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Primary structure of a collagenic tail peptide of *Torpedo* acetylcholinesterase: co-expression with catalytic subunit induces the production of collagen-tailed forms in transfected cells

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The asymmetric forms of cholinesterases are synthesized only in differentiated muscular and neural cells of vertebrates. These complex oligomers are characterized by the presence of a collagen-like tail, associated with one, two or three tetramers of catalytic subunits. The collagenic tail is responsible for ionic interactions, explaining the insertion of these molecules in extracellular basal lamina, e.g. at neuromuscular endplates. We report the cloning of a collagenic subunit from *Torpedo marmorata* acetylcholinesterase (AChE). The predicted primary structure contains a putative signal peptide, a proline-rich domain, a collagenic domain, and a C-terminal domain composed of proline-rich and cysteine-rich regions. Several variants are generated by alternative splicing. Apart from the collagenic domain, the AChE tail subunit does not present any homology with previously known proteins. We show that co-expression of catalytic AChE subunits and collagenic subunits results in the production of asymmetric, collagen-tailed AChE forms in transfected COS cells. Thus, the assembly of these complex forms does not depend on a specific cellular processing, but rather on the expression of the collagenic subunits.

Key words: acetylcholinesterase/asymmetric forms/collagen/cDNA sequence/*Torpedo marmorata*

Introduction

The asymmetric or collagen-tailed form of AChE consist of one to three catalytic tetramers, attached to the subunits of a triple helical collagenic tail, as clearly seen in electron micrographs (for a review, see Massoulié and Toutant, 1988). These complex molecules are of considerable physiological interest, because they are expressed only in the nervous system and in muscle tissue. Their appearance during embryogenesis also suggests that they are produced only by differentiated cells (see review by Toutant and Massoulié, 1988). In adult skeletal muscle, asymmetric AChE forms are generally localized specifically at neuromuscular junctions, and in some species (rat, chicken), they disappear after denervation, even though the levels of globular forms may either decrease (rat) or dramatically increase (chicken). The expression of asymmetric forms is thus controlled in a very specific manner, depending on cellular differentiation and activity.

Recently catalytic subunits of AChE and BuChE from various species have been cloned and sequenced. At least two types of catalytic subunits may be produced by alternative splicing. They possess a large common catalytic domain, but differ in their C-terminal region. One type of C-terminal peptide is partially cleaved and replaced by a glycophosphatidylinositol anchor, producing amphiphilic AChE dimers (Gibney *et al.*, 1988; Sikorav *et al.*, 1988; Maulet *et al.*, 1990). These forms exist in *Torpedo* electric organs, as well as in *Drosophila* nervous tissue and mammalian erythrocytes. Another type of C-terminal domain is retained in mature catalytic subunits incorporated into collagen-tailed forms of *Torpedo* AChE (MacPhee-Quigley *et al.*, 1985; Schumacher *et al.*, 1986; Sikorav *et al.*, 1987), and soluble tetramers of human plasma BuChE (Lockridge *et al.*, 1987; McTiernan *et al.*, 1987; Prody *et al.*, 1987). In the amphiphilic tetramers of AChE, which exist in the central nervous system of mammals, one of the dimers of catalytic subunits is associated through disulfide bonds with a structural 20 kd subunit which carries the hydrophobic domain (Gennari *et al.*, 1987; Inestrosa *et al.*, 1987). The similarity between the oligomeric organization of this molecule and that of the collagen-tailed forms strongly suggests that they contain the same type of catalytic subunit.

Because these catalytic subunits are assembled into various oligomeric forms, their expression cannot control the biosynthesis of asymmetric, collagen-tailed forms of AChE. The cloning of collagenic tail peptides has allowed us to show that the production of asymmetric forms simply requires the expression of catalytic and collagenic components, and does not result from specific processing by muscle or nerve cells.

Results

Purification of AChE A forms from Torpedo electric organs; isolation of the collagenic subunits and determination of peptide sequences

Asymmetric forms of AChE were purified from *Torpedo marmorata* electric organs and subjected to polyacrylamide gel electrophoresis in denaturing and reducing conditions. The peptide bands corresponding to collagenic subunits were identified by two criteria: (i) they were sensitive to collagenase treatment of the native enzyme (Figure 1), and (ii) they were not labeled in immunoblots with the monoclonal antibody TorME8, which is specific for the AChE catalytic subunits (Musset *et al.*, 1987; Bon *et al.*, 1987) (not shown). We observed a rather diffuse protein band corresponding to the collagenic tail subunits, which we named T_1. After storage, the tail subunits were partially

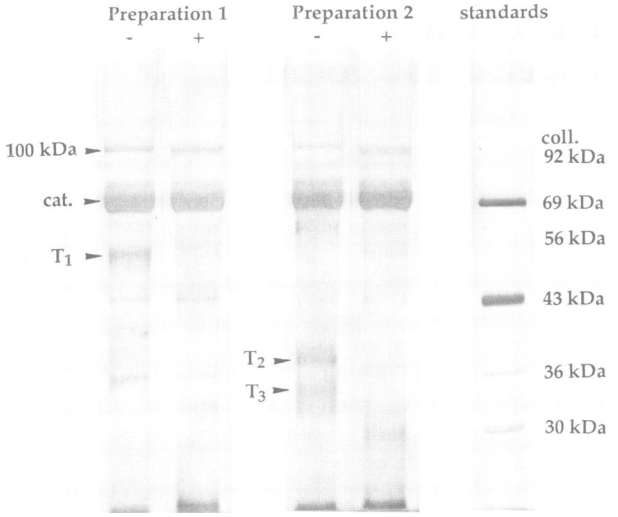

Fig. 1. Separation of subunits of AChE asymmetric forms from *Torpedo marmorata* electric organs by polyacrylamide gel electrophoresis. Preparations 1 and 2 differ by the extent of degradation of the tail subunits during storage. The 100 kd subunit and the catalytic subunit (cat.) are similar in both preparations, as well as minor bands corresponding to cleavage products of the catalytic subunit. In each case, control (−) and collagenase-treated samples (+) are compared. A minor band corresponding to collagenase (coll.) is visible in the treated samples. The protein bands corresponding to intact or cleaved collagenic tail subunits are indicated as T_1, T_2 and T_3. Note that the 100 kd subunit seems to be slightly altered after collagenase digestion.

