Quaternary Associations of Acetylcholinesterase

I. OLIGOMERIC ASSOCIATIONS OF T SUBUNITS WITH AND WITHOUT THE AMINO-TERMINAL DOMAIN OF THE COLLAGEN TAIL

Suzanne Bon and Jean Massoulie‡

From the Laboratoire de Neurobiologie Moléculaire et Cellulaire, Unité CNRS 1857, Ecole Normale Supérieure, 46 rue d’Ulm, 75005 Paris, France

(Received for publication, July 3, 1996, and in revised form, October 23, 1996)

We investigated the production of acetylcholinesterase of type T (AChE) in COS cells during transient transfection. When expressed alone, Torpedo AChE(T) remains essentially intracellular, forming dimers and tetramers; in contrast, rat AChE(T) is secreted and produces mostly amphiphilic monomers (G2) and dimers (G2), together with smaller proportions of nonamphiphilic (G2m) tetramers, amphiphilic tetramers (G2), and an unstable higher polymer (13.7 S). The latter two forms have not been described before. We show that secreted G2 and G2 forms differ from their cellular counterparts and that proteolytic cleavage occurs at the COOH terminus of “flagged” subunits. The binding proteins QN/HC and QN/stop are constructed by associating the NH2-terminal domain of the collagen tail (QN) with a functional or truncated signal for addition of a glycolipidic anchor (glycophosphatidylinositol). Coexpression with QN/stop recruits monomers and dimers to form soluble tetramers (G2), increasing the yield of secreted rat AChE and allowing secretion of Torpedo AChE. Using antibodies directed against QC, we showed that this COOH-terminal region could be removed by collagenase without disrupting the assembly of catalytic tetramers. This experiment suggested that AChE subunits were linked to the QN domain (8). We further showed that an isolated QN domain was sufficient to bind one AChE tetramer, by constructing a chimeric protein in which QN is fused to the COOH-terminal glycolipid (GPI) addition signal of the H subunit of Torpedo AChE (Hc). Coexpression of this QN/HC protein with catalytic T subunits of Torpedo, rat, or human AChE produces GPI-anchored AChE tetramers (8, 9).

We analyzed the enzyme produced by transfected COS cells expressing the rat AChE(T) subunit alone, exploring the effect of various parameters on activity and molecular forms. In agreement with a previous study (6), transfected COS cells expressing the rat AChE(T) subunit produce and secrete amphiphilic monomers and dimers of type II, G1 and G2 (10–12) and nonamphiphilic tetramers, G2m. We now show that the proportions of the various molecular forms may vary with culture, extraction, or storage conditions. We also show that, in addition to previously characterized molecules, the cells produce an amphiphilic tetramer, G2, as well as an unstable component of 13.7 S.

We then investigated the effect of cotransfection with vectors encoding the QN/HC protein, in which the attachment domain from AChE-associated collagenic subunits is fused to a GPI addition signal, or the QN/stop protein, which does not possess this signal. Heteromeric associations of AChE(T) with these binding proteins produced tetramers that were attached to the plasma membrane by a GPI anchor (GPI-G4) in the first case and secreted into the medium (Gm) in the second case. We therefore analyzed AChE in both cell extracts and culture media using sedimentation, non-denaturing electrophoresis, and immunofluorescence of the transfected COS cells. Although most experiments were performed with rat AChE(T), we obtained similar results with Torpedo AChE(T). We found that the presence of QN induces AChE monomers and dimers to form tetramers, with which the attachment protein remains associated, even after cleavage of the GPI anchor.
**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents were purchased from ProLabo (Paris, France) or from Sigma (St. Louis, MO, U. S. A.). PI-PLC from *Bacillus thuringiensis* was from Immunotech (Marseille, France). The M1 and M2 monoclonal antibodies directed against the "flag" epitope were from Eastman Kodak Co.

**Expression Vectors and Site-directed Mutagenesis**—The pEF-BOS expression vectors containing the coding sequence of the *Torpedo* QN subunit or of the chimeric QN/HC protein were described previously (9). In the Qn/Hc protein, the sequence encoding the QN domain (numbered 1–110) was attached by a linker containing a BamHI site (encoding the residues GI, not numbered) to the sequence encoding the COOH-terminal peptide of *Torpedo* AChET. Hc (numbered 532–566); the truncated Qn/stop protein was obtained by site-directed mutagenesis, inserting a TGA stop codon with the single strand method (13), at position 551 within the sequence encoding Hc. The cDNAs encoding *Torpedo* and rat AChET were also inserted in pEF-BOS. Insertion of a sequence encoding the flag peptidic epitope was performed by mutagenesis in QN/HC and in rat AChET. In the case of Qn/stop, the peptidic epitope was added at the end of the Qn domain, after the linker G residue.

**Transfection of COS Cells**—COS cells were transfected by the DEAE-dextran method, as reported (5), using 5 μg of DNA encoding the catalytic subunit AChEα and various amounts of DNA encoding the binding protein, as specified. In the case of *Torpedo* AChE, the cells were allowed to recover for 2 days at 37°C after transfection and then transferred to 27°C, to allow production of active enzyme, for 2–3 days. In the case of rat AChE, the cells were maintained at 37°C and extracted 2–4 days after transfection. The culture medium (7 ml/10-cm dish containing about 5 × 10⁶ cells) was collected after variable periods of time, as indicated, for analysis of released AChE activity. The extracts and culture media were stored at −80°C.

