both VLECC and ECC C-terminal peptides produced non-amphiphilic dimers and that the flag peptide was not removed upon secretion, in contrast with AChET.

Utilization of a Cryptic Upstream Donor Splice Site in Mammalian Cells—We performed RNase protection assays on mRNA extracted from COS and HEK cells transfected with cDNA/T, iAT, and imAT with two probes corresponding to the 3’ part of AChE/T or AChE/ECC (Fig. 11). We obtained the same results with the two types of transfected mammalian cells, indicating that they processed the primary transcripts in the same manner. In the case of iAT, we found that exon 4 was joined to exon T, as expected. In the case of imAT, we found that exon 4 was joined to pseudo-exon VLECC but also to another 3’ region, probably pseudo-exon ECC, in agreement with the results obtained in the RACE-3’ experiments and with the production of the flagged AChE/VLECC or AChE/ECC enzymes. Except in the case of cDNA, we also observed an unexpected heterogeneity at the junction of exon 3 and exon 4; in half of the protected fragments, exon 4 was not linked to the upstream exon 3. An analysis of the corresponding transcripts showed that splicing may occur from donor sites located in the 5’ region of exon 2, as illustrated in Fig. 2. This splicing deletes a large part of exon 2 and the totality of exon 3, e.g. most of the sequence encoding the catalytic domain, so that it cannot produce active AChE. Fig. 8 shows that splicing from the upstream donor sites, which are located in the sequence encoding the signal peptide and about 19 codons downstream of this sequence, does not respect the coding frame in either exon 4 or exon T.

Utilization of the Upstream Donor Sites and of the Pseudo-exons VLECC and ECC in Electrophorus Tissues?—Analyses of AChE from Electrophorus electric organs in sucrose gradients did not show the presence of non-amphiphilic molecules sedimenting at 7.3 S (data not shown), so that pseudo-exons VLECC and ECC do not seem to be utilized in vivo. This was confirmed by RACE-3’ experiments, which only characterized transcripts terminating with exon T, in this tissue. In addition, RNase protection assays performed with RNA extracted from Electrophorus spinal cord and brain showed that all transcripts contained exon 3, exon 4, and exon T (Fig. 11, left panel). We found no partially protected fragments that would suggest the use of alternative splicing, either upstream or downstream of exon 4 (Fig. 11, right panel), as observed in transfected mammalian cells with partial genomic constructions.

### TABLE II

|                | K<sub>m</sub> – NaCl | K<sub>m</sub> – NaCl | K<sub>m</sub> + 1 mM NaCl | K<sub>m</sub> + 1 mM NaCl | k<sub>cat</sub> |
|----------------|----------------------|----------------------|--------------------------|--------------------------|---------------|
|                | μM                   | mM                   | μM                      | mM                      | %             |
| WT (electric organs) | 104 ± 10<sup>a</sup> | 14.2 ± 1.5<sup>a</sup> | 282 ± 9<sup>b</sup>     | 20.3 ± 1.4<sup>b</sup>  | 100           |
| AChE/Eq (COS)     | 110 ± 5<sup>a</sup>  | 16.5 ± 1.6<sup>a</sup> | ND                      | ND                      | ND            |
| iAT (COS)         | 125 ± 7<sup>a</sup>  | 15.0 ± 1.1<sup>a</sup> | ND                      | ND                      | ND            |
| iAT (COS)         | 108 ± 5<sup>a</sup>  | 12.4 ± 0.9<sup>a</sup> | ND                      | ND                      | ND            |
| AChE/Eq (yeast)   | 108 ± 4<sup>a</sup>  | 18.6 ± 0.3<sup>a</sup> | 220 ± 13<sup>b</sup>    | 35 ± 9<sup>b</sup>     | 84 ± 10<sup>b</sup>|
| AChE/ECC (yeast)  | 106 ± 1<sup>a</sup>  | 23.5 ± 2.9<sup>a</sup> | 206 ± 7<sup>b</sup>     | 39 ± 7<sup>b</sup>     | 86 ± 13<sup>b</sup>|

<sup>a</sup> Data determined in duplicate, and values represent the means from three different experiments.