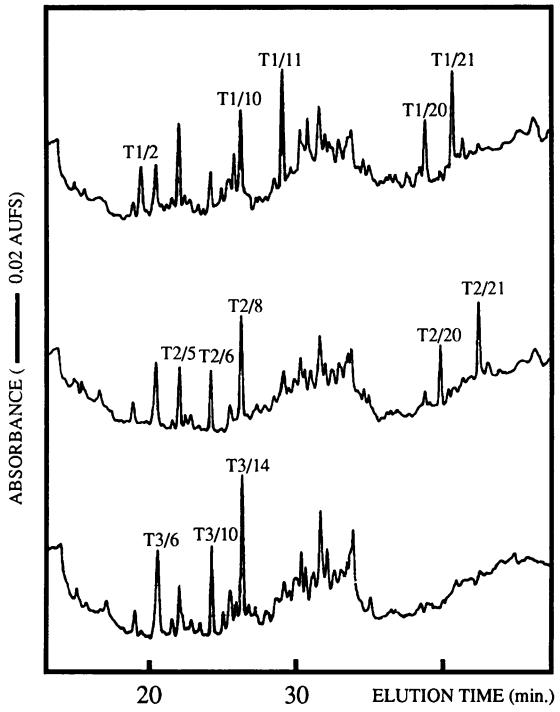

Fig. 2. HPLC elution profiles of tryptic peptides obtained from collagenic subunits. The profiles correspond to T_1, T_2 and T_3, identified in Figure 1. Peaks corresponding to well defined peptides are numbered. The sequences obtained are given in Table I (AUFS: Absorbancy Unit Full Scale).

degraded, yielding two faster migrating bands, T_2 and T_3, resembling the products obtained from T_1 after collagenase treatment (Figure 1).

After transfer onto Immobilon membranes, T_1, T_2 and T_3 were digested by trypsin, and the resulting peptides were separated by HPLC (Figure 2). Partial sequences of some of the peptides are shown in Table I. One of the peptides (T_3/8) was blocked. Most of the peptides were common to the different collagenic bands, T_1, T_2 and T_3. We obtained identical sequences for two distinct HPLC peaks (peaks 20 and 21 of T_1, and peaks 20 and 22 of T_2). These peptides, which all appeared identical in their sequence, could differ in their C-terminal tryptic cleavage sites, or in post-translational modifications such as glycosylation (see below).

Some of the sequences that we obtained correspond to a collagenic structure, with glycines every three amino acids, and a high proportion of prolines and hydroxyprolines. Some peptides, however, are not collagenic. None of the peptides could originate from the catalytic subunit.

Table I. Sequence of tryptic peptides derived from collagenic subunits of *Torpedo marmorata* asymmetric AChE

Torpedo collagenic subunit sequence	Polyacrylamide gel band and HPLC tryptic peptide
P_1 GMPGSP(E)SPGPIGPR	T_2/6, T_3/10
P_2 GEKGDIGLTGLP'GAR	T_1/10, T_2/8, T_3/14
P_3 GLP'GPVGR	T_3/6
P_4 SLYYR	T_2/5
P_5 DTVGWLPIQIAPIQQ	T_1/20, T_1/21, T_2/20, T_2/22 (absent from T_3)
P_6 SYLPGSYGELK	T_1/11
P_7 YFT	T_1/2

The peptides are numbered P_1-P_7 in the order of their appearance in the coding sequence of clone λACT_1. One peptide, T_3/8, was blocked. The peptides resulting from tryptic digestion of the protein bands are numbered in the order of their elution in HPLC. The amino acid residues are indicated in the one-letter code, P' indicating hydroxyproline. Residues in parentheses differ from those predicted in the coding sequence.

Isolation of probes and cDNA clones encoding collagenic subunits

To obtain specific probes for the collagenic subunits, we used the polymerase chain reaction (PCR) to amplify a fragment of the coding sequence corresponding to peptide P_5 (Figure 3). We thus prepared a less degenerate probe, which allowed us to identify positive clones in a cDNA library, in primary and secondary screenings. In this report, we describe the structure of one clone, λACT_1 (for Acetylcholinesterase Collagenic Tail). Further screening with a *Hin*dIII−*Hin*dIII fragment of λACT_1 led to the isolation of numerous clones, all encoding collagenic tail subunits, but presenting differences in their restriction patterns.

λACT_1 contains an open reading frame encoding 471 amino acids. The predicted protein includes all the tryptic peptides (underlined). The few discrepancies are partly due to the post-translational conversion of proline into hydroxyproline, and partly to sequencing uncertainties where cysteines were expected. This protein, therefore, unambiguously corresponds to a collagenic subunit from asymmetric AChE.

Characterization of collagen-tail mRNAs by Northern blotting

In Northern blots, a probe covering a large part of the coding sequence of clone λACT_1 (nucleotides 148−1258)

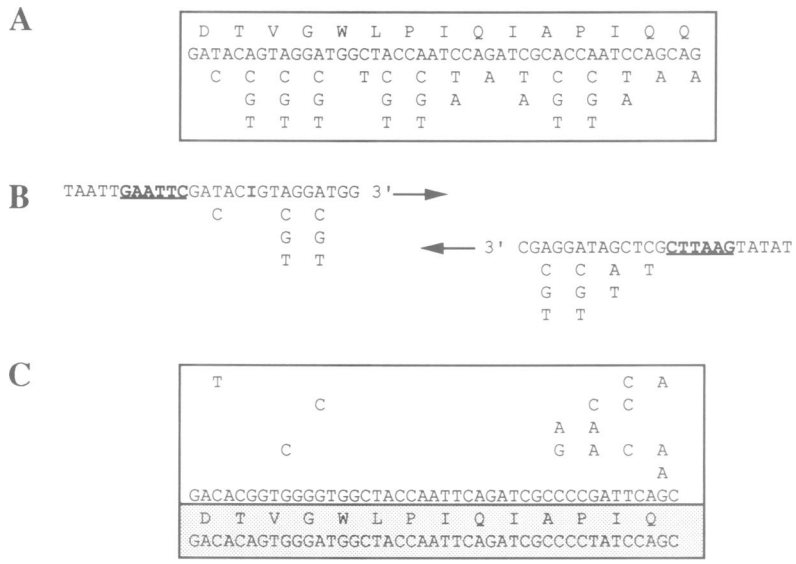

Fig. 3. Amplification of a genetic segment corresponding to a 15 amino acid peptidic sequence. (**A**) Peptidic sequence P_5 is shown on the first line above possible coding sequences; (**B**) structure of the families of primers: the *Eco*RI sites are underlined (note the presence of an inosine in one of the primers); (**C**) structure of several amplified genetic fragments and of the corresponding cDNA sequence (shaded).

produced a very strong hybridization signal around 2.8 kb, in the case of electric organs (Figure 4), but not in muscle (not shown). This indicates that collagenic tail peptides are abundantly expressed in electric organs, which contain a high level of AChE A forms, but not in tissues which do not contain these molecules, such as *Torpedo* muscle. The collagenic tail of AChE forms is not expressed independently of the production of asymmetric forms.

