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

Nathalie Duval, Jean Massoulié, and Suzanne Bon

Laboratoire de Neurobiologie, Centre National de la Recherche Scientifique UA 295, Ecole Normale Supérieure, 75005 Paris, France

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 glycoprophatidylinositol-anchored dimers of *Torpedo* electric organs, as well as a truncated T subunit (TA), lacking most of the COOH-terminal peptide. The transfected cells synthesized similar amounts of ACHE immunoreactive protein at 37° and 27°. 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 glyco- phosphatidylinositol-anchored dimers, resembling the corresponding natural ACHE form. The cells also released non-amphiphilic dimers G2m. The T polypeptides generated a series of active forms which are not produced in *Torpedo* electric organs: G1+, G3+, G4+, and G5m cellular forms and G2s and G4s secreted amphibilic forms appeared to correspond to type II forms (Bon, S., J. P. Toutant, K. Méfah, and J. Massoulié. 1988. J. Neurochem. 51:776-785; Bon, S., J. P. Toutant, K. Méfah, 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 TA 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, E. C. 3.117) 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 glycoprophatidylinositol (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, IIIr and IIIs, 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 IIIr 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 IIIe 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,

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1. Abbreviations used in this paper: A, asymmetric; ACHE, acetylcholinesterase; AChR, acetylcholine receptor; BuChE, butyrylcholinesterase; DOC, deoxycholate; DS, detergent soluble; G, globular; GPI, glycoprophatidylinositol; LSS, low-salt soluble.
All transfection experiments were performed in DME, with 10% Nu-serum, with DNA doubly purified by CsCl gradients. The Nu-serum was transferred at 27°C for 48 h.

and transfected the next day with 1 μg of DNA per plate, by the treatment with chloroquine (100 μM) and DMSO (0%). After incubation for 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 TCCACT-GGATCCCTGCTAGTCAAGGTTC.

Histochemical Staining of AChE Activity in Transfected COS Cells

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 medium (1-cm pathlength), and corresponds to the hydrolysis of 75 nmole of substrate per min. In the case of Torpedo AChE, 1 μg corresponds to ~50 Elman units (Vigny et al., 1978).

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

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 anti-proteolytic 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 MgCl2. 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 MgCl2, 1% Triton X-100.

Sedimentation Analyses

The 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% Na2 deoxycholate (DOC), as described previously (Bon et al., 1988b). 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°C or 27°C. The culture medium was removed, and the cells were incubated at the same temperature with 150 μCi of [35S]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, scrapped 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 at 15 min at 15,000 rpm/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 cell was divided into 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. (1991b), 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 [3H]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.

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 (Sikorav 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., 1991b). We therefore decided to analyze independent cDNA clones. A cDNA library from electric organ, which was described previously (Krejci et al., 1991a) was screened with a 1,208-bp AChE probe (Sikarow et al., 1988). We thus obtained a new clone, λAChEγ, 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, λAChEγ 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, λAChEγ 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 SI 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 λAChEγ 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 λAChEγ clone, and from a combination of λAChEγ, pACH2 (Sikarow et al., 1987), and λAChEβ (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, Duval et al. AChE Forms Expressed from H and T Torpedo Subunits 643
~0.5 10^-5 Ellman U/mg protein, corresponding to G1^* and G2^* (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 G2^*^ (10.5 S) and G1^*^ (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(T), CDM8-AChE(H) 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-

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**Figure 1.** The structure of S' non-coding regions of different Torpedo AChE cDNA clones. The sequences of clones λAChE3 (1) and λACh-E11 (2) of T. marmorata AChE (Sikorav et al., 1987) and two sequences from Z. californica (3 and 4) (Schumacher et al., 1986) were previously described. The new sequence reported here (λAChE(T)) (5) shows that T. marmorata possesses a S' region which had only been found hitherto in T. californica. The two different S' regions may correspond to distinct transcription origins.

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**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 S' and 3' non-coding sequences, were obtained, respectively, from λAChE(T) and from a combination of λAChE(T) and λAChE(H), 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 MCI061/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.
Karnovsky 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(G1), AChE activity corresponded almost exclusively to a G1 form (6.8 S in Triton X-100 and 4.7 S in Brij-96), accompanied by a trace of G2 form (7.5 S). Only 10% of G2 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 G2 form (Fig. 4 B, lower part), together with a trace of G4 form, most likely resulting from the secretion of endogenous AChE by the COS cells. The absence of any detectable G2 form indicated that the centrifuged and concentrated medium was not significantly contaminated by cell debris.

In the case of AChE(T), the cell extracts contained four AChE forms: G1 (5 S in Triton X-100 and 3.5 S in Brij-96), G2 (6.4 S in Triton X-100 and 3.5 S in Brij-96), G3 (10 S in Triton X-100 and 9 S in Brij-96) and G4 (11.3 S). The proportions of these forms were in the order of 30% (G1), 50% (G2), 5-10% (G3), and 10-20% (G4). 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 G1 and G2 forms were solubilized in the LSS fraction, together with a minority of G3. The DS fraction contained most of G1 and G2, together with the remaining G3 and G4 forms. The culture medium contained equivalent amounts of G1 and G2, with a trace of G3 (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 G1 and G2 forms (2.8 S and 4.5 S, in the presence of Brij-96), the cellular extract contained a single Torpedo AChE form, G3, that sedi-

**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-

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**Figure 3.** Histochemical staining of AChE activity in transfected cells. COS cells transfected with the CDM8-AChEr 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.
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 (--; 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-AChEt cell extract, indicating that the endogenous G, 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 G2a and GPI-anchored G2a from electric nerves. Lanes 1, 2, and 4 refer respectively to control, non-transfected cells, and to cells transfected with CDM8-AChEt and CDM8-AChEh.