<sup>b</sup> Data determined in duplicate, and values represent the means from five different experiments.

Hydrolysis of acetylthiocholine was analyzed as described under “Experimental Procedures,” at room temperature (20 °C).

Data determined in duplicate, and values represent the means from five different experiments.

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Utilization of Default Acceptor Sites, Upstream of Exon T: Pseudo-exons ECC and VLECC—To determine the 3′ structure of AChE transcripts produced in cells expressing the iAT and imAT constructions, we used the RACE-3′ and RT-PCR methods with mRNA extracted from transfected COS cells (Fig. 10). The sequence of the amplified DNA fragments showed that exon 4 had been spliced to acceptor sites located 78 or 72 bp upstream of exon T, creating novel C-terminal peptide sequences of only five or three residues, VLECC or ECC. Using reverse primers located upstream of these acceptor sites, we did not detect any other splicing product. Analysis by RT-PCR of mRNA isolated from cells transfected with the iAT construction, using as reverse primer either Ro or R<sub>o</sub> (within exon T), revealed a major amplification product corresponding to the splicing of exon 4 to exon T (Fig. 10). However, using a reverse primer located upstream of exon T (R<sub>e</sub>), after two successive amplifications we observed a weak band, corresponding to the splicing of exon 4 to the upstream splice acceptor (pseudo-exons VLECC or ECC), showing that they are used at a very minor level when exon T is present.

To verify that transcripts AChE/VLECC or AChE/ECC generated the non-amphiphilic dimers produced from the iAT construction or a mutated imAT construction, respectively (in which the acceptor VLECC site was mutated), we inserted the coding sequence of the flag epitope immediately before the stop codon. We found that the resulting cellular and secreted enzymes did carry this epitope, as shown by retardation with the M2 monoclonal antibody in non-denaturing gel electrophoresis (Fig. 7, lanes 7–18), showing that both VLECC and ECC C-terminal peptides produced non-amphiphilic dimers and that the flag peptide was not removed upon secretion, in contrast with AChET.
DISCUSSION

Homology with Other AChEs—The primary sequence deduced from cDNAs encoding Electrophorus AChE corresponds to a catalytic subunit of type T, containing all the residues that characterize acetylcholinesterases (catalytic triad, tryptophan and phenylalanine residues of the active site, and tryptophan residue of the peripheral site). The six cysteines that form three intracatenary disulfide bonds and the cysteine located near the C terminus which is involved in intercatenary linkage are conserved, but there is no free cysteine in the protein, as expected from previous chemical analyses (34).

Presence of a Non-conserved Peptide, with No Homology in Mammalian or Torpedo AChEs—The most conspicuous difference with Torpedo AChE and other previously studied AChEs is the presence of an additional hydrophilic peptide of 29 amino acids. Chicken AChE also contains a glycine-rich peptidic insertion, but larger and located at a different position. A homologous peptide insertion has recently been found in AChE from the zebrafish, Danio rerio (35). A three-dimensional model, based on the three-dimensional structure of Torpedo AChE (36), shows that the non-conserved peptide is located at the surface of the protein, opposite to the entry of the catalytic gorge. In agreement with this position, we found that its deletion did not modify the catalytic parameters of the enzyme (see below).

Molecular Weight and Glycosylation—There are six potential N-glycosylation sites in Electrophorus AChE as follows: one...
site is common with both Torpedo AChE and human butyrylcholinesterase (BChE, EC 3.1.1.8) (37), one site exists in Torpedo AChE, and three other sites exist in human BChE. Only one site has no equivalent in other ChEs (Asn-161 and His-159 in Torpedo). It is likely to be glycosylated, since it is located in a loop at the surface of the protein.