Sequence of a cDNA clone and primary structure of a collagenic subunit

In λACT_1 (Figure 5), the initiation codon is preceded by two stop codons in the same reading phase (at −9 and −36). The 5′-untranslated sequence of 161 nucleotides, however, contains two ATG codons, which would initiate short open reading frames, one of which overlaps the beginning of the collagenic subunit sequence, in a different phase. The collagenic protein can be divided into three main regions: an N-terminal domain of 117 residues (including the signal peptide), a central collagenic domain of 188 residues, and a C-terminal domain of 166 residues. It should be noted that all the sequenced peptides originate from the central collagenic region or from the C-terminal region. A search in data banks indicated that the collagenic tail subunit presents homology with collagens, as expected, but this is restricted to the collagenic domain and there is no significant homology with any other known protein.

The N-terminal region is largely hydrophobic, as expected for a signal peptide. The N-terminal extremity of the mature subunit has, however, not been identified yet, since all attempts to determine the N-terminal sequence of the electroblotted proteins have failed so far. It is possible that, besides cleavage of the signal peptide, the maturation process includes the removal of a procollagen peptide. The N-terminal domain contains 11 prolines, including a stretch of five consecutive residues.

The central collagenic region is characterized by the repetition of glycine every three residues. There are, however, several irregularities in the intervals between glycines. The size of this region, 188 amino acids including extreme glycines, predicts a triple helical collagenic structure

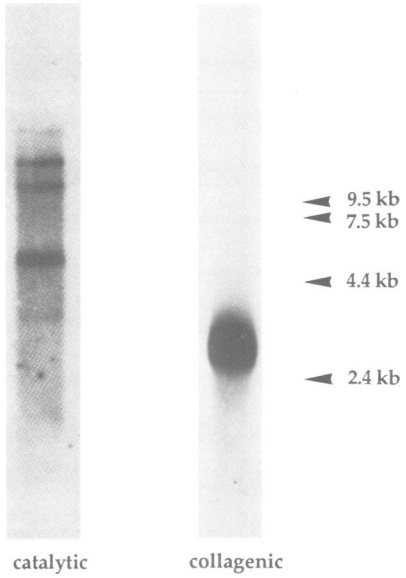

Fig. 4. Northern blots of collagenic subunit mRNAs in *Torpedo* electric organs. The blot was hybridized with probes corresponding to the catalytic subunits (left) and to the collagenic subunits (right). The collagenic probe hybridizes to an intense band of ~2.7 kb.

of ~550 Å. The collagenic domain is flanked by sequences containing two cysteine residues (CKITCIIG . . . and . . . GRCDC). These cysteines may be involved in disulfide bonds between each pair of the three subunits forming a triple helix, thus stabilizing the structure. On the other hand, the regular repetition of glycines every three residues is interrupted at several places, both by replacement with other residues (C, R, V), and by insertion of a short sequence (GSGHLMVG) near its C-terminal extremity. These irregularities, in both N- and C-terminal regions of the collagenic domain, may introduce weak points in the helical structure.

The C-terminal domain is completely maintained in the mature protein, since the terminal sequence corresponds to

