H and T Subunits of Acetylcholinesterase from *Torpedo*, Expressed in COS Cells, Generate All Types of Globular Forms

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Abstract. We analyzed the production of *Torpedo marmorata* acetylcholinesterase (AChE) in transfected COS cells. We report that the presence of an aspartic acid at position 397, homologous to that observed in other cholinesterases and related enzymes (Krejci, E., N. Duval, A. Chatonnet, P. Vincens, and J. Massoulié. 1991. *Proc. Natl. Acad. Sci. USA.* 88:6647–6651), is necessary for catalytic activity. The presence of an asparagine in the previously reported cDNA sequence (Sikorav, J. L., E. Krejci, and J. Massoulié. 1987. *EMBO* (Eur. Mol. Biol. Organ.) J. 6:1865–1873) was most likely due to a cloning error (codon AAC instead of GAC). We expressed the T and H subunits of *Torpedo* AChE, which differ in their COOH-terminal region and correspond respectively to the collagen-tailed asymmetric forms and to glycophosphatidylinositol-anchored dimers of *Torpedo* electric organs, as well as a truncated T subunit (TΔ), lacking most of the COOH-terminal peptide. The transfected cells synthesized similar amounts of AChE immunoreactive protein at 37° and 27°C. However AChE activity was only produced at 27°C and, even at this temperature, only a small proportion of the protein was active.

We analyzed the molecular forms of active AChE produced at 27°C. The H polypeptides generated glycophosphatidylinositol-anchored dimers, resembling the corresponding natural AChE form. The cells also released non-amphiphilic dimers G_2^{na}. The T polypeptides generated a series of active forms which are not produced in *Torpedo* electric organs: G_1^a, G_2^a, G_4^a, and G_4^{na} cellular forms and G_2^a and G_4^{na} secreted forms. The amphiphilic forms appeared to correspond to type II forms (Bon, S., J. P. Toutant, K. Méflah, and J. Massoulié. 1988. *J. Neurochem.* 51:776–785; Bon, S., J. P. Toutant, K. Méflah, and J. Massoulié. 1988. *J. Neurochem.* 51:786–794), which are abundant in the nervous tissue and muscles of higher vertebrates (Bon, S., T. L. Rosenberry, and J. Massoulié. 1991. *Cell. Mol. Neurobiol.* 11:157–172). The H and T catalytic subunits are thus sufficient to account for all types of known AChE forms. The truncated TΔ subunit yielded only non-amphiphilic monomers, demonstrating the importance of the T COOH-terminal peptide in the formation of oligomers, and in the hydrophobic character of type II forms.

THE molecular forms of acetylcholinesterase (AChE[1], E. C. 3117) are oligomers of catalytic subunits, associated in some cases with structural subunits. They have been classified as asymmetric forms (*A*) and globular forms (*G*), according to the presence or absence of a collagenic tail (Massoulié and Bon, 1982). The electric organs of *Torpedo* contain collagen-tailed forms and glycophosphatidylinositol (GPI)-anchored dimers, which incorporate two different types of catalytic subunits, differing in their COOH-terminal peptidic sequence. They are derived from a single gene, which generates multiple mRNAs by alternative splicing (Schumacher et al., 1986; Sikorav et al., 1987, 1988; Maulet et al., 1990).

Each coding sequence consists of three exons. The signal peptide and most of the primary structure of the mature protein are encoded by two common exons, I and II (Maulet et al., 1990). The COOH-terminal region is encoded by alternative exons, III_T and III_H, producing T and H polypeptides, generating respectively the GPI-anchored dimers and the collagen-tailed forms, in Torpedo electric organs (reviewed in Massoulié et al., 1992). In *Torpedo*, exon III_H encodes a 31-amino-acids peptide which is partially hydrophobic. In the mature GPI-anchored dimers, most of this COOH-terminal peptide is replaced by the glycolipidic anchor (Gibney et al., 1988). Exon III_T encodes a 40-amino acids peptide, which is conserved in the mature collagen-tailed forms. The cysteine residue located at the fourth position from the COOH-terminus (Cys-572 in *Torpedo* AChE) is involved in the formation of intersubunit disulfide bonds, linking catalytic subunits as dimers (MacPhee-Quigley et al., 1986).

Catalytic subunits of type T have been characterized in the soluble tetramers of human butyrylcholinesterase (BuChE,

1. *Abbreviations used in this paper*: A, asymmetric; AChE, acetylcholinesterase; AChR, acetylcholine receptor; BuChE, butyrylcholinesterase; DOC, deoxycholate; DS, detergent soluble; G, globular; GPI, glycophosphatidylinositol; LSS, low-salt soluble.

E.C. 3118) (Lockridge et al., 1987*a*,*b*) and in the hydrophobic-tailed tetramers of membrane-bound AChE from mammalian brain (Roberts et al., 1991).

In the present paper, we describe the production of *Torpedo marmorata* AChE in transfected COS cells. We analyze the AChE protein, its catalytic activity, and the molecular forms generated from the T and H subunits and from a truncated T subunit containing only four residues beyond the common catalytic domain.

Materials and Methods

Isolation of λAChE$_T$

We screened a cDNA library constructed in λZAP from poly-A$^+$ mRNA of *T. marmorata* electric organ (Krejci et al., 1991*a*) with the 1,208-bp probe previously described: 18 positive clones were examined by restriction analysis and by PCR, using oligonucleotides specific for the 5′ and 3′ ends of the mRNA as primers. Eight of these clones presented the pattern expected for a cDNA encoding the complete AChE$_T$ subunit. One of them, λAChE$_T$, was sequenced (part of this sequence is shown in Fig. 1).

H and T Expression Vectors

The structure of the expression vectors is illustrated in Fig. 2. λAChE$_T$ was excised and recovered in BlueScript SK$^-$, with its coding sequence in the KpnI to SacI orientation. A 2,800 bp, HindIII–NcoI fragment was introduced with adaptors into the BstXI site of CDM8 (Seed, 1987; Aruffo and Seed, 1987). One correctly oriented CDM8 recombinant was used for all transfection experiments after verification of the sequence.

CDM8-AChE$_H$ was constructed by combining pAChE2 (Sikorav et al., 1987) and λAChE9 (Sikorav et al., 1988), and replacing a BglII–NsiI restriction fragment (455 bp) containing an Asn residue at position 397 with the corresponding fragment from λAChE$_T$, containing Asp at the same position.

The T and H constructions were also introduced in the pEF-BOS vector (Mizushima and Nagata, 1990), using the strategy described above. The expressed cDNA is placed under the control of the human EF-1α promoter (Uetsuki et al., 1989). The yield of AChE activity was in general higher with pEF-BOS than with CDM8.

Construction of a Truncated T Sequence, TΔ

A 352-bp segment of the coding sequence of AChE$_T$, between nucleotides 1,345 and 1,696 (numbering from the first base of the initiation codon) was amplified by PCR, using an oligonucleotide primer which introduced a G to T mutation at position 1,690. This creates a TAG stop codon, terminating the protein after Asp-539, the fourth residue of the diverging COOH-terminal T sequence. The 5′ primer, corresponding to nucleotides 1,345–1,362, was ATATGCCCTTTGATGCAC; the 3′ primer was TCCACTGGATCCCTGCCTAGTCAATGGTCTC. The bold type A is the mutated residue (nucleotide 1,690), and the underlined nucleotides correspond to a BamHI restriction site, introduced for convenience. The amplification product was purified and subcloned in the PCR 2,000 vector (TA-cloning kit from InVitrogen, San Diego, CA). An NsiI and BamHI fragment was subcloned into the vector, pEF-AChE$_T$, digested by HindIII and NsiI. One of the transformants showing the correct restriction enzyme pattern of digestion was used to prepare DNA for transfection. This DNA was fully sequenced.

Transfections

All transfection experiments were performed in DME, with 10% Nu-serum, with DNA doubly purified by CsCl gradients. The Nu-serum was treated with soman (10^{-7} M), to inhibit all cholinesterase activity. Excess soman was hydrolyzed during storage at 4°C (at least 3 d), so that it did not interfere with AChE from the cultured cells. COS-7 cells were seeded at 10^6/10-cm plate, in 10 ml of DME supplemented with 10% Nu-serum, and transfected the next day with 1 μg of DNA per plate, by the DEAE/dextran transfection method (Selden et al., 1986). The cells were treated with chloroquine (100 μM) and DMSO (10%). After incubation for 24 to 48 h at 37°C, the medium was changed and the cells were generally transferred at 27°C for 48 h.

In most experiments, a control dish was transfected with the same amount of DNA of the β-galactosidase expression vector, PCH110 (Pharmacia LKB Biotechnology, Uppsala, Sweden). We found that 15 to 30% of the COS cells were successfully transfected, as shown by staining with the chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma Chemical Co.), as described by Herbomel et al. (1984).

Histochemical Staining of AChE Activity in Transfected COS Cells

COS cells were transfected in 35-mm plates. After incubation for 48 h at 37°C and 48 h at 27°C, the cells were washed twice with PBS (8 g/l NaCl, 2 g/l KCl, 14.4 g/l Na_2HPO_4, 2.4 g/l KH_2PO_4, pH 7.4), fixed with a solution of 4% paraformaldehyde and 0.4% glutaraldehyde for 15 min at room temperature. The cells were again washed in PBS, and treated as described by Karnovsky and Roots (1964).

AChE Activity and Protein Assays

Protein concentrations were determined by the method of Lowry et al. (1951). AChE activity was determined by the colorimetric method of Ellman et al. (1961), using acetylthiocholine as substrate, in the presence of dithiobisdinitrobenzoic acid. One Ellman unit is defined as the activity producing an increase in optical density of 1 OD unit per minute in 1 ml of assay medium (1-cm pathlength), and corresponds to the hydrolysis of 75 nmoles of substrate per min. In the case of *Torpedo* AChE, 1 μg corresponds to ~50 Ellman units (Vigny et al., 1978).

Preparation of Culture Media and Cellular Extracts, Assay, and Analysis of AChE Molecular Forms

Culture media were collected and centrifuged at 100,000 *g* for 1 h to remove cellular debris. The supernatant was submitted to isopycnic equilibration in a CsCl gradient (40 *g* CsCl for 100 ml; centrifugation in rotor [Ti50; Beckman Instruments Inc.] for 120 h, 47,000 rev/min, at 15°C). AChE activity was concentrated about 30 times in the pooled fractions. These fractions were diluted as required for sedimentation and electrophoretic analyses.

Culture dishes were cooled on ice and washed twice with cold TBS (25 mM Tris-HCl, pH 7.4, 150 mM NaCl). The cells were scraped in 2 ml of cold TBS, centrifuged 4 min at 3,500 rpm and the cellular pellet from each 10-cm dish was resuspended in 200 μl of extraction buffer containing antiproteolytic agents (1 mM EDTA, 0.1 mM benzamidine, 0.1 mg/ml bacitracin, 2.5 U/ml aprotinin, 5 μg/ml pepstatin A, 5 μg/ml leupeptin) and homogenized in a Potter glass teflon homogenizer. The homogenate was then centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant obtained was assayed for AChE activity and used for subsequent analyses. Low-salt soluble (LSS) fractions were obtained in 10 mM Tris-HCl, pH 7, 50 mM $MgCl_2$. Detergent-soluble (DS) fractions were obtained from the pellet resulting from low-salt extraction, by re-homogenizing in the same buffer, supplemented with 1% Triton X-100. Total extracts of all globular forms of AChE were obtained in one step, as low-salt detergent (LSD) fractions in 10 mM Tris-HCl, pH 7, 50 mM $MgCl_2$, 1% Triton X-100.