The catalytic subunit of Electrophorus AChE is progressively cleaved, upon storage, into fragments of approximately 60 and 30 kDa (38), which remain attached by a disulfide bond; the larger fragment carries the active site serine (labeled with diisopropyl fluorophosphate) and about twice the amount of carbohydrates as the smaller one (39). These features are consistent with the hypothesis that cleavage occurs within the non-conserved peptide and that all potential sites are glycosylated. The presence of these glycans may explain the fact that the apparent mass of Electrophorus AChE is higher than that of Torpedo AChE, 80 and 70 kDa, respectively; this difference does not appear in non-glycosylated polypeptides obtained by in vitro translation of electric organ mRNAs (40) and is essentially abolished after deglycosylation (41).

**Structure of the Electrophorus AChE Gene**

**Intron-Exon Structure: Introduction of the Peptide Insertion**—Electrophorus and mammalian AChE genes possess an additional intron, interrupting the coding sequence of the catalytic domain, but at a different position, indicating that the introduction of introns occurred independently in several lineages during evolution. The sequence encoding the additional peptide of Electrophorus AChE is located immediately upstream of this intron, suggesting that they were introduced together. This suggests that Electrophorus and Danio, which possess a homologous peptide insertion, belong to a Teleost lineage that did not give rise to terrestrial tetrapods.

**Absence of an Alternative H Exon**—Apart from the organization of exons encoding the catalytic domain, the vertebrate AChE genes differ by the possible existence of alternative exons encoding C-terminal peptides as follows: H or T in Torpedo and mammals (7), T or S in Bungarus, or only Thr in chicken. As mentioned above, we did not find evidence for the presence of alternative exons, in addition to exon T, in Electrophorus tissues. Expression of various constructs in transfected cells confirmed the absence of an H exon. However, deletion of the T exon, or mutation of its splicing acceptor site, revealed the possible use of additional intron acceptor sites, as discussed below.

**Abnormal Splicing of Electrophorus AChE Transcripts in Transfected Mammalian Cells**—In mammalian cells, splicing from the last exon corresponding to the catalytic domain to exon T was accompanied by splicing to upstream acceptor sites, leading to pseudo-exons, located about 80 bp upstream of exon T, encoding the C-terminal peptides VLECC and ECC. These events were minor in the presence of exon T but became dominant if exon T was deleted or if its splice acceptor site was mutated. In that case, the yield of active AChE was not reduced.

More surprisingly, about half of AChE transcripts were spliced from abnormal donor sites located in the first coding exon, down to the last catalytic exon (exon 4), ignoring the donor site of the conserved intron that precedes this exon (Fig. 11). The resulting protein is of course devoid of activity, since it lacks most of the catalytic domain. These observations show that cells may operate abnormal splicing, when expressing foreign genes.

**Expression of Recombinant Electrophorus AChE**

Expression and Secretion of Electrophorus AChE in Mammalian Cells and in the Yeast P. pastoris: Catalytic Parameters—When Electrophorus AChE was expressed in transfected mammalian cells, we observed that activity was low at 37 °C and was markedly increased at 27 °C. The effect of temperature on the folding of Electrophorus AChE therefore appears intermediate, compared with Torpedo AChE, which does not acquire any activity at 37 °C (17), and the Bungarus and mammalian enzymes, which yield more activity at 37 °C than at 27 °C (5).

The catalytic parameters of recombinant Electrophorus AChE, expressed in COS cells or in the yeast P. pastoris (16), were similar to those of the natural enzyme, extracted from Electrophorus electric organs. In addition, the deletion of the

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non-conserved peptide did not modify the yield of AChE activity or its secretion, in either mammalian cells or Pichia. Therefore, the presence of this peptide does not seem to affect the folding of Electrophorus AChE and does not modify its catalytic activity.

Characterization of the Electrophorus AChE T Subunit and of Abnormally Spliced AChE VLECC and AChE ECC: Quaternary Associations—The processing of Electrophorus AChE T subunits in mammalian COS, HEK, or CHO cells was the same and produced the same types of molecular forms as for AChE T subunits from other species, mostly amphiphilic dimers (G2a) together with both amphiphilic and non-amphiphilic tetramers (G4a and G4na). AChET subunits have previously been shown to generate dimers that were defined as amphiphilic forms of type II (22), and amphiphilic tetramers have also been obtained in transfected cells (30). The hydrophobic character of these mol-