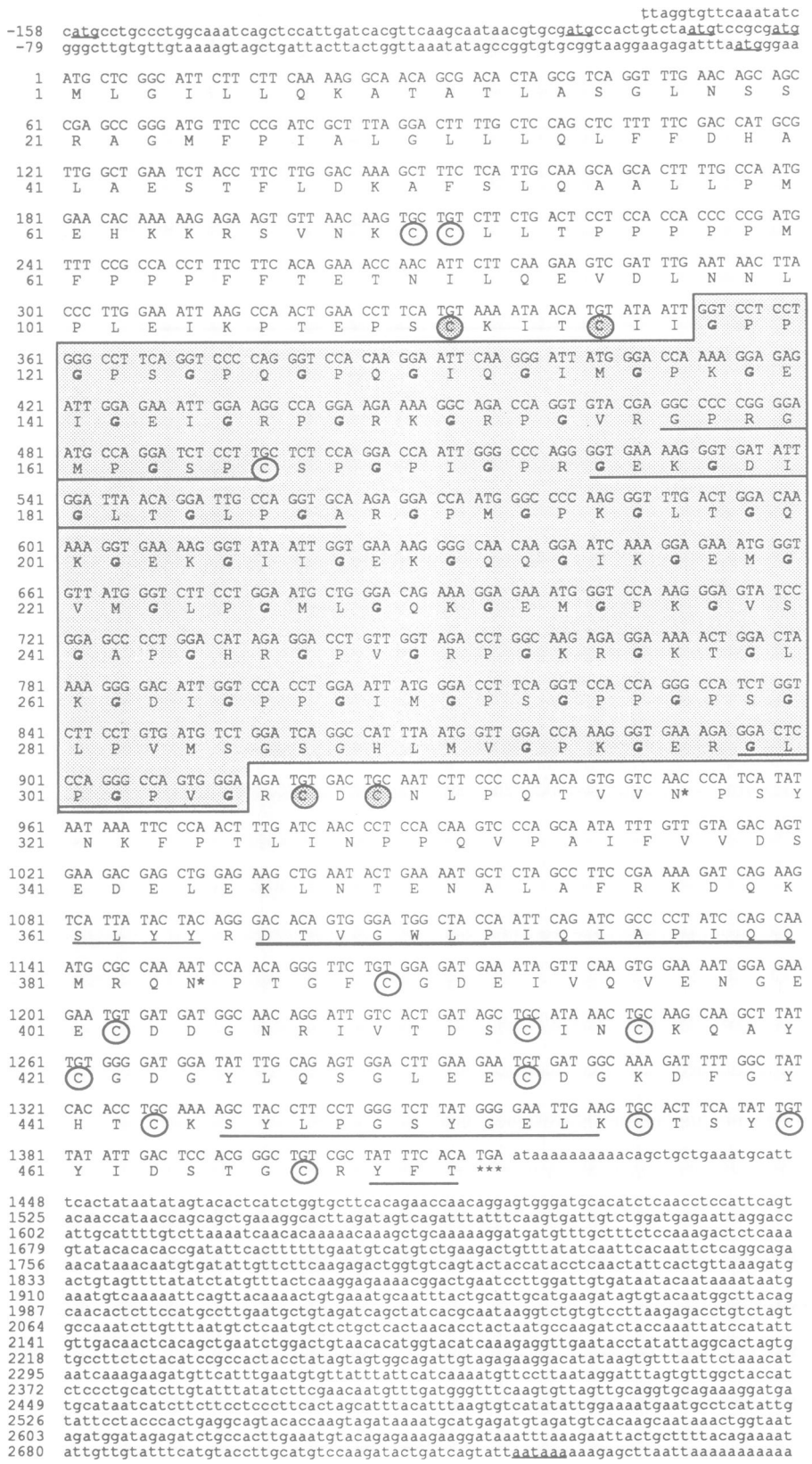

Fig. 5. Nucleotide sequence of clone λACT_1 and predicted primary structure of a collagenic tail peptide. The non-coding nucleotide sequence is shown in lower case letters and the coding sequence in capital letters. The 3′ untranslated sequence of λACT_1 was interrupted at nucleotide 1991; it was identical to that of a more extended clone, λACT_{29}, which was used to complement the sequence to the poly(A) stretch. The translated protein sequence is indicated below the coding sequence. Sequences corresponding to the tryptic peptides (Table I) are underlined. The peptide on which the PCR strategy used for obtaining genetic probes was based, is underlined in bold. Potential *N*-glycosylation sites are indicated by stars. Cysteines are encircled and those flanking the collagenic sequence are shaded. The regularly spaced glycine residues corresponding to the collagenic structure are in bold type. The ATG triplets preceding the proposed initiation codon are underlined.

one of the sequenced peptides (P_7, YFT). The first half of this domain contains nine prolines, and the second half contains ten cysteines. The cysteine-rich region contains a repeated motif (Figure 6). The C-terminal domain seems to present α and β secondary structures, according to the HCA plot (Figure 7) (Gaboriaud *et al.*, 1987; Lemesle-Varloot *et al.*, 1990). It is interesting to note that two potential glycosylation sites exist in the C-terminal region predicted by the λACT_1 cDNA clone. This is in good agreement with the fact that the collagenic tail is known to be glycosylated. Differences in glycosylation may explain why two HPLC peaks from both T_1 and T_2 bands yielded the same peptidic sequence, since in the predicted primary structure this sequence is followed very closely by a potential *N*-glycosylation site.

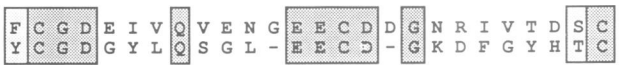

Fig. 6. Repeated protein motif in the C-terminal cysteine-rich region of the collagenic subunit.

Electron micrographs of Torpedo AChE A_{12} molecules

Figure 8 shows electron micrographs of representative A_{12} asymmetric AChE from *Torpedo* electric organs, obtained by negative staining as described previously in the case of *Electrophorus* (Cartaud *et al.*, 1975) and *Torpedo* (Rieger *et al.*, 1976). The length of the rod-shaped tail is ~500 Å. In some cases, the distal end of the tail seems to carry a globular structure (d, e, f in Figure 8).

Production of collagen-tailed forms in transfected cells

Using the CDM8 vector (Seed and Arrufo, 1987), we have transfected COS cells with a *Torpedo* AChE catalytic subunit, alone and in combination with the collagenic subunit. Control, non-transfected COS cells contain G_1 AChE, at a low level. When transfected with the A type of *Torpedo* AChE catalytic subunit, they produced active G_2 and G_4 forms (not shown). In the case of co-transfection with a vector containing the ACT_1 insert, we observed an additional form of AChE, sedimenting at 17S, exactly like the A_{12} form from *Torpedo* electric organs (Figure 9). After treatment with collagenase at 30°C, this form was