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 (G2a and the lytic forms G2a and G0a) 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., 1988b).

The G2a form produced by H-transfected cells resembled the nerve and electric organ GPI-anchored G2a 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 G2a form from the culture medium migrated slightly but significantly faster than the lytic form obtained from the cellular G2a form after digestion with PI-PLC (see next section).
In the case of T-transfected cells, the G₂⁺ forms from cellular extracts and from the culture medium differed in their electrophoretic migration (Fig. 4C, 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 G₂⁺ 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., 1991a). The G₂⁺ 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-G₂⁺ 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. 6A), and by non-denaturing electrophoresis in the presence of Triton X-100 (Fig. 6B).

PI-PLC converted the G₂⁺ form of an H extract into a non-amphiphilic G₂⁺⁺ form (lower part), without loss of activity. This lytic G₂⁺⁺ form migrated slightly slower, in non-denaturing electrophoresis, than the G₂⁺⁺ form released in the culture medium. The G₂⁺ obtained in H transfections was also sensitive to PLD from human serum, producing a G₂⁺⁺ 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 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.

**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-AChET; lower parts, extracts from cells transfected with CDM8-AChEH. (A) Sedimentation profiles of cellular extracts, in the presence of 1% Brij-96, after incubation without (○) 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.
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$_1^a$ form produced from subunit T was totally insensitive to PI-PLC (upper parts of Fig. 6, A and B) or PLD (not shown).

Conversion of Amphiphilic to Non-amphiphilic Forms by Proteolytic Digestion

The GPI-G$_2^a$ form of Torpedo (Bon et al., 1988b) and the mammalian hydrophobic-tailed G$_4^a$ form (Bon et al., 1991b) 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., 1988b). 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$_2^a$ which retained most of the original activity (Fig. 7, left part). This form appeared heterogeneous, probably because of a multiplicity of cleavage sites.

In the case of T-transfected cells, the G$_2^a$ form was activated under the same conditions. In contrast, the G$_4^a$ form was converted into a G$_4^n$ derivative, which appeared to differ in its electrophoretic migration from the G$_4^a$ 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°C, 37°C, or 42°C, in 0.4 M NaCl, 50 mM Tris-HCl, pH 7.5, 50 mM MgCl₂, 1% Triton X-100. In CDM8-AChE₇-transfected cell extracts, AChE activity was stable at 30°C and 37°C. In contrast, the AChE activity of CDM8-AChE₇-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-AChE₇-transfected cells for 4 min at 30°C (●—●), 37°C (□—□), and 42°C (△—△).

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°C, 37°C, and 42°C (Fig. 8, lower part). The sedimentation patterns show that the small molecular forms G₂ and G₄ were much more sensitive to thermal inactivation than the tetrameric forms G₄ and G₄.

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 [³⁵S]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-AChE₇ induced the production of a doublet of AChE protein of ~70–72 kD, while CDM8-AChE₇ 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
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 AChEa, 70–72 and 80–82 kD in AChEe) 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.

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 G2α 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°C or 27°C, irrespective of the production of AChE activity.
**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°C or 20°C (Claudio et al., 1988, 1989a; 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., 1989b; 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 Gc* 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 Gs* form, which was sensitive to PI-PLC, PLD, or proteolytic digestion, like natural *Torpedo* Gc* 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 Gb* form.

The T-transfected cells produced multiple molecular forms (Gc*, Gs*, Gv*, Gx*, Gs*), ~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 Gc* 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 Gc* and Gs* 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., 1988a,b). Such forms are abundant in T28 neuroblastoma cells, in rabbit muscles (Gx*) and in chick muscles (Gv*) (Bon et al., 1991a). The endogenous Gc* and Gs* AChE which were detected at a low level in untransfected COS cells possibly also belong to this category. Regarding the structure of the Gc* form produced in AChE-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 Gc* AChE from mammalian brain (Gennari et al., 1987; Inestrosa et al., 1987), although COS cells do not contain any detectable endogenous Gc*. On the other hand, the Gc* form may simply be a tetramer of T subunits. This is probably the case of small fraction of AChE Gc* form of *Torpedo* spinal cord, which is easily solubilized without detergent, and thus differs from the nerve Gc* form (Bon et al., 1988a,b).

The T-transfected cells released mainly non-amphiphilic Gb* and amphiphilic Gs* forms into the culture medium. The murine neural T28 cells also release an amphiphilic form, Gc* (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 TA 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 Gt* form from the TA 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 Gs* form of type II and the hydrophobic-tailed Gt* form. Although Gt* and Gv* 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 crystate (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-ampiphilic 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 co-migrated 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 inappropriate 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 kDa) was independent of glycosylation. Because it appeared identical in H and T subunits, it is likely to reside at the NH2-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 (Taljanidiz 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 kDa before deglycosylation and 75–77 kDa 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-kDa doublets in the case of AChEβ, and the 70-72-kDa doublet in the case of AChEγ) 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 sur-
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