**Sedimentation and Electrophoretic Analyses**—Centrifugation in 5–20% sucrose gradients was performed in a Beckman SW41 rotor, generally at 36,000 rpm, for 18 h at 7°C, as described previously (12). The gradients contained 1% Brij-96 or 0.2% Triton X-100, as indicated. Alkaline phosphatase (6.1 s) and β-galactosidase (16 s) from *Escherichia coli* were included as internal sedimentation standards. AChE was assayed by the colorimetric method of Ellman et al. (14).

Electrophoresis in nondenaturing polyacrylamide gels was performed as described previously (11). The gels contained 0.25% Triton X-100 and 0.05% deoxycholate and were electrophoresed for about 2–3 h under 15 volts/cm, with refrigeration at 15°C. AChE activity was revealed by the histochemical method of Karnovsky and Roots (15).

**Production of Anti-QN Antibser—**The rabbit polyclonal antiserum directed against the QN domain was prepared against a recombinant protein produced in *E. coli*, as described (9).

**Treatment with PI-PLC—**Samples of extracts (50 μl) were treated with 0.025 IU of PI-PLC for 1 h at 30°C.

**Immunofluorescence**—Immunofluorescence was performed with a rabbit antiserum directed against rat AChE (16), as described previously (17), except that the second antibody was an fluorescein isothiocyanate-conjugated Fab’ fragment of anti-rabbit IgG (Silenius, Australia). The cells were analyzed either intact or after permeabilization with 0.2% saponin.

**RESULTS**

**Quaternary Structures of Rat AChET Subunits Produced in COS Cells**

Expression of the Rat AChET Subunit in Transfected COS Cells: Variable Patterns of AChE Molecular Forms—We studied the production of AChE in COS cells expressing the rat AChET subunit. The activity obtained per culture dish increased with the cell density but plateaued at a maximal value around 10⁶ cells/10-cm dish, and we therefore used this density in all of our experiments. Following transfection, AChE activity was barely detectable in the cells after overnight incubation and then increased steadily for at least 5 days. It appeared slightly later in the culture medium, where its proportion increased from about 30% of the total activity after 30 h to 70% after 4 days. In addition, the yield of cellular activity showed a saturable Michaelis-type increase with the amount of recombinant plasmidic DNA used for transfection, reaching a plateau at about 10–15 μg/dish, with half-saturation around 5 μg of DNA/dish (not shown).

We analyzed the molecular forms of AChE in the cell extracts and in the culture medium, by sedimentation in sucrose gradi-
ents (Fig. 1, A and B). The sedimentation profiles did not change with the cell density or with the amount of DNA encoding AChE2/dish (not shown). In Fig. 1A, typical sedimentation profiles of cell extracts show the presence of monomers (G1; 3 S), dimers (G2; 4.5 S), amphiphilic tetramers (G4; 8.5 S), non-amphiphilic tetramers (G4na; 10.5 S), and a 13.7 S molecular species. The proportion of oligomers to monomers increased progressively during the first days following transfection, and the sedimentation pattern then remained essentially constant between 3 and 5 days. The sedimentation profiles were reproducible in a given series of experiments, but we observed a rather large variability in the proportions of molecular forms (i.e. in the relative proportion of monomers to dimers and higher oligomers) when comparing experiments performed over a long period of time, possibly because of variation in the batches of cells. The cause of this variability was not investigated systematically. It was less marked in the medium than in cell extracts (Fig. 1B); the medium generally contained about 35% G1, 45% G2, 20% G4na, together with small variable proportions of G2a and sometimes of the 13.7 S form. In some cases we observed the presence of a nonamphiphilic monomeric form (G1na), probably generated by a proteolytic process, after prolonged culture, e.g. 4 days (see below).

Fig. 1 illustrates the influence of detergents on the sedimentation of the different molecular forms: amphiphilic forms sediment faster in the presence of Triton X-100 than in the presence of Brij-96, whereas nonamphiphilic forms are unaffected. Both monomers (G1) and dimers (G2) are amphiphilic forms of type II (10–12), and the G4 form probably also belongs to this category, as evidenced by the influence of detergents on their electrophoretic migration (not shown) and on their sedimentation in the presence of Triton X-100 and Brij-96, respectively: 4.5 and 3 S (G2a), 6.5 and 4.5 S (G2na), 10 and 8.5 S (G4na).

Sedimentation profiles obtained in the presence of Triton X-100 or Brij-96 or without detergent showed that the 13.7 S component is not amphiphilic (Fig. 1A). Assuming that it is a globular protein composed exclusively of AChE subunits, the ratio of its mass to that of G4na tetramers, which is close to 1.5 ((13.7/10.5)3/2), suggests that it is hexameric. This component is relatively abundant in fresh cellular extracts that had been maintained in the cold, without detergent (Fig. 2). In the absence of detergent it dissociated at 37°C, mostly into monomers (G1). In the presence of Triton X-100, dissociation occurred even in the cold and produced amphiphilic tetramers (G2) as well as monomers (G1). This instability explains why the proportion of the 13.7 S peak was variable and why this component did not generally appear in the culture medium. We also observed some inactivation of the G1 form at 37°C, particularly in the presence of Triton X-100; the instability of this form probably contributes to the variability of its proportion in cell extracts, especially when analyzed by nondenaturing gel electrophoresis.