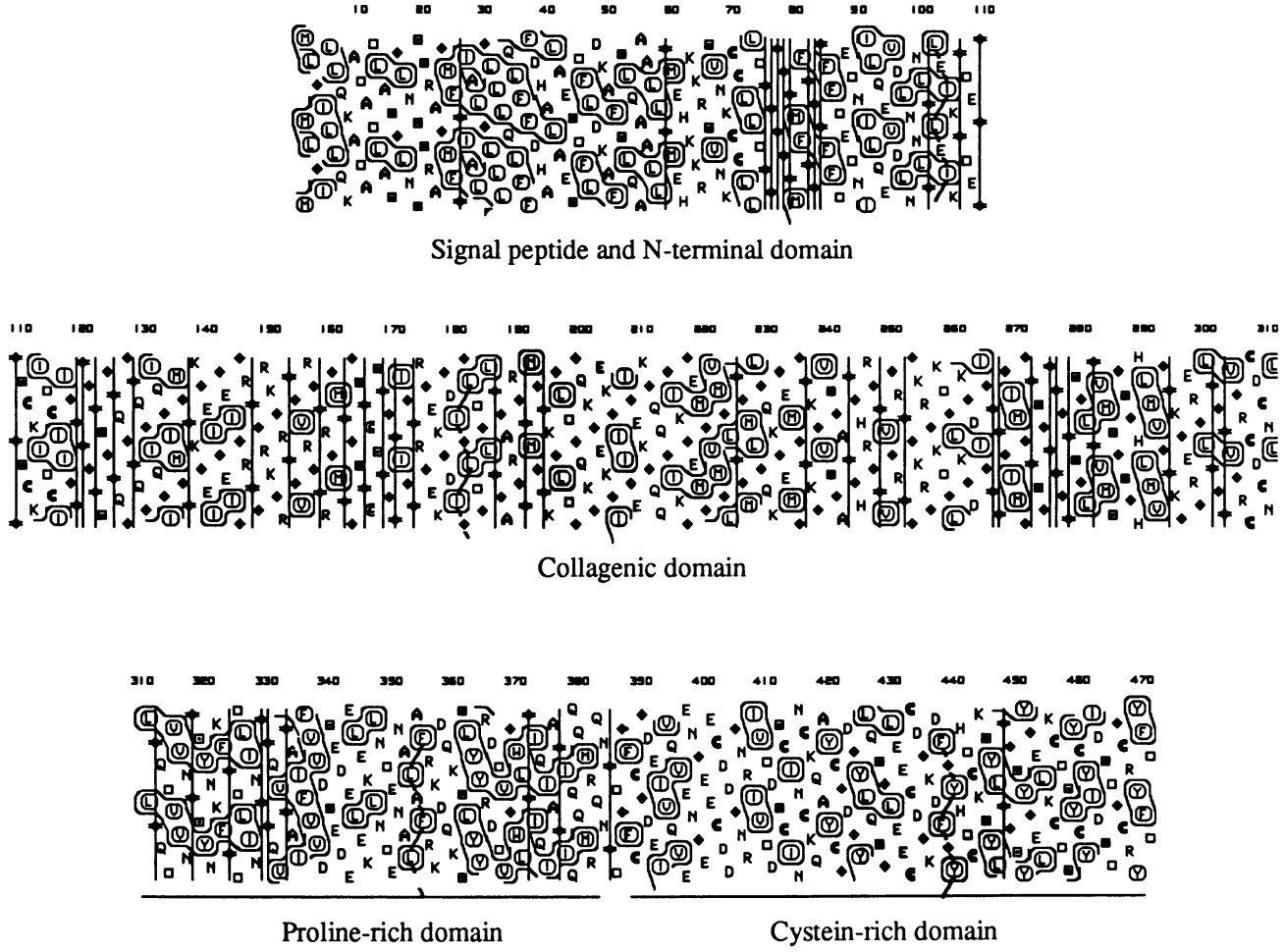

Fig. 7. Hydrophobic cluster analysis (HCA) of the collagenic subunit. In this graphic method, the amino acid residues are arranged as in an α helix, which is opened and flattened into a band and is repeated 2-fold side by side so as to show all potential neighbourhood relationships between residues (Gaboriaud *et al.*, 1987; Lemesle-Varloot *et al.*, 1990). Amino acid residues are represented in the one-letter code, except specific amino acids; glycines are represented by black diamonds, prolines by black stars and vertical lines, threonines and serines by dotted and empty squares, respectively. Clusters of hydrophobic residues, which tend to be grouped in the interior of the proteic structure, are encircled. The signal peptide contains a large hydrophobic cluster, as expected. Horizontal clusters, such as shown around residues 320 and 380, suggest the presence of α helices, whereas slanted hydrophobic groups, as around residues 290, 345 and 360, suggest β sheets. The top line contains the signal peptide and the putative N-terminal region. The middle row contains the collagenic domain, with the flanking pairs of cysteines; note the regular alignment of glycines (black diamonds). The third row contains the C-terminal region, with its proline-rich and cysteine-rich domains.

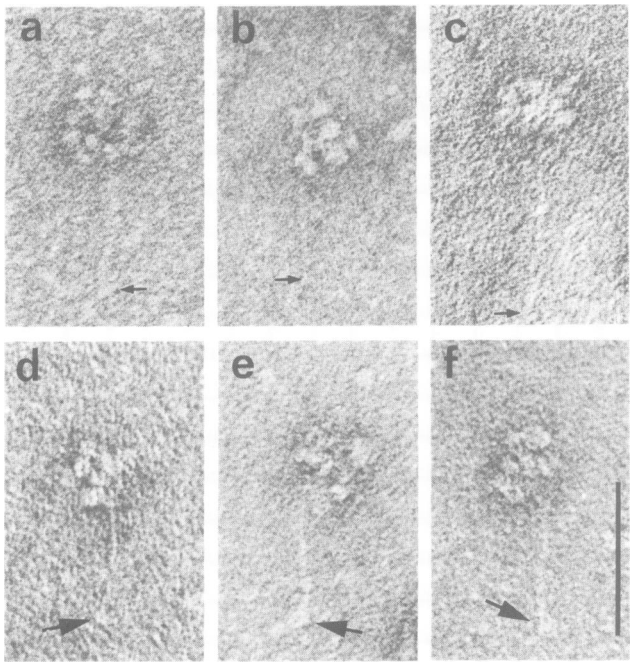

Fig. 8. Electron micrographs of *Torpedo* A_{12} AChE molecules. The vertical bar represents 50 nm. Arrows indicate the extremity of the rod-like tails; in d, e and f, larger arrows indicate the presence of globular structures which seem to terminate the tail.

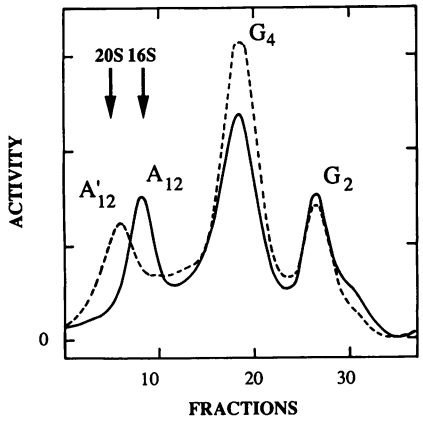

Fig. 9. Production of collagen-tailed forms in COS cells after co-transfection with *Torpedo* catalytic and collagenic subunits. Co-transfected COS cells contain a 17S form (solid line), which does not exist in cells expressing only the AChE catalytic subunit (not shown). After treatment with collagenase (dotted line), the sedimentation of this form was accelerated to 20S. The increase in the G_4 peak might result from the dissociation of some asymmetric molecules.

shifted to 20S, indicating without ambiguity that it possesses a collagenic tail (Bon and Massoulié, 1978).

Discussion

Structure of the collagenic tail and organization of the asymmetric AChE forms

The predicted primary structure of a collagenic subunit is entirely consistent with the organization of AChE asymmetric forms, as deduced from hydrodynamic analyses and visualized in electron micrographs, in the case of *Electrophorus* (Cartaud *et al.*, 1975) and *Torpedo* (Rieger *et al.*, 1976). In micrographs, asymmetric AChE from *Torpedo* appear somewhat different from *Electrophorus* molecules. Their rod-like tail is thinner and of variable length, and they are frequently found in head-to-tail pairs, with their tails closely associated along their entire length. The longer, presumably intact tails are ~500 Å in length, in excellent agreement with the expected dimension of the triple helix formed by the central collagenic domain. This collagenic structure may be stabilized at both extremities by disulfide bonds between each pair of subunits. Irregularities in the periodicity of glycines probably introduce local disorganizations, possibly explaining the presence of particular cleavage sites and the formation of intermediate products during digestion by collagenase (Bon and Massoulié, 1978).