Sedimentation Analyses

The cell extracts and the culture media were centrifuged in sucrose gradients (5–20% sucrose, wt/vol) in a centrifuge (SW41; Beckman Instruments, Inc.) at 4°C, 40,000 rpm. The gradients were prepared in the low-salt buffer, without detergent or supplemented with either 1% Triton X-100 or 1% Brij-96. Sedimentation coefficients were determined by comparison with β-galactosidase from *E. coli* (16 S), catalase from beef liver (11.3 S), alkaline phosphatase from calf intestine (6.1 S), which were included as internal standards in the gradients.

Non-denaturing Polyacrylamide Gel Electrophoresis

Electrophoresis in horizontal 7.5% polyacrylamide slab gels was performed in the absence of detergent, in the presence of 0.5% Triton X-100 or in the presence of 0.5% Triton X-100 plus 0.25% Na$^+$ deoxycholate (DOC), as described previously (Bon et al., 1988*b*). AChE activity was revealed after the migration by the histochemical staining method of Karnovsky and Roots (1964).

Antibodies against Torpedo AChE

The polyclonal serum Tor-152 (Sikorav et al., 1984) and the mAb Tor-ME8 (Musset et al., 1987) have been described previously. They recognize both T and H AChE subunits of *Torpedo* AChE, but do not react with mammalian AChE. The polyclonal antibody was preferable to obtain a quantitative immunoprecipitation, while the mAb was used in immunoblots, because of its exclusive specificity for *Torpedo* AChE.

Metabolic Labeling Experiments

After various periods of time following transfection (24 h at 37°C, or 24 h at 37°C followed by 2 h at 27°C), 35-mm dishes containing transfected COS cells were washed with Tris buffer saline and incubated 1 h in DME without serum and without methionine, at 37° or 27°C. The culture medium was removed, and the cells were incubated at the same temperature with 150 μCi of ^{35}S-L-methionine (Amersham, France) in 2 ml DME, without methionine and without serum, for 30 min. In some experiments, a chase was performed for 15 min, replacing the radioactive medium by 2 ml DME containing 10 mg/ml unlabeled methionine. The cells were then harvested, scraped in 1-ml extraction medium (0.1% SDS, 0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 100 μg/ml bacitracin), and centrifuged for 15 min at 15,000 rev/min at 4°C. An aliquot (10 μl) of the supernatant was used to count the TCA-precipitable radioactivity in a Betamatic counter (Kontron Analytical, Redwood City, CA). The TCA-precipitable radioactivity obtained at 27°C was between 50 and 60% of that obtained at 37°C. The rest of the sample was divided in two equal parts, one of which was incubated with 1/100 vol of the polyclonal anti-AChE antibody Tor-152 (Sikorav et al., 1984), and the other half with non-immune rabbit serum. After incubation overnight at 4°C, 2% of protein A-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) was added, and the samples were maintained at room temperature for 3 h under gentle agitation. After centrifugation (4 min at 4,000 rpm), the pellet was washed twice with 1% NP-40, 0.1 M NaCl, and 1 mM EDTA. The beads of protein A-Sepharose were then submitted to denaturation and reduction and the same volume of each sample was used for SDS-PAGE. The gels were stained with Coomassie brilliant blue, treated with Amplify (Amersham, France), and autoradiographed for 15 d at −70°C.

SDS-PAGE and Western Blots

SDS-PAGE and Western blots were performed, as described in Bon et al. (1991*b*), using a 1/250 dilution of ascites fluid containing the Tor-ME8 mAb and an anti-mouse IgG antibody coupled with peroxidase.

Digestion by N-glycanase

One unit of N-glycanase (Genzyme, Boston, MA) is defined as the amount of enzyme needed to hydrolyze one nanomole of [H^3]dansyl-fetuin glycopeptide per minute at 37°C. Cell or tissue extracts were incubated with N-glycanase (0.5 U per 100-μl sample) for different periods of time (6 h, or overnight) at 37°C, in 1.25% NP-40 (0.17% SDS, 0.2 M sodium phosphate, pH 8.6). The treated samples were then analyzed by SDS-PAGE and Western blotting.

Digestion and Solubilization by PI-PLC

The action of PI-PLC was assayed on whole cells and on cellular extracts. The cells from six culture dishes were washed in TBS, centrifuged at 3,500 rev/min for 4 min, resuspended in 600 μl TBS; 200-μl aliquots of this suspension were incubated at 30°C for 40 min, with or without 1/20 vol of PI-PLC from *Bacillus thuringiensis* (Sapporo Breweries Ltd., Funakoshi Pharmaceutical Co., Tokyo, Japan). Incubation was performed at 30°C to avoid loss of AChE at higher temperatures. The cells were then centrifuged as before. The AChE contents of the cells and of the supernatant were analyzed. The cellular pellet was homogenized with a glass-teflon Potter homogenizer, in the presence of 1% Triton X-100 and antiproteolytic agents, as indicated above. One third of the cell suspension was directly homogenized in this extraction medium, without prior incubation, to determine the total AChE content.

Cellular detergent extracts were incubated with PI-PLC, or with human serum containing PLD activity, under the same conditions used for the cell suspensions, to determine the sensitivity of solubilized AChE molecular forms.

Digestion by Proteinase K

Samples were incubated at 20°C for 30 min with 7 μg/ml proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany).

Results

Structure of AChE cDNA Clones and Construction of Expression Vectors

The sequence of a cDNA clone encoding the catalytic subunit of AChE from *Torpedo marmorata* was described previously (Sikarov et al., 1987). However, various expression vectors containing the corresponding coding sequence did not yield active AChE (results not shown). We suspected that this sequence might contain point mutations, probably introduced during the construction of the cDNA library. In particular, it presented an asparagine at position 397, instead of an aspartic acid as in other cholinesterases, including AChE from *Torpedo californica* (Schumacher et al., 1986), and other esterases presenting homology with cholinesterases (Krejci et al., 1991*b*). We therefore decided to analyze independent cDNA clones. A cDNA library from electric organ, which was described previously (Krejci et al., 1991*a*) was screened with a 1,208-bp AChE probe (Sikarov et al., 1988). We thus obtained a new clone, λAChE$_T$, encoding the AChE subunit of type T. The coding sequence of this clone differs from the previously published one by a single base, predicting the presence of Asp (codon GAC), instead of Asn (codon AAC) at position 397. This sequence produced active AChE in transfected cells, as shown in the present report.

In addition, λAChE$_T$ differs from the previously described cDNA structures in its non-coding sequences (Fig. 1). The 5′ non-coding sequence contains the 38 nucleotides sequence which was previously shown to be present or absent in cDNAs and probably represents a small alternatively spliced exon (Sikorav et al., 1987). Upstream of this sequence, λAChE$_T$ corresponds to an untranslated sequence which was previously described in *T. californica* (Schumacher et al., 1986). Its presence has not been directly demonstrated in *T. marmorata*, but was inferred from an analysis of S1 nuclease fragments (Sikorav et al., 1987). This structure confirms the complexity of the 5′ untranslated region of the AChE gene, possibly resulting in part from the existence of several transcription initiation sites.

The 3′ non-coding sequence of λAChE$_T$ differs only punctually from that described previously for the T subunit (Sikorav et al., 1987). It was sequenced only over 700 nucleotides, down to an EcoRI restriction site. The differences observed may result from errors in the construction of the libraries, or represent allelic variants in the *Torpedo* population.

CDM8 vectors expressing the T and H subunits (Fig. 2) were constructed as described in the Materials and Methods section, from the λAChE$_T$ clone, and from a combination of λAChE$_T$, pAChE2 (Sikorav et al., 1987), and λAChE8 (Sikorav et al., 1988), the latter encoding the COOH-terminal part of the H subunit.

Endogenous AChE in COS Cells

Non-transfected COS cells, or cells which had received a control CDM8 vector, contained very little AChE activity,

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1 GCACACACTCATCCACGCAGGTCTTGATGAAGTCGGTGACACCTGTTGCATATTCATTCAAGTCTATGGGCAGCAGGGATCTTTGCCTTCCTTGACAAGCTGGAGAGTTGCAAAGCAGACATGAGAGAAATG
2 GCACACACTCATCCACGCAGGTCTTGATGAAGTCGGTGACACCTGTTGCATATTCATTCAAGTCTATG.....GTTGCAAAGCAGACATGAGAGAAATG
3 CGCGGGTTGTATAGCTGACTGCTTCACCACCTCTGCAGTCCAGAGGGTGGACCGTCTGATTGCATACAGGCAGCAGGGATCCTTGCCTTCCTTGACAAGCTGGAGAGTTGCAAAGCAGACACGAGAGAAATG
4 CGCGGGTTGTATAGCTGACTGCTTCACCACCTCTGCAGTCCAGAGGGTGGACCGTCTGATTGCATACA.....TTGCAAAGCAGACACGAGAGAAATG
5 AGCTGACTGCTTCACCACCTCTGCAGTCCAGAGGGTGCACCGTCTGATTGCATACA.....GTTGCAAAGCAGACACGAGAGAAATG
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Figure 1. The structure of 5′ non-coding regions of different *Torpedo* AChE cDNA clones. The sequences of clones λAChE3 (*1*) and λAChE11 (*2*) of *T. marmorata* AChE (Sikorav et al., 1987) and two sequences from *T. californica* (*3* and *4*) (Schumacher et al., 1986) were previously described. The new sequence reported here ($\lambda AChE_T$) (*5*) shows that *T. marmorata* possesses a 5′ region which had only been found hitherto in *T. californica*. The two different 5′ regions may correspond to distinct transcription origins.

~0.5 10^{-4} Ellman U/mg protein, corresponding to G_1^a and G_2^a (respectively, 4.3 S and 6 S in the presence of Triton X-100, 2.8 S, and 4.5 S in the presence of Brij-96) (see Fig. 5). These cells secreted a low level of AChE, mostly G_4^{na} (10.5 S) and G_1^{na} (4.5 S).

The endogenous AChE activity was usually negligible under the assay conditions used to analyze AChE forms, except in some experiments where the cells failed to produce a high level of *Torpedo* AChE activity (see Fig. 5). In any case, the AChE activity originating from the *Torpedo* expression vectors could be specifically recognized by the mAb Tor-ME8 (Musset et al., 1987), or by the polyclonal antiserum Tor-152 (Sikorav et al., 1984).

Effect of Temperature on the Production of AChE Activity in Transfected COS Cells

COS cells were transfected with the CDM8-$AChE_H$, CDM8-$AChE_T$ or control CDM8 vectors, using the DEAE-dextran procedure, as described in the Materials and Methods section. In agreement with the report of Gibney and Taylor (1990), transfected COS cells did not produce any *Torpedo* AChE activity when grown at 37°C. After transferring the cultures to 27°C, significant activity appeared after 1 h. After 48 h at 27°C, the cells contained about 1–3 and 2–6 Ellman U/mg protein in the case of $AChE_T$ and $AChE_H$, respectively. This amount of active enzyme represented <5% of the AChE-immunoreactive protein (see below). The culture medium also presented a significant AChE activity, under these conditions. The amount of released activity was similar to that contained in the cells after 24 h at 27°C, and could be analyzed after adequate concentration (see Materials and Methods).