Distinction between Cellular and Secreted AChE Molecules—Although the cellular and released forms cannot be distinguished readily by their sedimentation, they migrate slightly differently in nondenaturing electrophoresis (Fig. 3), possibly because of differences in glycosylation, or proteolytic cleavage, or other post-translational modification. To investigate the last possibility, we introduced by mutagenesis the peptidic sequence DYKDDDDK (flag epitope) after the normal COOH terminus of the rat AChE2 subunit. The presence of this peptide at the COOH terminus did not significantly modify the production of active AChE, its distribution in the cellular and secreted fractions, or the proportions of its molecular forms. We found that although the cellular G2 forms carried the epitope, as shown by the effect of an anti-flag M2 monoclonal antibody on their sedimentation and electrophoretic migration, their secreted counterparts did not (not shown). This shows that proteolytic cleavage removes the flag peptide but not necessarily that it occurs within the COOH-terminal T peptide.

In fact, polyclonal antibodies raised against the last 10 residues of the T peptide of Torpedo AChE or of rat AChE (18) were able to retard the electrophoretic migration of secreted as well as cellular active molecules (G1a, G2a, G4a, and G4na), in the case of both species (not shown).

S. Bon, I. Cornut, J. Dufourcq, J. Grassi, and J. Massoulié, manuscript in preparation.
produced at different times in cells expressing rat AChET and the QN/stop or QN/HC binding proteins, while the amount of reduced to less than 10% of the control activity obtained when QN/HC; these patterns show that tetramers accumulate progressively after transfection, between 1 and 2 days, whereas the cellular activity was increased at low doses of DNA encoding the binding proteins QN/HC or QN/stop (QN/stop551).

Proteins QN/stop and QN/HC: Influence of the Levels of Expression—The structure of the binding proteins QN/HC, and QN/stop is shown in Fig. 4. In cotransfection experiments, we used a nonsaturating dose of DNA encoding the rat AChE subunit (5 μg/dish) together with various doses of DNA encoding the binding proteins QN/HC, or QN/stop (QN/stop551).

Coexpression with QN/HC Carries Rat AChET to the Cell Surface—Immunofluorescence of transfected COS cells, with or without permeabilization, showed that AChET was not associated with the cell surface when expressed alone. In contrast, a QN/HC protein that included a flag peptide epitope at its NH2-terminal extremity (see below) and could thus be visualized with a specific monoclonal antibody (M1), was exposed at the cell surface (not shown). When AChET was coexpressed together with QN/HC, it was carried with it to the cell membrane (Fig. 5) from where it could be released by PI-PLC (not shown), as in the case of GPI-anchored dimers generated from AChE homodimers (19).

The distribution of intracellular AChET was quite different in the two cases, showing an accumulation in vesicles in the presence of QN/HC, and a reticular pattern in its absence. This suggests that AChET transits differently from the endoplasmic reticulum to the external medium when associated with a GPI-anchored protein.

Conditions for Coexpression of Rat AChET and the Binding Proteins QN/stop and QN/HC: Influence of the Levels of Expression Vectors—Fig. 6 illustrates sedimentation profiles of AChE produced at different times in cells expressing rat AChE and QN/HC; these patterns show that tetramers accumulate progressively after transfection, between 1 and 2 days, whereas dimers and monomers increase little during the same period. This is entirely consistent with the fact that the metabolic half-life of monomers and dimers is much shorter than that of higher oligomers (20). To study the assembly of heteromeric tetrmers with QN/stop or QN/HC, we analyzed the molecular forms produced after a delay of 2–4 days following transfection.

We studied the effect of varying the amount of DNA encoding the QN/stop or QN/HC binding proteins, while the amount of DNA encoding AChET remained constant (Fig. 7, A and B). In both cases, the cellular activity was increased at low doses of DNA encoding the binding proteins, but the total AChE activity produced per dish, in the cells and in the medium, was considerably reduced at high doses. At 40 μg of DNA/dish, it was reduced to less than 10% of the control activity obtained when cells were only transfected with 5 μg of DNA encoding AChET/dish (not shown). In the case of QN/stop, the proportion of secreted activity was increased significantly at all concentrations of DNA (about 95% of the total activity compared with about 70% in control cells expressing AChET alone), in agreement with the production of soluble heterotetramers, and this was correlated with a much higher total activity/dish (Fig. 7B). In the case of QN/HC, which forms GPI-anchored tetramers attached to cell membrane, the proportion of secreted activity decreased, at least up to 1 μg of QN/HC DNA (Fig. 7A). The activity recovered in the culture medium increased, however, at higher doses of DNA, reflecting the release of nonamphiphilic monomers (G1na), as discussed below.