Cysteine residues which might be responsible for the attachment of the catalytic subunit exist in the N-terminal and C-terminal domains, although they are more numerous in the latter. We have not been able to determine the N-terminal extremity of the mature protein, so that we do not know whether the N-terminal domain is conserved or cleaved during maturation. Because we observed the presence of the C-terminal peptide, we know that the C-terminal domain is entirely maintained in the mature asymmetric forms.

In electron micrographs, the distal extremity of the tail did not systematically carry any additional structure. This suggests that the C-terminal domain is located at the proximal extremity and mediates the association of the tail with the catalytic tetramer. The proline-rich region of the C-terminal domain possibly constitutes a flexible link between the collagenic domain and the tetramer. The cysteine-rich region of the C-terminal domain presents a repeated motif and seems likely to interact with the two catalytic subunits of the internal dimer.

Distribution of mRNAs encoding collagenic tail subunits

Northern blots showed that mRNAs encoding the collagenic tail subunits are abundant in the electric organs. In contrast, they were not detectable in *Torpedo* dorsal muscle. This is entirely consistent with the fact that in this species, skeletal muscles contain only G_2 AChE, with only traces of A forms. In any case, it is clear that the collagenic tail subunits are not ubiquitous. The co-transfection experiments, as discussed below, suggest that the expression of these subunits is indeed specific to differentiated muscle and nerve cells and controls the production of asymmetric forms.

The broadness of the hybridized bands possibly reflects the existence of a multiplicity of mRNA variants, probably produced by differential splicing in their coding and non-coding regions, as previously observed in the case of the AChE catalytic subunit mRNAs (Sikorav *et al.*, 1987, 1988). Preliminary analyses of several cDNA clones indeed demonstrated a multiplicity of mRNA structures, suggesting the existence of a number of alternative primary structures.

Mode of association of catalytic and collagenic subunits

Denaturation of asymmetric AChE forms without reduction separates peripheral dimers of catalytic subunits from the central core of the molecule, in which the collagenic subunits remain associated with the internal catalytic dimers

(Rosenberry and Richardson, 1977; Anglister and Silman, 1978), and in the case of *Torpedo* AChE, the 100 kd subunits (Lee and Taylor, 1982; Lee *et al.*, 1982). The tail subunits are thus linked together, and associated with catalytic and 100 kd subunits, by disulfide bonds. The proteic region of the tail subunit which interacts with the catalytic and 100 kd subunits contains at least two cysteine residues, since each tail subunit may be linked to two catalytic subunits. In addition to the pairs of cysteines flanking the collagenic region, which may be responsible for the disulfide linkage of the three tail subunits, as mentioned above, we find a contiguous pair of cysteines in the N-terminal region subunit, and 10 cysteine residues in the C-terminal region of the tail subunit. Thus the tail may be attached to the other subunits of the hetero-oligomeric asymmetric forms, either through its N-terminal proline-rich region or through the larger, probably globular C-terminal region.

It is noteworthy that reduction of intersubunit disulfide bonds may be achieved, in the native molecules, without dissociating the hetero-oligomeric structure (Bon and Massoulié, 1976). Thus, quaternary interactions are sufficient to maintain the association of the tail subunits with the catalytic AChE subunits, and also probably with the 100 kd subunits in the case of *Torpedo* A forms. This suggests that the different subunits of these hetero-oligomers might be able to self-assemble.

Significance of a multiplicity of collagenic subunits

The existence of different types of collagenic subunits raises interesting questions. These subunits may form hetero- as well as homo-trimeric tail structures. Distinct subunits might not be equivalent in their association with catalytic subunits and/or 100 kd subunits. This might explain the existence of a variety of combinations of 100 kd and catalytic subunits (Lee *et al.*, 1982), in the case of *Torpedo* AChE. In other species, AChE asymmetric forms have not been found to contain non-catalytic globular subunits similar to the *Torpedo* 100 kd. The existence of tail subunits presenting reduced affinity for catalytic subunits might, however, be responsible for the formation of molecules containing only one tetramer (A_4) or two tetramers (A_8), together with the A_{12} form in which each tail peptide is associated with a catalytic tetramer.

It is quite possible that not all of this collagen is linked with AChE catalytic subunits, but that some exists either isolated, or associated with other proteins such as the 100 kd subunits. This would be consistent with the abundance of the corresponding mRNAs in electric organs. Thus, collagenic tails would be specifically expressed in differentiated cell types, producing AChE A forms, but might be engaged in a variety of associations, in addition to these complex molecules.

A related question concerns the specificity of the tail subunits associated with AChE and BuChE subunits. The existence of hybrid asymmetric forms containing both types of catalytic subunits (Tsim *et al.*, 1988) would be compatible with the possibility of interaction of the same tail subunits with either AChE or BuChE. Tsim *et al.* (1988), however, found that hybrid AChE–BuChE A forms occurring in chicken embryos and adult AChE A forms possess distinct tail structures, differing in their interaction with a monoclonal antibody. They proposed a model in which embryonic tails, which could associate with BuChE subunits, would be replaced during development by a specific AChE tail. It is entirely possible that these various tail subunits could be generated from the same gene by alternative splicing.

Possible homology between cholinesterase associated domains

In addition to asymmetric forms, vertebrate cholinesterases are known to be engaged in other hetero-oligomeric structures. The membrane-bound form of AChE from mammalian brain consists of two disulfide-linked dimers, one of which is linked to the 20 kd subunit which carries the hydrophobic domain and mediates the attachment to the membrane (Inestrosa *et al.*, 1987). This organization is remarkably similar to that of the asymmetric molecules. It appears quite likely that the domains of the collagenic tail subunits and of the hydrophobic 20 kd subunits which associate through quaternary interactions and disulfide bonds with cholinesterase catalytic subunits are homologous in their structure. It is even possible that in a given species, both hydrophobic and collagenic cholinesterase-associated subunits could be produced from a single gene, through alternative splicing.

Co-expression of collagenic and catalytic AChE subunits is sufficient to generate asymmetric forms

COS cells are derived from kidney cells and do not produce any asymmetric AChE forms. When co-transfected with both *Torpedo* AChE catalytic (type A) and collagenic subunits (ACT_1), they produced an A_{12} form, clearly identified by its sedimentation coefficient and specific sensitivity to collagenase.