The transfected COS cells, producing active AChE at 27°C, were stained by the histochemical method of Kar-

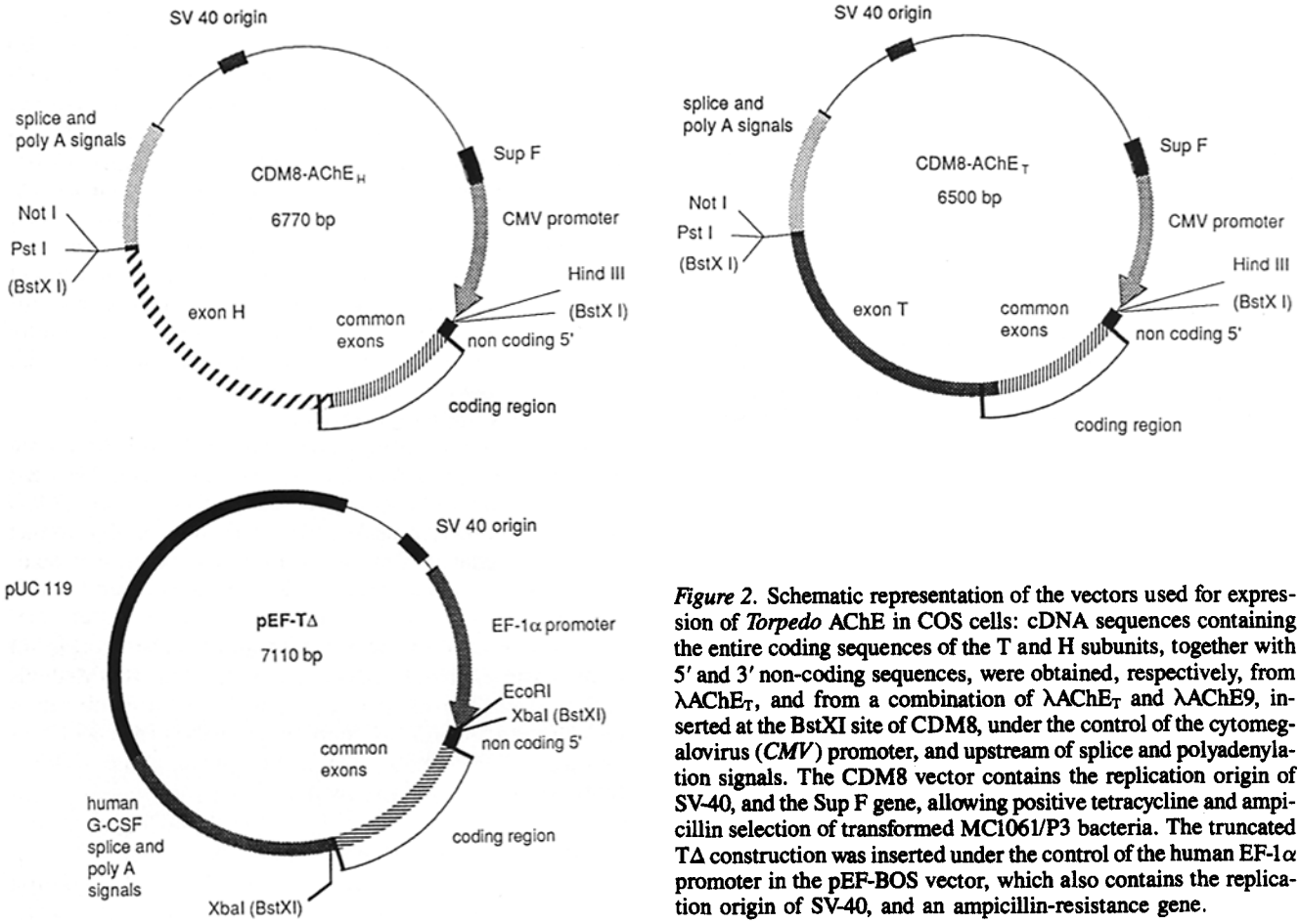

Figure 2. Schematic representation of the vectors used for expression of *Torpedo* AChE in COS cells: cDNA sequences containing the entire coding sequences of the T and H subunits, together with 5′ and 3′ non-coding sequences, were obtained, respectively, from $\lambda AChE_T$, and from a combination of $\lambda AChE_T$ and λAChE9, inserted at the BstXI site of CDM8, under the control of the cytomegalovirus (*CMV*) promoter, and upstream of splice and polyadenylation signals. The CDM8 vector contains the replication origin of SV-40, and the Sup F gene, allowing positive tetracycline and ampicillin selection of transformed MC1061/P3 bacteria. The truncated TΔ construction was inserted under the control of the human EF-1α promoter in the pEF-BOS vector, which also contains the replication origin of SV-40, and an ampicillin-resistance gene.

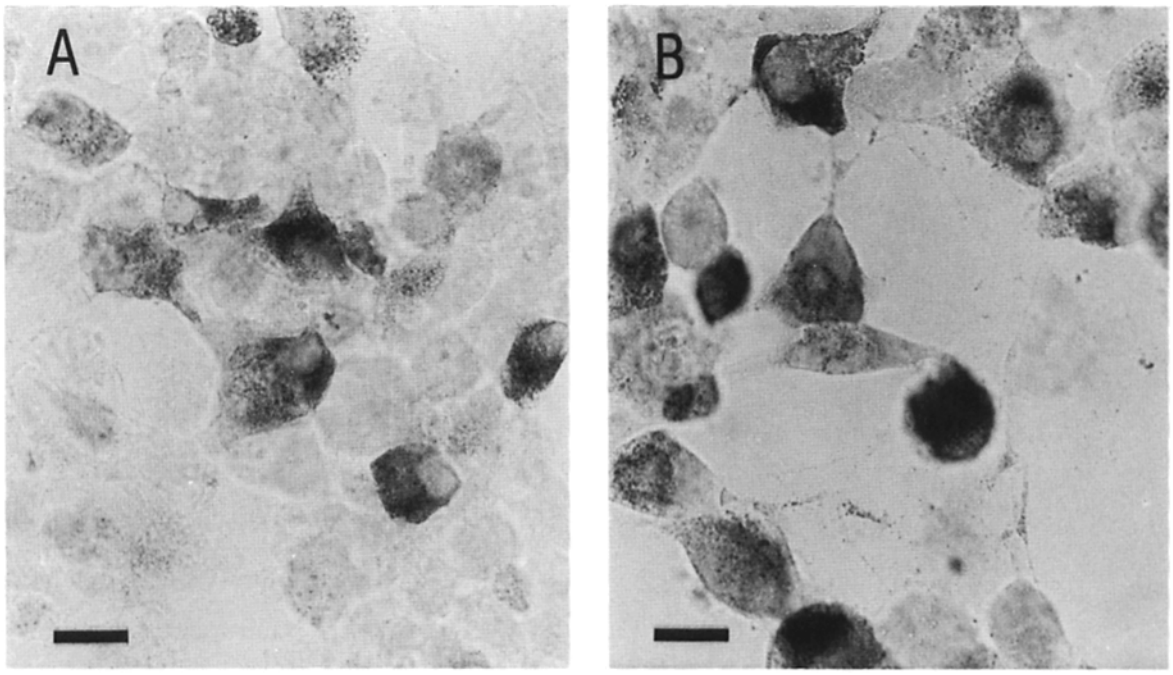

Figure 3. Histochemical staining of AChE activity in transfected cells. COS cells transfected with the CDM8-AChE$_T$ vector, and incubated at 27°C for 48 h, as indicated in Methods, were stained by the method of Karnovsky and Roots (1964). *A* and *B*, different cell densities. Bar, 10 μm.

novsky and Roots (1964), as shown in Fig. 3. The proportion of the cells which displayed active AChE was similar to that of cells producing β-galactosidase activity in control transfections with a β-galactosidase expression vector (see Materials and Methods). The morphology of the cells depended on their density: they extended long processes when dispersed on the substrate. The AChE expressing cells did not seem to differ in their morphology from the negative cells.

Sedimentation Analysis of Active AChE in Transfected Cells

Active AChE forms were analyzed by sedimentation in extracts from transfected cells, maintained at 27°C for 48 h (Fig. 4 *A*) and in the culture medium (Fig. 4 *B*). AChE was solubilized either in a single step, in the presence of 1% Triton X-100 (low salt/detergent extract), or in two successive extractions, first without detergent (LSS fraction) and subsequently in the presence of 1% Triton X-100 (DS fraction). Analysis of the low salt/detergent extracts in sucrose gradients, containing either Triton X-100 or Brij-96, revealed the presence of amphiphilic forms whose sedimentation coefficients were lower in the presence of Brij-96, and non-amphiphilic forms whose sedimentation coefficients were identical in both detergents.

In the case of AChE$_H$ (Fig. 4 *A*, *lower part*), AChE activity corresponded almost exclusively to a G_2^a form (6.8 S in Triton X-100 and 4.7 S in Brij-96), accompanied by a trace of G_2^{na} form (7.5 S). Only 10% of G_2^a was solubilized in the LSS fraction, the bulk of this amphiphilic form being recovered in the DS fraction. The culture medium contained essentially a non-amphiphilic G_2^{na} form (Fig. 4 *B*, *lower part*), together with a trace of G_4^{na} form, most likely resulting from the secretion of endogenous AChE by the COS cells. The absence of any detectable G_2^a form indicated that the centrifuged and concentrated medium was not significantly contaminated by cell debris.

In the case of AChE$_T$ (Fig. 4 *A*, *upper part*), the cell extracts contained four AChE forms: G_1^a (5 S in Triton X-100 and 3.5 S in Brij-96), G_2^a (6.4 S in Triton X-100 and 3.5 S in Brij 96), G_4^a (10 S in Triton X-100 and 9 S in Brij-96) and G_4^{na} (11.3 S). The proportions of these forms were in the order of 30% (G_1^a), 50% (G_2^a), 5–10% (G_4^a), and 10–20% (G_4^{na}). They varied, however, depending on the experiment (compare for example the profiles illustrated in Fig. 4 *A* and in Fig. 5 *A*). Most of the G_1^a and G_4^{na} forms were solubilized in the LSS fraction, together with a minority of G_2^a. The DS fraction contained most of G_4^a and G_2^a, together with the remaining G_1^a and G_4^{na} forms. The culture medium contained equivalent amounts of G_2^a and G_4^{na}, with a trace of G_4^a (Fig. 4 *B*).

COS cells expressing the truncated T subunit, TΔ, contained only about a third of the activity obtained with the complete T subunit, in parallel transfections using the same vector. Besides the endogenous G_1^a and G_1^{na} forms (2.8 S and 4.5 S, in the presence of Brij-96), the cellular extract contained a single *Torpedo* AChE form, G_1^{na}, that sedimented at 4.8 S and reacted with the anti-*Torpedo* AChE polyclonal antiserum Tor-152 (Fig. 5). In the culture medium, we did not detect any additional component, other than those obtained with non-transfected cells (not shown).