\[ \text{FIG. 10. PCR analysis of AChE transcripts in COS cells, after transfection with the partial genomic constructs iT and iAT.} \]

\[ \text{The structure of the constructs is shown at the top, with the positions of the oligonucleotide primers (the dashed line corresponds to the vector sequence, located between the insert and the polyadenylation site).} \]

\[ \text{The mRNA was reverse-transcribed with (dT)17-Ri-Ro and amplified with F3 and various reverse primers (Ro, R3, R5 or R6). The products obtained after reamplification with F4 and Ri, R3, R5, or R6 are as illustrated.} \]

\[ \text{The structure of the amplified products corresponding to each lane is schematically shown at the bottom. Lane m corresponds to size markers (1-kilobase pair ladder, Life Technologies, Inc.).} \]

\[ \text{FIG. 11. RPA of AChE transcripts obtained in COS and HEK cells and in Electrophorus tissues.} \]

\[ \text{The probes contained a fragment of vector DNA, exon 3, exon 4 and either exon T (probe P1, left panel) or pseudo-exon VLECC (probe P2, right panel), as schematically indicated.} \]

\[ \text{The lanes correspond to the intact probes and to protected fragments obtained with transcripts from constructs iT, T(cDNAa), and iAT (see Fig. 2), expressed in COS cells (C) or HEK cells (H), and with mRNA from Electrophorus brain (Br) and spinal cord (SC). The structure of the protected fragments is indicated.} \]

\[ \text{Note that AChET transcripts are the only ones found in the case of the cDNA construct (T) and in the tissues.} \]

\[ \text{In the case of COS and HEK cells transfected with iT, they represent about half of the transcripts, another major component corresponding to transcripts lacking exon 3, derived from abnormal upstream splice donor sites (see Fig. 2).} \]

\[ \text{In the case of iAT, the upper two bands seen with probe P2 correspond to the use of VLECC and ECC acceptor sites.} \]

\[ \text{The lower bands were produced from abnormal upstream donor sites, with removal of exon 3.} \]
ecules probably derives from the amphiphilic α-helical structure of the T peptide (7); amphiphilic and non-amphiphilic tetramers may thus differ by the exposure or occlusion of this peptide motif. Like Torpedo and mammalian AChE subunits (17, 30, 42), the Electrophorus AChE subunits presented the capacity to form heteromeric quaternary associations, since they were organized into tetramers in the presence of a QN binding domain (30).

The proportion of tetramers was relatively higher in the medium than in the cell extracts, as also observed in the case of rat AChE. In contrast with rat AChE, however, we found only traces of monomers, suggesting that Electrophorus subunits have a greater tendency to form dimers or are unstable in the monomeric form. In fact, the AChECL and AChEECC subunits, which are obtained by abnormal splicing in mammalian cells and terminate with short peptides, VLECC or ECC, were found to produce non-amphiphilic dimers, probably linked by disulfide bridges via their C-terminal cysteines. Therefore, the T peptide is not required for dimerization of AChE.

The sedimentation coefficients of the secreted G2α forms were slightly but significantly higher than those of the corresponding cellular molecules. This shows that the release of AChE into the medium does not reflect leakage from lysed cells but actually represents a true secretory process, which is accompanied by post-translational modifications. As previously observed in the case of rat AChE, we found that a flag peptide epitope, added by mutagenesis at the end of the T peptide, could be recognized by the specific M2 monoclonal antibody in secreted molecules, indicating that cleavage does not occur within the flag peptide itself. Thus, proteolysis appears to take place in the T peptide upon secretion, but it certainly does not remove the amphiphilic α-helix, in which the conserved aromatic residues form a hydrophobic patch (7), since the secreted molecules retain their interaction with detergents.

In conclusion, the cloning and expression of Electrophorus AChE allows interesting comparisons with other AChE genes, illustrating general evolutionary processes, in particular the introduction of introns that may be partially integrated into coding sequences and the loss or acquisition of alternative exons. The production of recombinant Electrophorus AChE shows that its processing and quaternary associations are similar but not identical to those of AChEs from other species.

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