These observations demonstrate that the tail of asymmetric AChE may be assembled as a homo-trimer of the described collagenic subunit. In addition, they demonstrate that the co-expression of catalytic and tail subunits suffices to generate asymmetric forms. Thus the restricted presence of collagen-tailed forms in muscle and nerve cells cannot be explained by a specific biosynthetic capacity of these cells, but probably only by the expression of the collagenic subunits. The present results suggest that these hetero-oligomers probably assemble spontaneously. This process has been shown to take place in the Golgi apparatus (Rotundo, 1984).

Thus, the presence of AChE asymmetric forms in muscle and nerve cells only depends on the expression of the collagenic subunits. It will be interesting to analyze the factors which control the expression of these subunits in differentiated cells.

Materials and methods

Protein purification and sequencing

Electric organs of *T.marmorata* were subjected to a sequential extraction procedure, as described previously (Bon and Massoulié, 1980; Bon *et al.*, 1986). Following extractions in low salt without detergent and with 1% Triton X-100, the tissue was extracted in high salt (1 M NaCl, 50 mM $MgCl_2$, 10 mM Tris–HCl, pH 7). The A forms, recovered in this high salt soluble fraction, were purified by affinity chromatography on *N*-methylacridinium Sepharose (Vallette *et al.*, 1983). Because the amount of protein required to obtain peptidic sequences from the collagenic subunits was relatively important, we had to accumulate the products obtained from a number of purified preparations, which were stored frozen. The purified enzyme was dialyzed against a buffer containing 10 mM Tris–HCl, 0.4 M NaCl, 40 mM $MgCl_2$ and concentrated to ~10–15 mg/ml by centrifugation in Centricon filters. The subunits were separated by polyacrylamide gel electrophoresis, following the method of Laemmli (1970).

Analytical aliquots were digested with 500 U/ml of collagenase ABC form III (Advanced Biofactures Corporation, Lynbrook, NY) in 100 mM Tris−HCl, pH 8, 5 mM $CaCl_2$ for 4 h at 20°C or 1 h at 37°C.

The protein was blotted by electrophoretic transfer onto Immobilon membranes. The bands corresponding to collagenic subunits were cut out, digested by trypsin, and the resulting peptides were separated by HPLC and sequenced in an Applied Biosystems sequencer (Biosystems Inc., 470 A), as described previously (Bauw *et al.*, 1987).

Electron micrographs

Electron micrographs were performed by negative staining, as previously indicated (Cartaud *et al.*, 1975; Rieger *et al.*, 1976).

RNA purification and cDNA library construction

Poly(A)$^+$ RNA from *T.marmorata* electric organs was purified using a standard procedure (Cathala *et al.*, 1983). Double-stranded cDNA was synthesized according to the method of Gubler and Hoffman (1983) using a cDNA synthesis kit (Amersham). Adaptors [$5'_{(OH)}$AATTCGGAGGATT-CGAAGGATCCGGGTACCATGG and $5'_{(P)}$CCTAGGTACCCGGTA-CCTTCGAATCCTCC) were used to introduce cDNA into dephosphorylated, *Eco*RI cut, λZAP vector (Stratagene). A library of 2×10^6 recombinants was obtained.

Production of a DNA probe by PCR

PCR was performed with double-stranded cDNA as template and a set of degenerate oligonucleotide primers corresponding to five amino acids at the N-terminal and C-terminal extremities of the non-collagenic peptide P_5 (Table I), respectively in the coding and non-coding orientations. At degenerate coding positions, allowing all four bases, we introduced inosines in the primers, except near the 3′ extremity, where the different bases were used. The oligonucleotides carried *Eco*RI sites at their 5′ end. The amplification reaction was performed according to a standard procedure (Saiki *et al.*, 1988). The reaction mixture (100 μl) was subjected to 35 cycles of denaturation (92°C, 1 min), hybridization (55°C, 1 min) and extension (70°C, 2 min). The PCR products were analyzed and purified by electrophoresis in a 2% agarose gel. Fragments of ~60 nucleotides were subjected to further amplification and purification, digested with *Eco*RI and cloned in M13mp19. Sequences of the PCR products were determined by the dideoxy chain termination method (Sanger *et al.*, 1977), using Sequenase (USB). The sequences of several clones were determined. It is noteworthy that the amplified DNA segments generally contained G at positions corresponding to inosines. Other degenerate positions contained various bases. The amplified sequence, located between the two primers, encoded the expected central part of the peptide.

Library screening

A PCR probe (Schowalter and Sommer, 1989) was generated from one SS M13 recombinant with two degenerate primers and used to screen 100 000 recombinants of the cDNA library. Hybridization was performed in 0.5 M Na phosphate, pH 7.2, 7% SDS, 100 μg/ml denatured herring sperm DNA, at 55°C overnight. The filters (Genescreen, NEN) were washed with 0.2 M Na phosphate, 1% SDS at 55°C, twice for 15 min and autoradiographed at −70°C with an intensifying screen. Two positive plaques were purified, pBluescript plasmids were rescued by superinfection with R408 helper phage (Short *et al.*, 1988) and subjected to DNA analysis.

Nucleotide sequence analysis

cDNA clones were sequenced by the method of exonuclease III nested deletions (Henikoff, 1987), restriction fragment cloning into M13 and specific oligonucleotide primers. The programs written by Bellon (1988) were used to compile sequence data and restriction maps. Screening protein sequence data banks (Genepro and NBRF) was done by connecting to CITI 2 (Paris).

Northern blots

Northern blots were performed as previously described (Sikorav *et al.*, 1987). Approximately equivalent amounts of poly(A)$^+$ mRNA from different tissues were used.

Transfection experiments

A cDNA clone containing the complete coding sequence of a catalytic subunit of type A (asymmetric forms), $\lambda AChE_{15\text{-}2}$, was obtained from the same library as λACT_1 by hybridization with a specific probe (Sikorav *et al.*, 1987). The inserts of λACT_1 and $\lambda AChE_{15\text{-}2}$ were cut, filled in, ligated with *Bst*XI adaptors and introduced into the CDM8 vector. The orientation of the inserts was ascertained by restriction analysis. Plasmid DNA was prepared from MC1061/P3 cells and purified twice in CsCl gradients. Petri dishes of 10 cm were plated with 10^6 COS cells and left overnight. After washing with PBS, 3 ml of DMEM containing 10% Nuserum (Collaborative Res. Inst.) was added. The AChE activity of Nuserum had been inactivated with 10^{-7} M soman. The cells were transfected with 10 μg of each vector DNA (catalytic and collagenic subunits). The cells were incubated for 48 h at 37°C, 48 h at 28°C, then washed with PBS, and collected by scraping. The pellet obtained from three dishes was extracted with 300 μl of detergent−saline buffer (1% Triton X-100, 1 M NaCl, 10 mM Tris−HCl, pH 7) containing antiproteolytic agents, as indicated previously (Vallette *et al.*, 1990). The supernatant (2 mg of protein/ml) hydrolyzed 15 nmol of acetylthiocholine/min/mg of protein in the case of control COS cells, 125 nmol/min/mg in the case of cells transfected with the catalytic AChE subunit, and 170 nmol/min/mg in the case of cells co-transfected with catalytic and collagenic subunits. The molecular forms of AChE were analyzed by centrifugation in sucrose gradients, containing 1% Triton X-100, 0.4 M NaCl, 10 mM Tris−HCl pH 7, in SW41 Beckman rotors, 34 000 r.p.m. for 17 h at 6°C. Control COS cells produced only G_1 AChE, while cells transfected with the *Torpedo* catalytic subunit produced G_2 and G_4 AChE (not shown).