Non-denaturing Electrophoretic Analysis of AChE Molecular Forms

We confirmed the amphiphilic character of some AChE forms by charge-shift electrophoresis: the AChE forms obtained in the T and H transfections were analyzed in non-denaturing polyacrylamide gels containing either Triton X-100 alone, Triton X-100 and deoxycholate, or no deter-

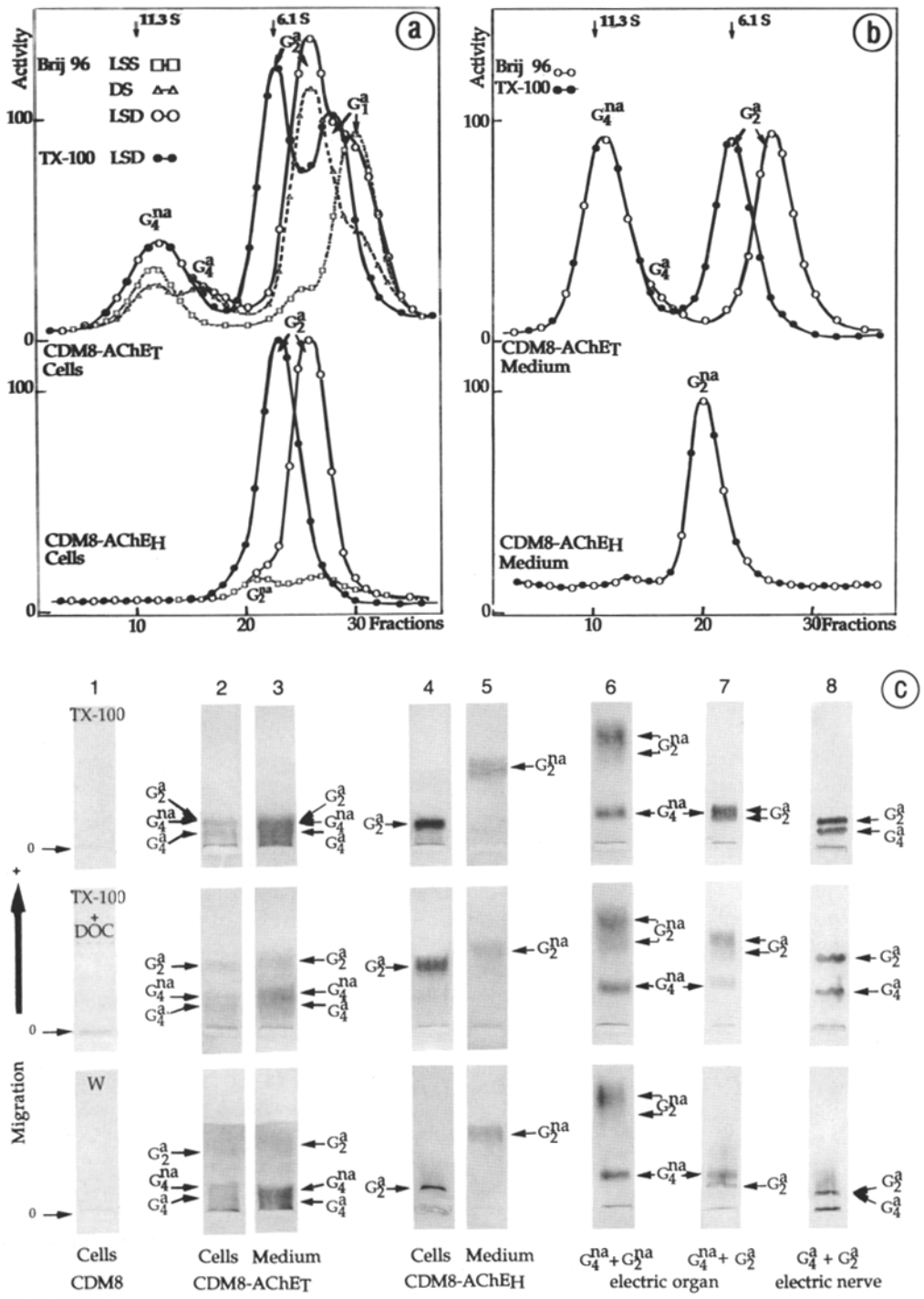

Figure 4. Sedimentation and electrophoretic analyses of active *Torpedo* AChE produced in COS cells expressing the T and H subunits. (*A*) Sedimentation profiles of cellular extracts, in the presence of 1% Triton X-100 (*black symbols*) or 1% Brij-96 (*open symbols*): LSS fraction (□···□); DS fraction (△—△); total LSD extract (○—○, ●—●). Samples of extracts were layered on the gradients; 150-μl aliquots of each fraction were assayed in 1 ml of Ellman reaction medium, for 2–6 h at room temperature in the case of cellular extracts, and overnight at 4°C in the case of concentrated culture media. The activity is plotted on an arbitrary scale. Note the absence of any visible activity peak around 4 S, in the CDM8-AChE$_H$ cell extract, indicating that the endogenous G_1 form is not visible in these assay conditions. (*B*) Sedimentation profiles of culture media, in the presence of 1% Triton X-100 (*closed symbols*) or 1% Brij-96 (*open symbols*). (*C*) Non-denaturing gel electrophoresis of cell extracts (lanes *1*, *2*, and *4*), and culture media (lanes *3* and *5*). AChE from *Torpedo* tissues are illustrated in lanes *6*, *7*, and *8* for comparison: lane *6* corresponds to lytic, non-amphiphilic forms obtained by proteolysis of electric organ AChE, lane *7* to native GPI-anchored dimers from electric organs (note the presence of two electromorphs), and lane *8* to natural G_4^{na} and GPI-anchored G_2^a from electric nerves. Lanes *1*, *2*, and *4* refer respectively to control, non-transfected cells, and to cells transfected with CDM8-AChE$_T$ and CDM8-AChE$_H$. The absence of any detectable AChE activity in lane *1* indicates that the bands seen in the other lanes all correspond to the transfected *Torpedo* enzyme. The migration was performed in the presence of 0.5% Triton X-100 (*TX-100, upper panel*), in the presence of 0.25% Na^+ deoxycholate and 0.5% Triton X-100 (*TX-100 + DOC, middle panel*), and in the absence of detergent (*W, lower panel*). The different molecular forms are identified by arrows.

gent, and compared with the corresponding forms extracted from *Torpedo* electric organ (G_2^a and the lytic forms G_2^{na} and G_4^{na}) and nerves (Fig. 4 *C*, lanes *6–8*). The mobility of the AChE forms produced by transfected COS cells was similar to that of their homologs from *Torpedo* tissues, but not identical, probably because of differences in glycosylation such as those observed between different tissues (Bon et al., 1988*b*).

The G_2^a form produced by H-transfected cells resembled the nerve and electric organ GPI-anchored G_2^a forms, in the three electrophoretic conditions (Fig. 4 *C*, compare lanes *4* and *5*). In particular, they formed a thin line in the absence of detergent, indicating their aggregation. The G_2^{na} form from the culture medium migrated slightly but significantly faster than the lytic form obtained from the cellular G_2^a form after digestion with PI-PLC (see next section).

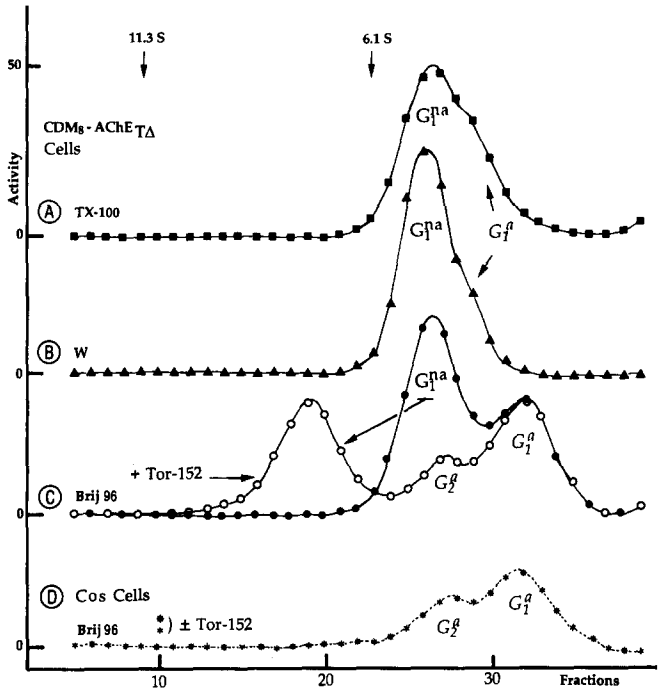

Figure 5. Sedimentation analysis of AChE forms obtained in COS cells expressing the truncated TΔ subunit. Total low salt detergent extracts of transfected (*A*, *B*, and *C*) and control (*D*) COS cells were analyzed in sucrose gradients, in the presence of Triton X-100 (*A*), without detergent (*B*), and Brij-96 (*C* and *D*). In *C* and *D*, samples of the extracts (o—o, *—*) were incubated for 4 h at 20°C with 1/100 vol of antiserum Tor-152, previously treated with soman as indicated in the Materials and Methods section. The extracts from untransfected cells were prepared at the same protein concentration and analyzed in the same conditions as those of transfected cells, so that the activities are directly comparable in the figure.

In the case of T-transfected cells, the G_2^a forms from cellular extracts and from the culture medium differed in their electrophoretic migration (Fig. 4 *C*, lanes *2* and *3*), but were both subject to charge shift, indicating their amphiphilic character. When solubilized in the presence of Triton X-100, the cellular G_2^a form aggregated in electrophoresis without detergent, because it retained Triton X-100 micelles, as previously described for other amphiphilic forms of type II (Bon et al., 1991*a*). The G_1^a form of T extracts was not detected in these analyses, probably because its activity was not stable in the conditions of electrophoresis (pH 8.9) or of staining (pH 5.2).

Subunits H Form Glycolipid-anchored Dimers in Transfected COS Cells

The GPI-G_2^a AChE form of *Torpedo* electric organs presents a characteristic sensitivity to PI-PLC (Futerman et al., 1983), as well as GPI-anchored proteins synthesized in COS cells (Moran et al., 1991). We therefore examined the effect of PI-PLC on *Torpedo* AChE forms produced in COS cells. Total cellular extracts were treated with PI-PLC at 30°C and analyzed by sedimentation in sucrose gradients (Fig. 6 *A*), and by non-denaturing electrophoresis in the presence of Triton X-100 (Fig. 6 *B*).