Sedimentation profiles of corresponding cell extracts and culture media are shown in Fig. 8. The activity of G1, G2, and 13.7 S decreased in the cells and in the medium with the dose of DNA encoding either QN/HC or QN/stop. With increasing doses of QN/stop, the activity of heteromeric tetramers, G4na, increased in the cells and in the medium with a concomitant decrease of G1 and G2, reaching a maximum around 2–3 μg of DNA encoding the binding protein/dish (Fig. 8, C and D); the decrease observed at higher doses reflected the decrease in total cellular AChE activity. In the case of QN/HC, we observed a similar evolution of G1na in the cells (Fig. 8A) and in the culture media, which coadsorbed with G2 in the presence of Brij-86. This form was abundant in the experiment illustrated in Fig. 8, A and B, but its proportion was variable, and it was not always observed in other batches of COS cells. It probably results from a lytic process in the metabolic pathway of the GPI-anchored molecules, since it did not appear in the case of parallel cotransfections with QN/stop, even at high doses of DNA (Fig. 8, C and D).

Fig. 7 also illustrates the variation of the ratio R of heteromeric forms to total cellular or secreted AChE activity: R varies with the dose of DNA encoding QN/stop (d), as R = R(0) + Rmaxd/d(E + d), where R(0) is the ratio obtained with AChET alone, and Rmax is the maximal ratio obtained with a saturating dose of DNA. The parameter E thus defines an overall "efficiency" of interaction between the AChET subunits and the binding proteins in the secretory pathway. Under specific experimental conditions, this parameter allows an evaluation of such interactions in living cells. In the case of QN/stop (Fig. 7B),

---

3 S. Bon, F. Coussen, and J. Massoulié, manuscript in preparation.
E is smaller for secreted than for cellular AChE, in agreement with the fact that the G4 na form is exported more efficiently than the G1 a and G2 a forms, whereas in the case of QN/HC, the values of E were approximately equal for the cellular and secreted compartments (Fig. 7 A).

In the following experiments we generally used 5 μg of DNA encoding AChET and 9 μg of DNA encoding QN/HC per dish containing about 10^6 cells. The proportion of GPI-anchored tetramers (G4 a) increased markedly in cellular extracts between 1 day (○) and 2 days (△) after transfection.

To examine whether the complete binding protein remained associated in the hetero-oligomers, we also introduced the flag peptide at the NH2-terminal and at the COOH-terminal extremities of the QN domain.

In any case, this showed that the QN domain was included not only in the GPI-anchored G4 a molecules but also in the protein containing this domain. This antibody, anti-QN, increased the sedimentation coefficient of both cell-associated and released tetramers produced by cells expressing Torpedo AChET together with QN/HC by about 1 S unit, whereas the G2 peak was not affected (not shown). The effect of the antiserum was more marked in nondenaturing electrophoresis, possibly because dilution was less important in this case (Fig. 9): a fraction of the G4 form, secreted by cells coexpressing rat AChET and either QN/HC or QN/stop, was retarded, indicating that the released tetramers contained the QN binding domain.

To examine whether the complete binding protein remained associated in the hetero-oligomers, we also introduced the flag peptide at the NH2-terminal and at the COOH-terminal extremities of the QN domain.

In the first case, the epitope was placed between residues Ala-42 and Glu-43 of the precursor sequence in the QN/HC chimeric protein ("N-flagged" QN/HC), corresponding to the most likely cleavage site of the signal peptide (21). By coexpression of N-flagged QN/HC with rat AChET, we obtained cell-associated GPI-anchored G4 a and released G4 na, with the same apparent affinity as with QN/HC, demonstrating that the presence of the flag epitope did not interfere with the heteromeric association of QN and AChET subunits. The sedimentation of these molecules was not affected visibly by the M1 monoclonal antibody, probably because its affinity was not sufficient to withstand dilution in the gradients, but they were both partially retarded by M1 during electrophoretic migration in nondenaturing polyacrylamide gels (Fig. 10 A). Only a fraction of the molecules was retarded, as also observed in the case of anti-QN, perhaps because accessibility of the epitope was restricted by the presence of an associated tetramer of catalytic subunits. Alternatively, it is possible that several sites of cleavage coexist or that the flag sequence introduced a new cleavage site, which eliminated the epitope from the mature protein, so that only a fraction of hetero-oligomers possessed a complete epitope.

In any case, this showed that the QN domain was included not only in the GPI-anchored G4 a molecules but also in the

![Image](https://example.com/image.png)

**Fig. 5.** Immunofluorescence staining of transfected COS cells expressing rat AChET alone and together with the binding protein QN/HC. Left panels, AChET alone; right panels, AChET + QN/HC; panels a and b, fields; panels c–h, details of individual cells; panels a–d, nonpermeabilized cells; panels e–h, permeabilized cells. Note that AChE is externalized when coexpressed with QN/HC and that the pattern of intracellular staining is markedly different in the case of AChET alone and of AChET + QN/HC. The magnification factor is approximately 50 for panels a and c, 80 for panels b and d, and 200 for panels e–h. The scale bar (panel a) corresponds to about 100 μm in panels a and c (objective × 25), 60 μm in panels b and d (× 40), and 25 μm in panels e–h (× 100). Note that the size of transfected cells was highly variable, i.e. the two cells shown in panel d.