Supernatants obtained from co-transfected cells were incubated with collagenase type III (Advance Biofactors Corp., Lynbrook, NY), 10 U/100 μl, for 9 min at 30°C.

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References

Anglister,L. and Silman,I. (1978) *J. Mol. Biol.*, **125**, 293−311.
Bauw,G., De Loose,M., Inzé,D., Van Montagu,M. and Vandekerckhove,J. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 4806−4810.
Bellon,B. (1988) *Nucleic Acids Res.*, **16**, 1837−1876.
Bon,S. and Massoulié,J. (1976) *FEBS Lett.*, **71**, 273−278.
Bon,S. and Massoulié,J. (1978) *Eur. J. Biochem.*, **89**, 89−94.
Bon,S. and Massoulié,J. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 4464−4468.
Bon,S., Cartaud,J. and Massoulié.J. (1978) *Eur. J. Biochem.*, **85**, 1−14.
Bon,S., Chang,J.Y. and Strosberg,A.D. (1986) *FEBS Lett.*, **209**, 206−212.
Bon,S., Méflah,K., Musset,F., Grassi,J. and Massoulié,J. (1987) *J. Neurochem.*, **49**, 1720−1731.
Cartaud,J., Rieger,F., Bon,S. and Massoulié.J. (1975) *Brain Res.*, **88**, 127−130.
Cathala,G., Savouret,J.F., Mendez,B., West,B.L., Karin,M., Martial,J.A. and Baxter,J.D. (1983) *DNA*, **2**, 329−335.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6−13.
Gaboriaud,C., Bissery,V., Benchetrit,T. and Mornon,J.-P. (1987) *FEBS Lett.*, **224**, 149−155.
Gennari,K., Brunner,J. and Brodbeck,U. (1987) *J. Neurochem.*, **49**, 12−18.
Gibney,G., MacPhee-Quigley,K., Thompson,B., Vedvick,T., Low,M.G., Taylor,S.S. and Taylor,P. (1988) *J. Biol. Chem.*, **263**, 1140−1145.
Gubler,U. and Hoffman,B.J. (1983) *Gene*, **25**, 263−269.
Henikoff,S. (1987) *Methods Enzymol.*, **155**, 156−165.
Inestrosa,N.C., Roberts,W.L., Marshall,T. and Rosenberry,T.L. (1987) *J. Biol. Chem.*, **262**, 4441−4444.
Laemmli,U.K. (1970) *Nature*, **227**, 680−685.
Lee,S.L. and Taylor,P. (1982) *J. Biol. Chem.*, **257**, 12292−12301.
Lee,S.L., Heinemann,S. and Taylor,P. (1982) *J. Biol. Chem.*, **257**, 12283−12291.
Lemesle-Varloot,L., Henrissat,B., Gaboriaud,C., Bissery,V., Morgat,A. and Mornon-J.P. (1990) *Biochimie*, **72**, 555−574.
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.
MacPhee-Quigley,K., Taylor,S.S. and Taylor,P. (1985) *J. Biol. Chem.*, **260**, 12185−12189.
Massoulié,J. and Toutant,J.P. (1988) *Handbook Exp. Pharmacol.*, **86**, 167−224.
Maulet,Y., Camp,S., Gibney,G., Rachinsky,T., Ekstrom,T.J. and Taylor,P. (1990) *Neuron*, **4**, 289−301.

McTiernan,C., Adkins,S., Chatonnet,A., Vaughan,T.A., Bartels,C.F., Kott,M., Rosenberry,T.L., La Du,B.N. and Lockridge,O. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 6682–6686.
Musset,F., Frobert,Y., Grassi,J., Vigny,M., Boulla,G., Bon,S. and Massoulié.J. (1987) *Biochimie*, **69**, 147–156.
Prody,C.A., Zevin-Sonkin,D., Gnatt,A., Goldberg,O. and Soreq,H. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3555–3559.
Rieger,F., Bon,S., Massoulié,J., Cartaud,J., Picard,B. and Benda,P. (1976) *Eur. J. Biochem.*, **68**, 513–521.
Rosenberry,T.L. and Richardson,J.M. (1977) *Biochemistry*, **16**, 3550–3558.
Rotundo,R.L. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 479–483.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Higuchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–491.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Schowalter,D.B. and Sommer,S.S. (1989) *Anal. Biochem.*, **177**, 90–94.
Schumacher,M., Camp,S., Maulet,Y., Newton,M., MacPhee-Quigley,K., Taylor,S.S., Friedman,T. and Taylor,P. (1986) *Nature*, **319**, 407–409.
Seed,B. and Arrufo,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 3365–3369.
Short,J.M., Fernandez,J.M., Sorge,J.A. and Huse,W.D. (1988) *Nucleic Acids Res.*, **16**, 7583–7600.
Sikorav,J.L., Krejci,E. and Massoulié.J. (1987) *EMBO J.*, **6**, 1865–1873.
Sikorav,J.L., Duval,N., Anselmet,A., Bon,S., Krejci,E., Legay,C., Osterlund,M., Reimund,B. and Massoulié.J. (1988) *EMBO J.*, **7**, 2983–2993.
Toutant,J.P. and Massoulié.J. (1988) *Handbook Exp. Pharmacol.*, **86**, 225–265.
Tsim,K.W., Randall,W.R. and Barnard,E.A. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 1262–1266.
Vallette,F.M., Marsh,D.J., Muller,F., Massoulié,J., Marçot,B. and Viel,C. (1983) *J. Chromatogr.*, **27**, 285–296.

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