PI-PLC converted the G_2^a form of an H extract into a non-amphiphilic G_2^{na} form (lower part), without loss of activity. This lytic G_2^{na} form migrated slightly slower, in non-denaturing electrophoresis, than the G_2^{na} form released in the culture medium. The G_2^a obtained in H transfections was also sensitive to PLD from human serum, producing a G_2^{na} form which migrated slightly slower than that produced by PI-PLC, in agreement with the fact that it does not retain the phosphate group, and therefore possesses a smaller

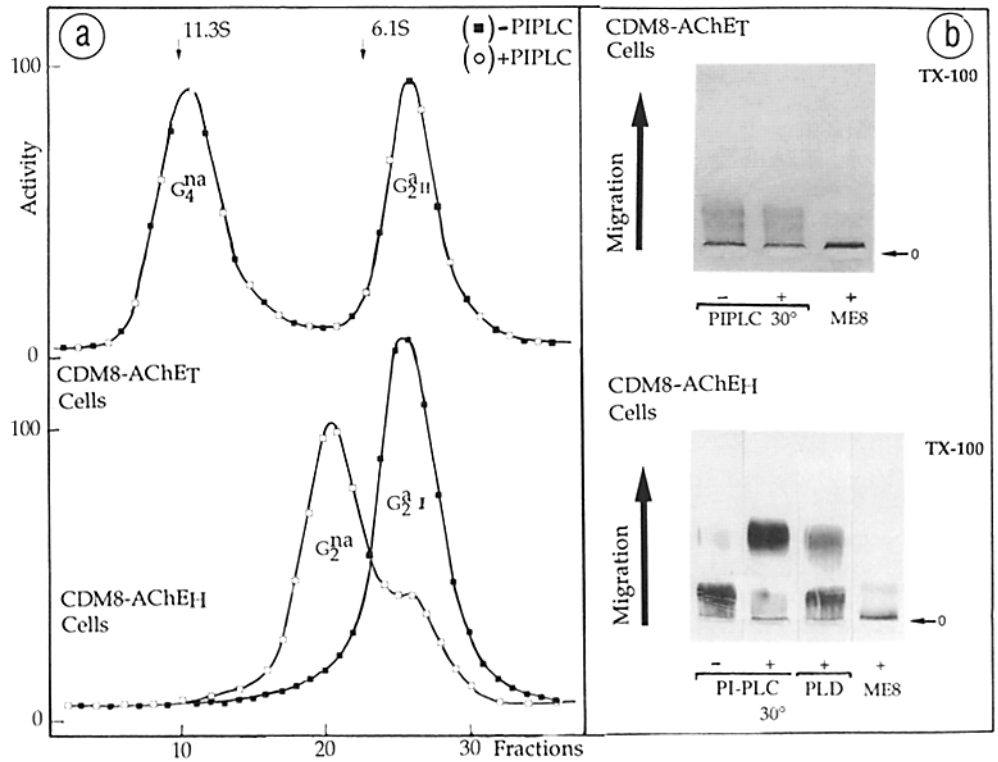

Figure 6. Sensitivity to PI-PLC of active *Torpedo* AChE produced in transfected COS cells: sedimentation and electrophoretic analyses. Upper parts of the figure, extracts from cells transfected with CDM8-$AChE_T$; lower parts, extracts from cells transfected with CDM8-$AChE_H$. (*A*) Sedimentation profiles of cellular extracts, in the presence of 1% Brij-96, after incubation without (□) or with PI-PLC from *B. thuringiensis* (■). (*B*) Non-denaturing gel electrophoresis in the presence of 0.5% Triton X-100. Extracts were incubated without phospholipase (−) or with (+) PI-PLC or PLD, as indicated. Preincubation with the mAb anti-*Torpedo* AChE Tor-ME8 before electrophoresis prevented migration of AChE into the gels (*right lanes*), indicating that all active bands correspond to transfected *Torpedo* AChE.

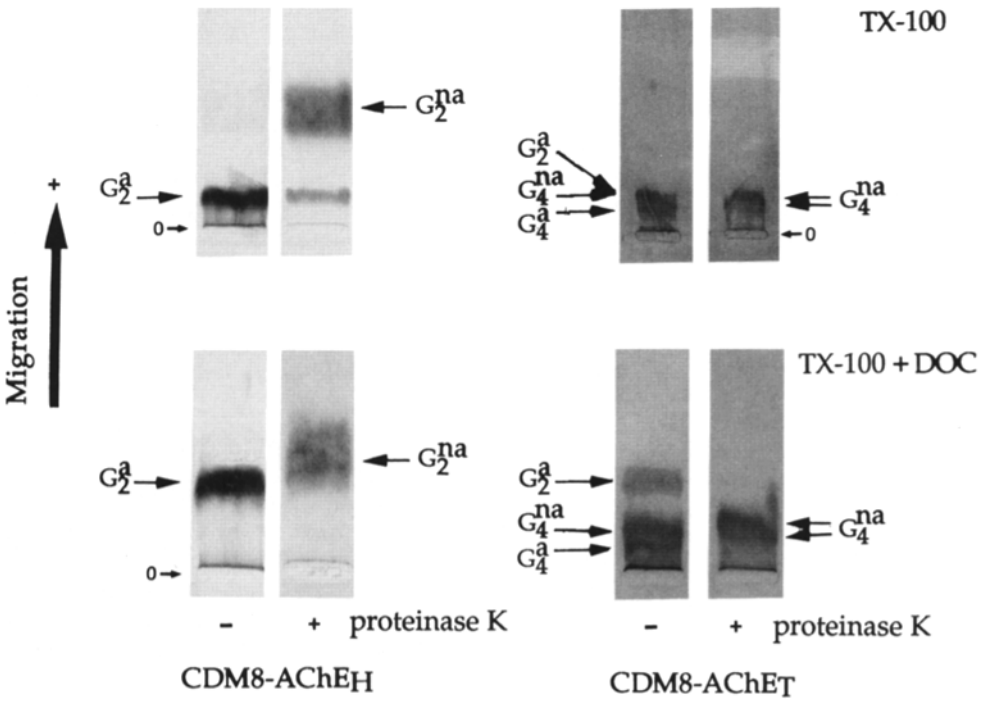

Figure 7. Effect of proteinase K on the amphiphilic character of *Torpedo* molecular forms produced in COS cells. Extracts of transfected cells were incubated with proteinase K (7 μg/ml in 100 mM Tris-HCl, pH 8; $MgCl_2$ 50 mM; 1% Triton X-100, 30 min at 20°C). Control and treated samples were analyzed by nondenaturing electrophoresis in the presence of Triton X-100 (*TX-100*), with or without deoxycholate (*DOC*). In the CDM8-$AChE_H$ extract (*left*), the GPI-anchored G_2^a form was nearly totally converted into a non-amphiphilic derivative, G_2^{na}, which appeared heterogeneous. In the CDM8-$AChE_T$ extract (*right*), the activity of the G_2^a form (type II) was lost, and the G_4^a form was converted into a non-amphiphilic G_4^{na} form, different from that originally present in the cell extract.

negative charge. The fact that the G_2^a form produced from subunit H was sensitive to PI-PLC and PLD clearly demonstrates that it is GPI anchored.

In contrast, the G_2^a form produced from subunit T was totally insensitive to PI-PLC (upper parts of Fig. 6, *A* and *B*) or PLD (not shown).

Accessibility of AChE to the External Medium in Transfected Cells

To examine the accessibility of the enzyme to the external medium, a suspension of transfected cells in TBS was incubated at 30°C for 40 min, in the presence or absence of *B. thuringiensis* PI-PLC (see Materials and Methods). After centrifugation, the supernatants were assayed for AChE activity. In the case of $AChE_T$, 10% of total cellular AChE activity was recovered in the supernatant, with or without PI-PLC. The released enzyme consisted of G_4^{na}, G_2^a, and G_1^a, but not G_4^a. In the case of H-transfected cells, the control supernatant contained ~5% of the cellular activity, without PI-PLC, and 20–40% after incubation with PI-PLC. In this case, the PI-PLC–solubilized enzyme was exclusively a non-amphiphilic G_2^{na} form. The remaining cell-associated G_2^a form was found to be sensitive to PI-PLC, after solubilization by Triton X-100. These experiments indicate that the H-transfected GPI-G_2^a form is largely exposed at the cell surface.

We also determined the proportion of externally accessible activity by comparing the hydrolysis of acetylthiocholine in the presence of intact cells incubated in the Ellman assay medium, supplemented with 0.15 M NaCl to avoid lysis, and after lysis by addition of Triton X-100. These experiments indicated that a significant proportion of the activity is external (about 40–50%), in the case of both H and T transfections (not shown).

Conversion of Amphiphilic to Non-amphiphilic Forms by Proteolytic Digestion

The GPI-G_2^a form of *Torpedo* (Bon et al., 1988*b*) and the mammalian hydrophobic-tailed G_4^a form (Bon et al., 1991*b*) may be efficiently converted into non-amphiphilic forms by limited proteolytic digestion. Under similar conditions, however, the G_1^a and G_2^a forms of type II lose most of their activity (Bon et al., 1988*b*). To compare the transfected and natural enzymes, we treated transfected cell extracts with proteinase K, at 20°C, a temperature at which all forms of AChE are stable.

The GPI-G_2^a form from H-transfected cells was readily converted into a non-amphiphilic form G_2^{na}, which retained most of the original activity (Fig. 7, *left part*). This form appeared heterogenous, probably because of a multiplicity of cleavage sites.

In the case of T-transfected cells, the G_2^a form was inactivated under the same conditions. In contrast, the G_4^a form was converted into a G_4^{na} derivative, which appeared to differ in its electrophoretic migration from the G_4^{na} form originally present in the extract (Fig. 7, *right part*). Unfortunately, this could not be analyzed further, because the activity of the G_4 components was too low to allow their isolation, either before or after digestion.

Thermal Stability of the AChE Forms Produced in Transfected Cells

Because COS cells produced only inactive *Torpedo* AChE at 37°C, we wondered whether the active enzyme obtained at 27°C was thermally stable at 37°C. Fig. 8 shows thermal inactivation curves of AChE in T- and H-transfected cell extracts. The G_2^a form produced by H-transfected cells was stable at 37°C, and inactivated at 42°C with a half life of ~15

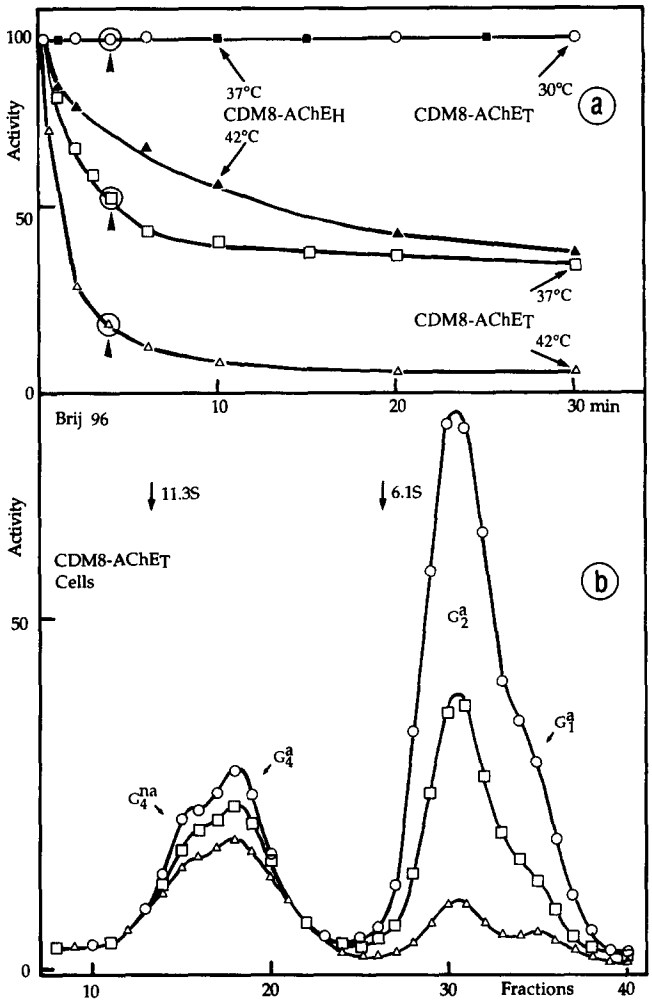

Figure 8. Thermal stability of *Torpedo* AChE forms produced in COS cells. (*A*) The residual activity of total extracts of transfected cells is plotted as a function of incubation time at 30°, 37°, or 42°C, in 0.4 M NaCl, 50 mM Tris-HCl, pH 7, 50 mM $MgCl_2$, 1% Triton X-100. In CDM8-$AChE_H$-transfected cell extracts, AChE activity was stable at 30° and 37°C. In contrast, the AChE activity of CDM8-$AChE_T$-transfected cells was partially inactivated even at 37°C. The molecular forms corresponding to the indicated samples (*circles* and *arrows*) were analyzed in B. (*B*) Sedimentation analysis of the residual active molecular forms of AChE after thermal inactivation of an extract of CDM8-$AChE_T$-transfected cells for 4 min at 30°C (○—○), 37°C (■—■), and 42°C (△—△).