**Fig. 6.** Sedimentation patterns of AChE in COS cells expressing AChET and QN/HC; effect of time after transfection. Transfection was performed with 5 μg of DNA encoding AChET and 9 μg of DNA encoding QN/HC per dish containing about 10^6 cells. The proportion of GPI-anchored tetramers (G4 a) increased markedly in cellular extracts between 1 day (○) and 2 days (△) after transfection.
FIG. 7. Cotransfection of rat AChET with QN/HC or QN/stop: effect of the dose of DNA encoding the binding protein on the production and release of AChE activity, and on the proportion of heteromeric forms. Panel A, QN/HC; panel B, QN/stop. The cells were transfected with 5 µg of DNA encoding AChET and the indicated amount of DNA encoding the binding protein. Cells and culture media were collected 3 days after transfection. ●, cellular activity; ■, secreted activity; ▲, total activity. The proportion R of heteromeric forms in the cellular (□) and secreted (○) AChE activity was calculated from the sedimentation profiles shown in Fig. 8. The curves were fitted by equations of the form

\[ R = R(0) + R_\infty \frac{d(E + d)}{d} \]

In the case of QN/HC, the variation of R in the medium only tends to plateau if we include the lytic nonamphiphilic G1na form (\( R = (G_{1na}^a + G_{1na}^b)/\text{total secreted activity} \)), clearly suggesting that G1na was derived from heteromeric GPI-anchored tetratomers. The values of E used for fitting the data were 0.7 for both cells and medium in the case of QN/HC, 1 for cells and 0.15 for medium in the case of QN/stop.

released G1na. In addition, it indicated that the cleavage of the signal peptide occurred after Ala-42 at least in a fraction of the protein, since the M1 antibody is considered to recognize the flag epitope only in an NH2-terminal position. Glu-43 is therefore the likely NH2-terminal extremity of the mature QN domain that binds an AChET tetramer.

The flag epitope was also added immediately after the QN domain, instead of Hc. When this construction (QN/flag) was cotransfected with rat AChET, we obtained the same result as with QN/stop, i.e., a large production and release of G2na, at the expense of G1 and G2. In the medium the released G2na molecules contained the QN/flag protein, as shown by the M2 antibody, which recognizes the epitope in a COOH-terminal position; it induced a shift in sedimentation (not shown) and retarded migration in nondenaturing electrophoresis (Fig. 10B). The presence of two distinct bands that react with the antibody suggests that, at high doses of DNA, QN may also be associated with dimers of AChET subunits, in addition to tetratomers.

Thus, the presence of a flag epitope, either in an NH2-terminal or a COOH-terminal position, did not prevent the production of the heteromorphic AChE tetratomers. The M1 antibody was able to recognize the NH2-terminal flag in the lytic cellular and released soluble tetratomers (G2na) derived from GPI-anchored G2. Soluble tetratomers obtained in the presence of QN/stop carrying an NH2- or COOH-terminal flag were recognized by M1 and M2, respectively, showing that they also contained the binding protein.

**DISCUSSION**

*The Fate of AChET Subunits Produced in COS Cells: Quaternary Associations; Secretion—* In the first part of this study we analyzed the production of AChE by transfected COS cells expressing rat AChET subunits alone. As shown previously, the cells contained monomers (G2), dimers (G2na), and tetratomers (G4) (6). The G2 and G2na forms correspond to amphiphilic molecules of class II, which interact with detergent and lipid micelles, probably through an amphiphilic α-helical region in the COOH-terminal T peptide\(^2\) (1). The production of AChE increased with the quantity of DNA used for transfection, in a saturable manner, but the proportions of the different forms were not modified. AChE activity increased with time after transfection, with an accumulation of tetratomers over monomers and dimers, in agreement with the fact that the metabolic turnover rate of the latter is more rapid (20).

We show here the existence of amphiphilic tetratomers (G4) as well as nonamphiphilic tetratomers (G2na). As shown in the case of butyrylcholinesterase (22, 23), the G4 component probably represents homotetramers of AChET subunits, in which the amphiphilic helices of the T peptides interact with each other. In contrast, the structure of G2 might represent another type of quaternary organization in which all or part of the amphiphilic helices is exposed. The fact that this molecular form can be solubilized readily in the absence of detergent indicates that it differs from the membrane-bound G2 AChE of mammalian brain, which contains a 20-kDa hydrophobic anchor (24–26).

COS cells also produced a nonamphiphilic, unstable component sedimenting at 13.7 S, which may be an hexamer of catalytic subunits, unless it contains other subunits. In spite of its instability, the 13.7 S form was sometimes observed in the culture medium, although in a minor proportion, suggesting that it does not incorporate intracellular resident proteins. It was also found in *Xenopus* oocytes expressing the rat AChET subunit.\(^4\) This component readily dissociates at 37°C, producing mostly monomers (G2) in the absence of detergent, but also dimers and amphiphilic tetratomers (G2na) in the presence of Triton X-100. Interaction with detergent micelles may stabilize a quaternary conformation of tetratomers in which hydrophobic surfaces are exposed.