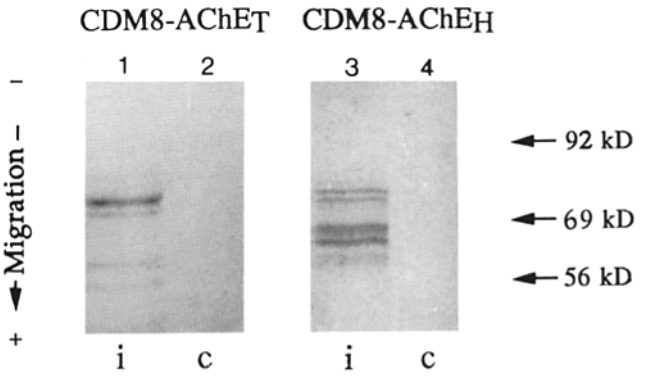

Figure 9. Metabolic labeling of *Torpedo* AChE-immunoreactive protein in transfected COS cells at 27°C. [^{35}S]Methionine was incorporated for a period of 30 min, followed by a chase of 15 min (a similar result was obtained in the absence of chase). The cellular extracts were immunoprecipitated with the anti-*Torpedo* AChE polyclonal rabbit serum Tor-152 (lanes *1* and *3*) or with a non-immune control serum (lanes *2* and *4*), and analyzed by SDS-PAGE and autoradiography. In the case of CDM8-$AChE_T$ (lane *1*) and CDM8-$AChE_H$ (lanes *3*), the labeled AChE protein formed doublets of ~70–72 and 66–68 kD, respectively. These doublets were accompanied by components of smaller molecular weight, and in the case of $AChE_H$, by a doublet of higher molecular weight of ~80–82 kD. The patterns observed after labeling at 37°C were identical.

min, similar to that of the natural AChE from electric organs (not shown).

The AChE activity produced in T-transfected cells presented a more complex inactivation pattern: ~60% of this activity was rapidly inactivated at 37°C, the rest being essentially stable at this temperature. Approximately 95% of the activity was rapidly inactivated at 42°C. We examined the molecular forms remaining after 4 min at 30°, 37°, and 42°C (Fig. 8, *lower part*). The sedimentation patterns show that the small molecular forms G_1^a and G_2^a were much more sensitive to thermal inactivation than the tetrameric forms G_4^a and G_4^{na}.

Analysis of the AChE-immunoreactive Polypeptides

The production of *Torpedo* AChE protein was examined in different culture conditions (24 h of culture at 37°C, followed or not by further incubation at 27°C), by two different methods. In one series of experiments, the synthesis of AChE protein was analyzed by metabolic incorporation of [^{35}S]methionine (30 min, followed or not by a chase period of 15 min), and immunoprecipitated with the rabbit polyclonal antiserum Tor-152. In another series of experiments, the content of the cells in AChE-immunoreactive protein was analyzed by immunoblotting with the mAb Tor-ME8.

The metabolic labeling experiments showed that incorporation was ~50–60% lower at 27°C, compared to 37°C, both in total TCA-precipitable protein, and in AChE-immunoreactive material. However, we obtained similar autoradiographic patterns of AChE-immunoreactive bands in SDS-PAGE at the two temperatures (Fig. 9). They did not change after a chase period of 15 min. CDM8-$AChE_T$ induced the production of a doublet of AChE protein of ~70–72 kD, while CDM8-$AChE_H$ induced the production of two doublets, of ~66–68 and 80–82 kD, the two bands of each doublet differing by 2 to 3 kD. The doublets of 70–72 and 66–68 kD corresponded to the mass expected, according to the coding sequences, and were comparable to the bands obtained with the subunits of natural enzymes (Fig. 10).

The patterns of Western blots were the same when the cells had been maintained at 37°C, or cultured at 27°C (Figs. 10 and 11). They closely resembled the patterns obtained by metabolic labeling, except that the bands appeared more diffuse. This shows that all protein components are essentially stable in the cells.

Treatment with N-glycanase indicated that the main AChE-immunoreactive bands were glycosylated (Fig. 10). The presence of some intermediates was visible at 6 h, but the deglycosylation reaction appeared complete after 16 h. The decrease in apparent molecular weight of the major AChE bands was similar to that observed for the subunits of

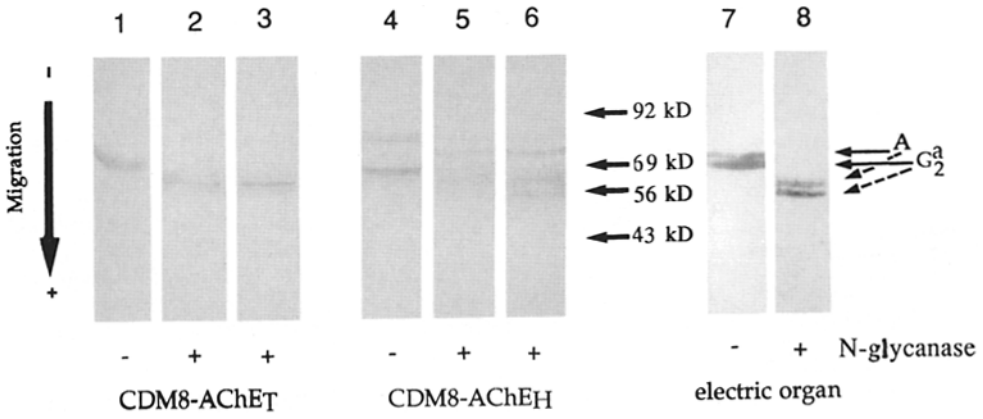

Figure 10. Effect of N-glycanase on *Torpedo* AChE-immunoreactive protein in transfected COS cells and electric organs. Extracts were incubated without N-glycanase (lanes *1*, *4*, and *7*), or with N-glycanase for 6 h (lanes *2*, *5*, and *8*) or for 16 h (lanes *3* and *6*), at 37°C, and analyzed by immunoblotting as in Fig. 8. Lanes *7* and *8* correspond to natural AChE from *Torpedo* electric organ, and show that deglycosylation was essentially complete after 6 h, under the conditions used; this is also demonstrated in the case of the transfected enzymes by comparison of the two incubation periods. The major bands (66–68 kD in $AChE_H$, 70–72 and 80–82 kD in $AChE_T$) were displaced, indicating the presence of N-linked carbohydrates, and the deglycosylated proteins still appeared as doublets. Minor bands appeared to be insensitive to deglycosylation.

the A and G_2^a forms of AChE from *Torpedo* electric organ, indicating a comparable content of N-glycans. The presence of doublets did not result from heterogeneity of the carbohydrate chains, since the difference in mass was maintained after deglycosylation. Thus, the main AChE-immunoreactive components, observed both in Western blots and in metabolic labeling experiments, correspond to glycosylated protein. Since remodeling of the carbohydrate chains occurs over a longer period of time than that used for metabolic labeling, it may introduce some microheterogeneity in the carbohydrate chains, and thus explain the broadening of the bands in Western blots.

In the absence of reduction, most of the AChE immunoreactive material remained at the top of the gel, as disulfide-linked homo- or hetero-aggregates. A small proportion of H polypeptides appeared as dimers, and a small proportion of T polypeptides as monomers. The active subunits would be expected to be included in disulfide-linked dimers in the polymeric G_2 and G_4 forms.

Proportion of Active AChE in the AChE-immunoreactive Protein Synthesized at 27°C

We examined the relationship between the AChE activity and the intensity of immuno-staining, for extracts of transfected cells, producing active AChE at 27°C, and fresh extracts from *Torpedo* electric organ, containing both A and G_2^a forms (Fig. 11). For a similar AChE activity, the intensity of staining was much stronger in the transfected cells extracts than in the electric organ extract. Although quantification is difficult, the proportion of active AChE subunits may be estimated as <5% of the AChE protein synthesized in the COS cells at 27°C. This active enzyme would not by itself yield any detectable staining in the immunoblots, under the experimental conditions used here. It is therefore clear that the staining pattern corresponds to inactive AChE protein, and this explains why we obtained the same pattern after incubation of the cells at 37° or 27°C, irrespective of the production of AChE activity.

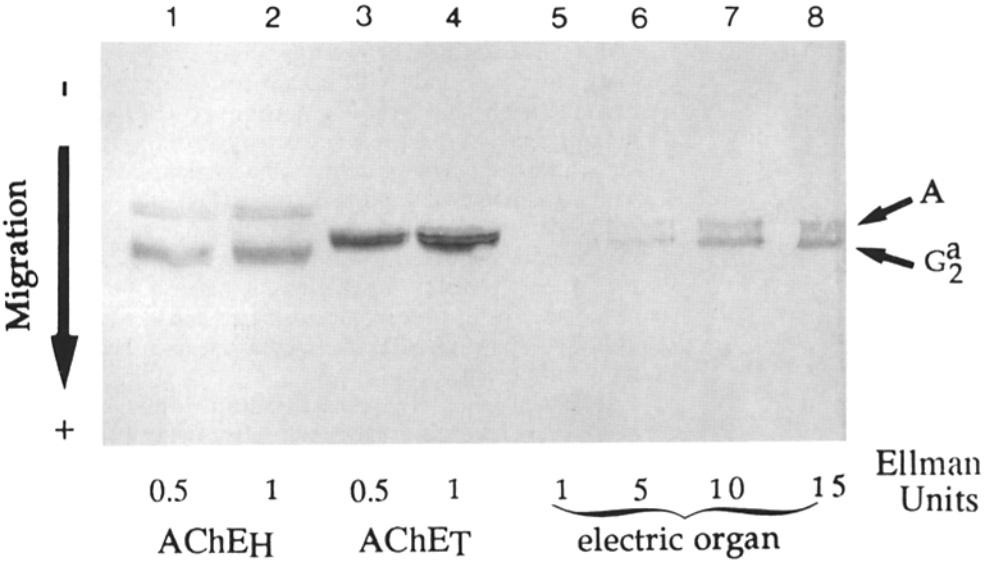

Figure 11. Comparison of AChE activity and immunostaining intensity: demonstration of the presence of inactive AChE protein in transfected cells at 27°C. Aliquots of extracts from transfected COS cells (lanes *1* and *2*, $AChE_H$; lanes *3* and *4*, $AChE_T$) and from electric organ (lanes *5* to *8*), containing the indicated amounts of AChE (expressed in Ellmann units), were analyzed by Western blotting (cf., Fig. 10). Samples containing the same AChE activity were stained much more intensely in the case of the transfected cells, particularly with $CDM8\text{-}AChE_T$.

Discussion

Expression of Active AChE in Transfected Cells, Influence of Temperature, Production of GPI-anchored Dimers and Amphiphilic Forms of Type II

Transfections of COS cells with CDM8 vectors carrying inserts encoding catalytic subunits of *Torpedo* AChE induced the synthesis of AChE-immunoreactive protein at 37°C, but no AChE activity was produced at this temperature, in agreement with the findings of Gibney et al. (1990). It was previously reported that the active conformation of the *Torpedo* proteins, such as the acetylcholine receptor (AChR), was only obtained at a lower temperature, 26° or 20°C (Claudio et al., 1988, 1989*a*; Paulson and Claudio, 1990; Paulson et al., 1991). These authors showed that the misfolded α, β, γ, and δ subunits remained sequestered in the ER as polydispersed aggregates (Claudio et al., 1989*b*; Paulson et al., 1991). In the case of *Torpedo* AChE, Gibney et al. (1990) performed the transfection at 37°C and then lowered the temperature to 27°C. We obtained active *Torpedo* AChE under similar conditions, from constructions expressing subunits H and T, as well as a truncated T subunit, TΔ. The level of activity obtained was sufficient to analyze the molecular forms produced in transfected cells.

In parallel experiments, the activity was generally higher in H than in T transfections, and lower in TΔ transfections. This may be related to the fact that the H subunit generated a GPI-anchored G_2^a form, which was similar to that formed in electric organs, and appeared equally stable, whereas T and TΔ subunits generated molecules which are not found in vivo. Some of these forms were unstable at 37°C, possibly because the active conformation failed to be adequately stabilized by glycosylation, disulfide bonding, or oligomeric interactions. In any case, the fact that at least some of the active AChE formed at 27°C remains stable at 37°C indicates that the folding process is limiting, rather than the stability of the mature protein.

As previously shown by Gibney and Taylor (1990), the COOH-terminal sequence of the *Torpedo* H subunit may be correctly processed in COS cells into a GPI-anchored G_2^a form, which was sensitive to PI-PLC, PLD, or proteolytic digestion, like natural *Torpedo* G_2^a of type I. Nearly half of this enzyme was exposed at the surface of the cells, according to both AChE activity and PI-PLC sensitivity of intact cells. The cells secreted a non-amphiphilic G_2^{na} form.

The T-transfected cells produced multiple molecular forms (G_4^{na}, G_4^a, G_2^a, G_1^a), ~20–40% of the activity being exposed at the cell surface towards the external medium. It is not surprising to observe the synthesis of a G_4^{na} form, since T subunits constitute the non-amphiphilic tetramers of human serum BuChE (Lockridge et al., 1987). The presence of amphiphilic forms is more intriguing. The G_1^a and G_2^a forms are not GPI anchored and thus correspond to type II forms, as observed in some *Torpedo* tissues, but not in electric organs (Bon et al., 1988*a*,*b*). Such forms are abundant in T_{28} neuroblastoma cells, in rabbit muscles (G_1^a) and in chick muscles (G_2^a) (Bon et al., 1991*a*). The endogenous G_1^a and G_2^a AChE which were detected at a low level in untransfected COS cells possibly also belong to this category. Regarding the structure of the G_4^a form produced in $AChE_T$-transfected cells, two possibilities may be considered. This form may incorporate a structural hydrophobic component, such as the 20-kD subunit of the hydrophobic-tailed G_4^a AChE from mammalian brain (Gennari et al., 1987; Inestrosa et al., 1987), although COS cells do not contain any detectable endogenous G_4^a. On the other hand, the G_4^a form may simply be a tetramer of T subunits. This is probably the case of small fraction of AChE G_4^a form of *Torpedo* spinal cord, which is easily solubilized without detergent, and thus differs from the nerve G_4^a form (Bon et al., 1988*a*,*b*).

The T-transfected cells released mainly non-amphiphilic G_4^{na} and amphiphilic G_2^a forms into the culture medium. The murine neural T_{28} cells also release an amphiphilic form, G_1^a (Lazar et al., 1984). In both cases, the released forms were found to differ slightly in their electrophoretic migration from the corresponding cellular molecules. These forms may thus undergo some modification of their hydrophobic domain upon release, which would render them more soluble without losing the capacity to bind detergent micelles.

Structure and Significance of Amphiphilic Forms of Type II: Role of the COOH-terminal T Peptide

The fact that a truncated TΔ subunit produced a monomeric form is entirely consistent with the involvement of Cys-572 in intersubunit disulfide bonds: Velan et al. (1991) recently showed that only monomers were produced after replacement of the COOH-terminal cysteine by alanine in the human AChE T subunit. In addition, the fact that we obtained a non-amphiphilic G_1^{na} form from the TΔ subunit shows that the hydrophobic domain of amphiphilic forms of type II must be carried by the T peptide.

In mammalian brain, only T subunits of AChE are synthesized (Li et al., 1991), producing mostly the G_1^a form of type II and the hydrophobic-tailed G_4^a form. Although G_1^a and G_2^a forms of type II have not been detected in electric organs, they may exist at a low level, as precursors of the tetramers which constitute the collagen-tailed molecules. If this is the case, the hydrophobic character of the T monomers and dimers must not result from any irreversible modification of the catalytic subunits, since these subunits are intact, particularly at their COOH terminus, in collagen- and hydrophobic-tailed forms.

We may consider two hypotheses about the structure of the hydrophobic domain of type II forms: (*a*) posttranslational addition of hydrophobic residues; (*b*) exposure of hydrophobic residues of the unfolded COOH-terminal peptide. According to the first hypothesis, we could consider palmitoylation or isoprenylation. Indeed, it has recently been reported that amphiphilic forms, obtained in transfected cells producing the mouse T subunit, incorporated palmitate (Randall, W. R., manuscript in preparation). On the other hand, the COOH-terminal peptide of subunit presents a cysteine (Cys-572) at the fourth position from its extremity (CAEL), and resembles somewhat an isoprenylation signal (Hancock et al., 1989). However, palmitoylation as well as isoprenylation are known to operate on cytosolic proteins, and not on proteins which are synthesized within the endoplasmic reticulum.

According to the second hypothesis, exposure of the COOH-terminal peptide would be sufficient to explain the amphiphilic properties of such forms. This hypothesis relies on the fact that, although the COOH-terminal T peptide does not present the hydrophobic character of a transmembrane

segment, it contains hydrophobic residues, e.g., tryptophans, which may bind a detergent micelle. These residues would be buried in the collagen-tailed forms and in soluble non-amphiphilic tetramers.

Processing of AChE in Transfected and Normal Cells: Presence of a Large Pool of Inactive AChE Protein

A comparison of the intensity of immunostaining with that of natural *Torpedo* AChE from electric organs, showed that even after incubation of the cells at 27°C, the active subunits constituted <5% of the total immunoreactive AChE subunits, and would not be detectable in immunoblots. Thus, immunoblots and metabolic labeling revealed only inactive AChE, and it is not certain that the active subunits comigrated with AChE-immunoreactive bands in SDS-PAGE.

Active AChE subunits contain three intracatenary disulfide loops (MacPhee-Quigley et al., 1986). Inactive subunits may thus be trapped in an inadequate arrangement of disulfide bonds. Indeed, the immuno-staining patterns obtained in denaturing, non-reducing conditions, showed that most of the AChE polypeptides were included in disulfide-linked high molecular weight aggregates.

Metabolically labeled AChE subunits appeared as doublets, whose difference (2 to 3 kD) was independent of glycosylation. Because it appeared identical in H and T subunits, it is likely to reside at the NH_2-terminal extremity, and could represent an uncleaved signal peptide. There is good evidence that the signal peptide is retained in a subset of mammalian dopamine-β-hydroxylase (Taljanidisz et al., 1989).

In addition to AChE subunits of the expected molecular weight, the H-transfected cells produced a higher molecular weight doublet, of about 80–82 kD before deglycosylation and 75–77 kD after deglycosylation. Since this is specific to the H subunit, it must be related to the processing of COOH-terminal sequence. The foreign *Torpedo* COOH-terminal sequence might be incompletely processed in COS cells. The situation would thus be similar to that of the decay acceleration factor mutants, in which modifications of the cleavage/attachment site result in incomplete processing and produce protein doublets (Moran et al., 1991).

The fact that metabolic labeling and Western blots produced very similar patterns of AChE-immunoreactive protein indicates that the main components (the 66–68- and 80–82-kD doublets in the case of $AChE_H$, and the 70–72-kD doublet in the case of $AChE_T$) are stable in the transfected cells. They do not therefore represent normal folding intermediates, but rather misfolded dead-end products.

The importance of misfolding, and the existence of thermally unstable molecules which may represent intermediates in the stabilization of conformation are particularly obvious in the expression of *Torpedo* AChE in mammalian cells. These processes may be similar to the normal maturation of endogenous AChE, as suggested by the existence of a time lag between synthesis of the protein and acquisition of activity (Lazar et al., 1984), and by the existence in culture and in vivo, of a significant fraction of inactive AChE subunits (Rotundo et al., 1989; Vallette et al., 1991).

Conclusion

Mammalian cells correctly process the *Torpedo* H subunits into GPI-anchored dimers, and expose them at the cell surface (although the untransfected COS cells do not produce this AChE form). The expression of T subunits generates a high proportion of amphiphilic monomers and dimers, resembling the amphiphilic forms of type II which exist in some *Torpedo* tissues and are abundant in the nervous tissue and muscles of higher vertebrates (Bon et al., 1991*a*). A partial deletion of the T COOH-terminal peptide has shown that it is responsible for the formation of oligomers and for the hydrophobic character of amphiphilic forms of type II.

In transfected cells, H subunits produce only GPI-G_2^a dimers, whereas T subunits are extremely versatile, generating all other known types of AChE molecular forms: amphiphilic forms of type II (G_1^a, G_2^a, and possibly G_4^a), hydrophobic-tailed G_4^a, and collagen-tailed A forms. This is why we use distinct letters for the alternative exons and the corresponding subunits (H and T), on one hand, and for the two groups of quaternary structures (A and G) that they can generate, on the other hand. The existence of additional distinct COOH-terminal regions has been predicted from the structures of cDNAs (Sikorav et al., 1987; Li et al., 1991), but these sequences have not yet been observed in AChE forms, and it is not certain that they correspond to active AChE. It is noteworthy that invertebrates seem to possess only H subunits. T subunits probably appeared in the ancestors of vertebrates, allowing a much greater variety of AChE attachments.

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References

Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573–8577.

Bon, S., J. P. Toutant, K. Méflah, and J. Massoulié. 1988*a*. Amphiphilic and nonamphiphilic forms of *Torpedo* cholinesterases: I. Solubility and aggregation properties. *J. Neurochem.* 51:776–785.

Bon, S., J. P. Toutant, K. Méflah, and J. Massoulié. 1988*b*. Amphiphilic and nonamphiphilic forms of *Torpedo* cholinesterases: II. Existence of electrophoretic variants and of phosphatidylinositol phospholipase C-sensitive and -insensitive forms. *J. Neurochem.* 51:786–794.