Finally, we illustrate the fact that, although the characteristics of the molecular forms are well defined, their proportions

\(^4\) E. Krejci, personal communication.
are variable among different experiments, especially over long periods of time, using different batches of COS cells. Such variations may result from differences in the biosynthetic capacity of the cells, in the culture medium, or in the extraction and storage of the enzyme, especially in view of the instability of some of the molecular forms. The fact that the pattern of molecular forms may be modified in a temperature-dependent manner by detergent (Triton X-100) indicates that it does not exactly reflect the state of quaternary associations of AChE in the intact cell.

COS cells expressing Torpedo AChET subunits produce a very small proportion of monomers, but mostly dimers, G2a, together with minor G2a and G4na forms. This suggests that, unlike rat AChET, the Torpedo AChET subunits are unstable in the monomeric state.

The release of Torpedo and rat AChE in the culture medium differed markedly. The culture medium of COS cells expressing rat AChET subunits contained about 30% of the total AChE activity after 2 days and 60% after 3 days following transfection, with similar proportions of amphiphilic dimers (G2a) and monomers (G1a) of type II, together with a smaller proportion of nonamphiphilic tetramers (G4na). Although their sedimentation properties appeared identical, the migration of the cellular and released G2a and G4na forms was clearly different in nondenaturing electrophoresis. This suggests that post-translational modifications accompany the release of rat G1a and G2a AChE, which

![Figure 8](image-url)
Torpedo was secreted readily at 27°C. It seems, therefore, that the anti-QN antiserum. The culture medium of COS cells expressing rat AChE<sub>r</sub> together with Q<sub>N</sub>/H<sub>C</sub> or Q<sub>N</sub>/stop was analyzed by non-denaturing electrophoresis, without (−) or with the anti-QN antiserum (+). Whereas nonimmune serum had no effect, the anti-QN antiserum retarded the migration of a fraction of the G<sub>2</sub><sup>a</sup> form. In this experiment, we used Q<sub>N/stop111</sub>, in which a stop codon was added at position 111 within the QN domain (see Ref. 31). Asterisks indicate the position of complexes with the anti-QN antibodies.

Therefore represents a true secretory process and does not result from cell lysis or other damage. These modifications may include maturation of glycans, proteolysis, or possibly palmitoylation (27).

When a flag peptidic epitope was added by mutagenesis at the COOH terminus of the rat AChE<sub>T</sub> subunit, it did not modify the production, oligomeric assembly, or secretion of the enzyme. The epitope could be detected by a specific monoclonal antibody (M2) in the cellular but not in the secreted molecules, indicating that secretion could be accompanied by proteolytic cleavage. If cleavage does occur within the T peptide, however, only few residues are removed, since antibodies raised against its last 10 amino acids recognize both cellular and secreted molecules. This is consistent with the fact that the secreted monomers and dimers retain their amphiphilic character, which probably depends on the presence of an amphiphilic α-helix, constituted by the first 20 amino acids of the T peptide.\(^2\) Note, however, that Liao et al. (17) observed that soluble monomers from bovine brain did not react with their anti-COOH-terminal peptide. We also found that media recovered after several days of culture could contain nonamphiphilic monomers (G<sub>4</sub><sup>na</sup>), probably resulting from proteolysis of the T peptide, as already observed by Velan et al. (28), in the case of HEK 293 cells expressing the human AChE<sub>T</sub> subunit. Taken together, these observations suggest the existence of multiple cleavage sites. It will be interesting to investigate whether secreted dimers (G<sub>2</sub><sup>a</sup>) conserve the cysteine residue that is located at position −4 from the COOH terminus and forms an intersubunit disulfide bond in tetramers.

Cells expressing Torpedo AChE<sub>T</sub> subunits produced the G<sub>2</sub><sup>a</sup> form but no G<sub>1</sub><sup>a</sup> form and released very little AChE activity. This difference could not be accounted for by the fact that the cultures were maintained at 37 °C in the case of rat AChE, but transferred to 27 °C to produce Torpedo AChE in an active form. When rat AChE was expressed at 27 °C, the total activity was reduced to about one-third of its value at 37 °C, but the ratio of secreted to cellular enzyme was approximately the same (not shown). Moreover, the heteromeric G<sub>4</sub><sup>na</sup> form of Torpedo AChE, obtained in coexpression with a Q<sub>N</sub>/stop protein, was secreted readily at 27 °C. It seems, therefore, that the Torpedo G<sub>2</sub><sup>a</sup> form cannot be transported efficiently to the membrane and released into the medium.