Bon, S., T. L. Rosenberry, and J. Massoulié. 1991*a*.Amphiphilic, glycophosphatidylinositol-specific phospholipase C (PI-PLC)-insensitive monomers and dimers of acetylcholinesterase. *Cell. Mol. Neurobiol.* 11:157–172.

Bon, S., A. Lamouroux, A. Vigny, J. Massoulié, J. Mallet, and J. P. Henry. 1991*b*. Amphiphilic and non amphiphilic forms of bovine and human dopamine-β-hydroxylase. *J. Neurochem.* 57:1100–1111.

Claudio, T., H. L. Paulson, D. S. Hartman, S. M. Sine, and F. J. Sigworth. 1988. Establishing a stable expression system for studies of acetylcholine receptors. *Curr. Top. Membr. Transp.* 33:219–247.

Claudio, T., H. L. Paulson, W. N. Green, A. F. Ross, D. S. Hartman, and D. A. Hayden. 1989*a*. Fibroblasts transfected with *Torpedo* acetylcholine receptor β, γ, δ, subunit cDNAs express functional AChRs when infected with a package retroviral-α-recombinant. *J. Cell Biol.* 108:2277–2290.

Claudio, T., D. S. Hartman, W. N. Green, A. F. Ross, H. L. Paulson, and D. A. Hayden. 1989*b*. Stable expression of multisubunit protein complexes in mammalian cells. *In* NATO ASI Series. Vol. H32. "Molecular Biology of Neuroreceptors and Ion Channels." A. Maelicke, editor. Springer-Verlag, Berlin/Heidelberg. 469–480.

Ellman, G. L., K. D. Courtney, V. Andres, and R. M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7:88–95.

Futerman, A. H., M. G. Low, and I. Silman. 1983. A hydrophobic dimer of acetylcholinesterase from *Torpedo californica* electric organ is solubilized by phosphatidylinositol-specific phospholipase C. *Neurosci. Lett.* 40:85–89.

Gennari, K., J. Brunner, and U. Brodbeck. 1987. Tetrameric detergent-soluble acetylcholinesterase from human caudate nucleus: subunit composition and number of active sites. *J. Neurochem.* 49:12–18.

Gibney, G., K. MacPhee-Quigley, B. Thompson, T. Vedvick, M. G. Low, S. S. Taylor, and P. Taylor. 1988. Divergence in primary structure between the molecular forms of acetylcholinesterase. *J. Biol. Chem.* 263:1140–1145.

Gibney, G., and P. Taylor. 1990. Biosynthesis of *Torpedo* acetylcholinesterase in mammalian cells. Functional expression and mutagenesis of the glycophospholipid-anchored form. *J. Biol. Chem.* 265:12576–12583.

Hancock, J. F., A. I. Magee, J. E. Childs, and C. J. Marshall. 1989. All *ras* proteins are polyisoprenylated but only some are palmitoylated. *Cell.* 57:1167–1177.

Herbomel, P., B. Bourachot, and M. Yaniv. 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. *Cell.* 39:653–662.

Inestrosa, N. C., W. L. Roberts, T. Marshall, and T. L. Rosenberry. 1987. Acetylcholinesterase from bovine caudate nucleus is attached to membranes by a novel subunit distinct from those of acetylcholinesterase in other tissues. *J. Biol. Chem.* 262:4441–4444.

Karnovsky, M. J., and L. Roots. 1964. A direct-coloring thiocholine method for cholinesterases. *J. Histochem. Cytochem.* 12:219–222.

Krejci, E., F. Coussen, N. Duval, J. M. Chatel, C. Legay, M. Puype, J. Vandekerckhove, J. Cartaud, S. Bon, and J. Massoulié. 1991*a*. Primary structure of a collagenic tail subunit of *Torpedo* acetylcholinesterase: coexpression with catalytic subunit induces the production of collagen-tailed forms in transfected cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 10:1285–1293.

Krejci, E., N. Duval, A. Chatonnet, P. Vincens, and J. Massoulié. 1991*b*. Cholinesterase-like domains in enzymes and structural proteins: functional and evolutionary relationships; identification of a catalytically essential aspartic acid. *Proc. Natl. Acad. Sci. USA.* 88:6647–6651.

Lazar, M., E. Salmeron, M. Vigny, and J. Massoulié. 1984. Heavy isotope labeling study of the metabolism of monomeric and tetrameric acetylcholinesterase forms in the murine neuronal-like T28 hybrid cell line. *J. Biol. Chem.* 259:3703–3713.

Li, Y., S. Camp, T. L. Rachinsky, D. Getman, and P. Taylor. 1991. Gene structure of mammalian acetylcholinesterase: alternative exons dictate tissue-specific expression. *J. Biol. Chem.* 266:23083–23090.

Lockridge, O., C. F. Bartels, T. A. Vaughan, C. K. Wong, S. E. Norton, and L. L. Johnson. 1987*a*. Complete amino-acid sequence of human serum cholinesterase. *J. Biol. Chem.* 262:549–557.

Lockridge, O., S. Adkins, and B. N. La Du. 1987*b*. Location of disulfide bonds within the sequence of human serum cholinesterase. *J. Biol. Chem.* 262:12945–12952.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:365–375.

MacPhee-Quigley, K., T. S. Vedvick, P. Taylor, and S. Taylor. 1986. Profile of the disulfide bonds in acetylcholinesterase. *J. Biol. Chem.* 261:13565–13570.

Massoulié, J., and S. Bon. 1982. The molecular forms of cholinesterase in vertebrates. *Annu. Rev. Neurosci.* 5:57–106.

Massoulié, J., L. Pezzementi, S. Bon, E. Krejci, and F. M. Vallette. 1992. Molecular and cellular biology of cholinesterases. *Prog. Neurobiol.* In press.

Maulet, Y., S. Camp, G. Gibney, T. Rachinsky, T. J. Ekstrom, and P. Taylor. 1990. Single gene encodes glycophospholipid-anchored and asymmetric acetylcholinesterase forms: alternative coding exons contain inverted repeat sequences. *Neuron.* 4:289–301.

Mizushima, S., and S. Nagata. 1990. pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.* 18:5322.

Moran, P., H. Raab, W. J. Kohr, and I. W. Caras. 1991. Glycophospholipid membrane attachment: molecular analysis of the cleavage/attachment site. *J. Biol. Chem.* 266:1250–1257.

Musset, F., Y. Frobert, J. Grassi, M. Vigny, G. Boulla, S. Bon, and J. Massoulié. 1987. Monoclonal antibodies against acetylcholinesterase from electric organs of *Electrophorus* and *Torpedo*. *Biochimie.* 69:147–156.

Paulson, H. L., and T. Claudio. 1990. Temperature-sensitive expression of all-*Torpedo* and *Torpedo*-rat hybrid AChR in mammalian muscles cells. *J. Cell Biol.* 110:1705–1717.

Paulson, H. L., A. F. Ross, W. N. Green, and T. Claudio. 1991. Analysis of early events in acetylcholine receptor assembly. *J. Cell Biol.* 113:1371–1384.

Randall, W. R., K. W. K. Tsim, J. Lai, and E. A. Barnard. 1987. Monoclonal antibodies against chicken brain acetylcholinesterase; their use in immunopurification and immunochemistry to demonstrate allelic variants of the enzyme. *Eur. J. Biochem.* 164:95–102.

Randall, W. R. 1991. Cellular expression of murine AChE from a cloned brain transcript. *In* Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology. J. Massoulié, F. Bacou, E. A. Barnard, A. Chatonnet, B. P. Doctor, and D. M. Quinn, editors. American Chemical Society, Washington D.C. 152–156.

Roberts, W. L., B. P. Doctor, J. D. Foster, and T. L. Rosenberry. 1991. Bovine brain acetylcholinesterase primary sequence involved in intersubunit disulfide linkages. *J. Biol. Chem.* 266:7481–7487.

Rotundo, R. L., K. Thomas, K. Porter-Jordan, R. J. J. Benson, C. Fernandez-Valle, and R. E. Fine. 1989. Intracellular transport, sorting, and turnover of acetylcholinesterase. Evidence for an endoglycosidase H-sensitive form in Golgi apparatus, sarcoplasmic reticulum, and clathrin-coated vesicles and its rapid degradation by a non-lysosomal mechanism. *J. Biol. Chem.* 264:3146–3152.

Schumacher, M., S. Camp, Y. Maulet, M. Newton, K. MacPhee-Quigley, S. S. Taylor, T. Friedman, and P. Taylor. 1986. Primary structure of *Torpedo californica* acetylcholinesterase deduced from cDNA sequence. *Nature (Lond.).* 319:407–409.

Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature (Lond.).* 329:840–842.

Selden, R. F., K. Burke-Howie, M. E. Rowe, H. M. Goodman, and D. D. Moore. 1986. Human growth hormone as a reporter gene in regulation studies employing transient gene expression. *Mol. Cell. Biol.* 6:3173–3179.

Sikorav, J. L., J. Grassi, and S. Bon. 1984. Synthesis *in vitro* of precursors of the catalytic subunits of acetylcholinesterase from *Torpedo marmorata* and *Electrophorus electricus*. *Eur. J. Biochem.* 145:519–524.

Sikorav, J. L., E. Krejci, and J. Massoulié. 1987. cDNA sequences of *Torpedo marmorata* acetylcholinesterase: primary structure of the precursor of a catalytic subunit; existence of multiple 5′-untranslated regions. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1865–1873.

Sikorav, J. L., N. Duval, A. Anselmet, S. Bon, E. Krejci, C. Legay, M. Osterlund, B. Reimund, and J. Massoulié. 1988. Complex alternative splicing of acetylcholinesterase transcripts in *Torpedo* electric organ: primary structure of the precursor of the glycolipid-anchored dimeric form. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2983–2993.

Taljanidisz, J., L. Stewart, A. J. Smith, and J. P. Klinman. 1989. Structure of bovine adrenal dopamine-β-monooxygenase, as deduced from cDNA and protein sequencing: evidence that the membrane-bound form of the enzyme is anchored by an uncleaved signal peptide. *Biochemistry.* 28:10054–10061.

Uetsuki, T., A. Naito, S. Nagata, and Y. Kaziro. 1989. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1α. *J. Biol. Chem.* 264:5791–5798.

Vallette, F. M., S. De la Porte, and J. Massoulié. 1991. Regulation and distribution of acetylcholinesterase molecular forms *in vivo* and *in vitro*. *In* Cholinesterases: Structure, Function, Mechanism, Genetics, and Cell Biology. J. Massoulié, F. Bacou, E. A. Barnard, A. Chatonnet, B. P. Doctor, and D. M. Quinn, editors. American Chemical Society, Washington, D. C. 98–102.

Velan, B., H. Grosfeld, C. Kronman, M. Leitner, Y. Gozes, A. Lazar, Y. Flashner, D. Marcus, S. Cohen, and A. Shafferman. 1991. The effect of elimination of intersubunit disulfide bonds on the activity, assembly, and secretion of recombinant human acetylcholinesterase: expression of acetylcholinesterase Cys-580→Ala mutant. *J. Biol. Chem.* 266:23977–23984.

Vigny, M., S. Bon, J. Massoulié, and F. Leterrier. 1978. Active-site catalytic efficiency of acetylcholinesterase molecular forms in *Electrophorus*, *Torpedo*, rat and chicken. *Eur. J. Biochem.* 85:317–323.