The structure of AChE<sub>T</sub> is remarkable because these various oligomeric states of the enzyme are not in equilibrium; extracted or secreted molecules may form aggregates under specific conditions but were never observed to assemble into well defined oligomers such as those produced in the cells. The nature of the enzyme has a crucial influence on the proportions of oligomeric and monomeric forms produced in cells; this depends on the alternative COOH-terminal peptides as shown by the difference between rat AChE<sub>r</sub> and AChE<sub>h</sub> in rat basoleukemia cells (17), but also on the species. Thus, Torpedo AChE preferentially produces dimers, and rat AChE mostly produces monomers. These proportions also depend on the nature and state of differentiation of the cell; for example, it varies during development of the nervous system (for review, see Ref. 1), and AChE<sub>r</sub> subunits are expressed in a tissue-specific manner in Xenopus embryos, even under the control of a viral promoter (29, 30). In culture, the yield of AChE activity was markedly less in rat basoleukemia cells than in COS cells, in parallel transient transfections (17). We show here that, depending on their state, the COS cells themselves produce variable patterns of molecular forms from AChE<sub>r</sub> subunits. In the case of the rat enzyme, the cells may contain almost exclusively G<sub>2</sub><sup>a</sup>, or equal amounts of G<sub>2</sub><sup>a</sup> and G<sub>2</sub><sup>na</sup>, together with significant proportions of G<sub>4</sub><sup>a</sup>, G<sub>4</sub><sup>na</sup>, and 13.7 S oligomers. Thus, the cellular environment somehow controls the formation of oligomers.

Interaction between the Q<sub>N</sub> Domain and AChE<sub>r</sub> in Cotransfected COS Cells: Production of Heteromeric Tetramers—Cotransfection of COS cells with rat or Torpedo AChE<sub>r</sub> subunits, together with an isolated Q<sub>N</sub> domain (Q<sub>N/stop</sub>) or with a Q<sub>N</sub>...
domain associated with a GPI addition signal (Q_N/H_C), produced heteromeric molecules, G^{na}_na or GPI-G_4^{na}. In the case of Torpedo AChE, this allowed an active exportation and release of tetramers, whereas dimers were almost entirely retained within the cells. Immunofluorescence of permeabilized cells showed that the enzyme appeared distributed in the reticulum when expressed alone, but concentrated into subcellular bodies, perhaps secretory vesicles, when coexpressed with Q_N/H_C.

At low doses of DNA encoding the binding proteins, we observed an increase in the production of total AChE (cellular and secreted), in agreement with the incorporation of monomeric G_4^{na} and dimeric G_2^{na} forms, which present a rapid metabolic turnover rate (20) into more stable tetramers. However, the total yield of AChE was decreased at high doses of DNA encoding Q_N/stop and more markedly Q_N/H_C, probably because of competition between the production of catalytic and structural subunits.

The heteromeric G^{na}_na molecules obtained by association of Q_N/stop with AChE_T tetramers were soluble and secreted, resulting in a high yield of AChE activity in the culture medium. In contrast, the GPI-anchored molecules obtained by expression of Q_N/H_C alone or in combination with AChE_T were mostly attached to the cellular surface, as shown by immunofluorescence of intact cells. Thus, the presence of Q_N/H_C induced the assembly of GPI-anchored tetramers of AChE_T. Coexpression with Q_N/H_C also produced some lytic G_4^{na}; in addition, at high doses of DNA encoding Q_N/H_C, and in some batches of COOH-terminal cells, we also observed the production of G^{na}_na molecules, both in cell extracts and in the medium. Since this G^{na}_na form was not observed in parallel cotransfections with Q_N/stop, the production of these lytic molecules possibly occurs in the metabolic pathway of GPI-anchored proteins, e.g., after reinternalization from the cell surface. Note, however, that cells expressing only AChE_T also produced a G^{na}_na form after several days in culture, as also observed in HEK 293 cells (28), but this may result from another type of cleavage.

We considered the possibility that a Q_N domain might induce the formation of AChE_T tetramers, without necessarily participating in the final quaternary structure. This hypothesis seemed consistent with the fact that homotetramers are formed at a low level, in the absence of binding proteins, and that the pair of vicinal cysteines of the Q_N domain could be mutated without affecting the production of G_4 molecules (31). To detect the presence of Q_N in G_4 molecules, we used an antisera raised against this domain. We found that the sedimentation of released G_4 was shifted in the presence of this antisera to the same extent as that of PI-PLC-treated molecules derived from GPI-anchored G_4, indicating that Q_N was associated with the released G_4 tetramers. The presence of Q_N in the secreted soluble G_4 form, as well as in the GPI-anchored cellular G_4 form, was confirmed by inserting a flag epitope in NH_2-terminal position (N-flagged Q_N/stop and Q_N/H_C), and in COOH-terminal position (Q_N/flag). The M_1 monoclonal antibody reacted only with a fraction of heteromeric tetramers when a flag epitope was introduced between residues 42 and 45 of the precursor of Q_N (both in Q_N/stop and Q_N/H_C), possibly because it could be cleaved during maturation. Although limited, this reaction indicated that cleavage of the signal peptide occurs at the indicated position, that the mature protein probably starts at Glu-43, and that the flag peptide does not prevent interaction with AChE_T.

In this study we showed that AChE_T subunits, when expressed in COS cells, are able to generate a number of various more or less stable oligomeric associations. Coexpression with binding proteins derived from the NH_2-terminal domain of the collagenic tail induces the preferential formation of heteromeric molecules in which AChE_T tetramers are assembled with a binding domain. This appears to facilitate the transport of AChE_T subunits in the secretory pathway and exportation to the cell surface, in the case of GPI-anchored molecules, or their secretion, in the case of soluble molecules. The recruitment of AChE_T subunits depends on the dose of binding protein, in a saturable manner. It is therefore possible to analyze these protein-protein interactions quantitatively in the secretory pathway of living cells.

Acknowledgments—We thank Anne Le Goff and Rizwana Nawaz for expert technical assistance; Dr. Françoise Coussen and Dr. Jacques Grassi for preparation of the rabbit polyclonal anti-Q_N antisera; Prof. Bent Nørnghed-Pedersen, Prof. Urs Brodbeck, and Dr. Nicola Boschetti for the anti-bovine COOH-terminal peptide antisera; and Jacqueline Pons for typing the manuscript.

REFERENCES

1. Massoulie, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F. M. (1993) Prog. Neurosci. 41, 31–91
2. Rosenberry, T. L., and Richardson, J. M. (1977) Biochemistry 16, 3550–3558
3. Anglister, L., and Silman, I. (1978) J. Mol. Biol. 125, 293–311
4. Lee, S. L., Heinemann, S., and Taylor, P. (1982) J. Biol. Chem. 257, 12292–12299
5. Krejci, E., Coussen, F., Duval, N., Chatel, J. M., Legay, C., Puyje, M., Vandekerckhove, J., Cartaud, J., Bon, S., and Massoulie, J. (1991) EMBO J. 10, 1285–1293
6. Legay, C., Bon, S., Vernier, P., Coussen, F., and Massoulie, J. (1993) J. Neurochem. 60, 337–346
7. Massoulie, J., Sussman, J., Bon, S., and Silman, I. (1993) Prog. Brain Res. 98, 139–146
8. Camp, S., Bon, S., Li, Y., Getman, D. K., Engel, A. G., Massoulie, J., and Taylor, P. (1995) J. Clin. Invest. 95, 333–340
9. Duval, N., Krejci, E., Grassi, J., Coussen, F., Massoulie, J., and Bon, S. (1992) EMBO J. 11, 3255–3261
10. Bon, S., Toutant, J. P., Mefik, and Massoulie, J. (1988) J. Neurochem. 51, 576–585
11. Bon, S., Toutant, J. P., Mefik, K., and Massoulie, J. (1988) J. Neurochem. 51, 157–172
12. Bon, S., Rosenberry, T. L., and Massoulie, J. (1991) Cell. Mol. Neurobiol. 11, 157–172
13. Kinkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
14. Eillman, G. L., Courtney, K. D., Andreas, V., and Featherstone, R. M. (1961) Biochem. Pharmacol. 7, 88–95
15. Karnovsky, M. J., and Roots, L. (1964) J. Histochem. Cytochem. 12, 219–232
16. Marsh, D., Grassi, J., Vigny, M., and Massoulie, J. (1984) J. Neurochem. 43, 204–213
17. Coussen, F., Bonnerot, C., and Massoulie, J. (1995) Eur. J. Cell Biol. 67, 254–260
18. Liao, J., Boschetti, N., Mortensen, V., Jensen, S. P., Koch, C., Norgaard-Pedersen, B., and Brodbeck, U. (1994) J. Neurochem. 63, 1446–1453
19. Gibney, G., and Taylor, P. (1990) J. Biol. Chem. 265, 12576–12583
20. Lazaer, M., Salmeron, E., Vigny, M., and Massoulie, J. (1984) J. Biol. Chem. 259, 3703–3713
21. von Heijne, G. (1986) Nucleic Acids Res. 14, 4683–4690
22. Lockridge, O., Adkins, S., and La Du, B. N. (1987) J. Biol. Chem. 262, 19429–19452
23. Lockridge, O., Bartels, C. F., Vaughan, T. A., Wong, C. K., Norton, S. E., and Johnson, L. L. (1987) J. Biol. Chem. 262, 549–557
24. Inestrosa, N. C., Roberts, W. L., Marshall, T. L., and Rosenberry, T. L. (1987) J. Biol. Chem. 262, 4441–4444
25. Gennari, K., Brunner, J., and Brodbeck, U. (1987) J. Neurochem. 49, 12–18
26. Roberts, W. L., Doctor, B. P., Foster, J. D., and Rosenberry, T. L. (1991) J. Biol. Chem. 266, 7481–7487
27. Randall, W. R. (1994) J. Biol. Chem. 269, 12374–12374
28. Velan, B., Grosfeld, H., Kronman, C., Leitner, M., Gozes, Y., Lazar, A., Flashner, Y., Marcus, D., Cohen, S., and Shafferman, A. (1991) J. Biol. Chem. 266, 23977–23984
29. Ben Aziz-Aloya, R., Seidman, S., Timberg, R., Sternfeld, M., Zakt, H., and Soreq, H. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2471–2475
30. Seidman, S., Sternfeld, M., Ben Aziz-Aloya, R., Timberg, R., Kaufer-Nachum, D., and Soreq, H. (1995) Mol. Cell. Biol. 1555, 2993–3002
31. Bon, S., Coussen, F., and Massoulie, J. (1997) J. Biol. Chem. 272, 3016–3021