Identical N-terminal peptide sequences of asymmetric forms and of low-salt-soluble and detergent-soluble amphiphilic dimers of *Torpedo* acetylcholinesterase

Comparison with bovine acetylcholinesterase

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We have determined partial N-terminal sequences of acetylcholinesterase (AChE) catalytic subnunits from *Torpedo marmorata* electric organs and from bovine caudate nucleus. We obtain identical sequences (23 amino acids) for the soluble ('low-salt-soluble' or LSS fraction) and particulate ('detergent-soluble', or DS fraction) amphiphilic dimers (G_2 form) and for the asymmetric, collagen-tailed forms ('high-salt-soluble', or HSS fraction, A_{12} + A_8 forms). There are two amino acid differences, at position 3 (Asp/His) and 20 (Ile/Val), with the sequences obtained for *T. californica* by MacPhee-Quigley et al. [(1985) J. Biol. Chem. 260, 12185–12189] for the soluble G_2 form and the lytic G_4 form which is derived from asymmetric AChE. The bovine sequence (12 amino acids) presents an identity of 4 amino acids (Glu-Leu-Leu-Val) with that of *Torpedo*, at positions 5–8 (*Torpedo*) and 7–10 (bovine). There is also a clear homology with the sequence of human butyrylcholinesterase [(1986) Lockridge et al. J. Biol. Chem., in press] indicating that these enzymes probably derive from a common ancestor.

Acetylcholinesterase *N-terminal sequence* *Molecular form* (Torpedo, *Bovine brain*)

1. INTRODUCTION

Two distinct cholinesterases, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase, or pseudocholinesterase (BuChE, EC 3.1.1.8), appear to coexist in every vertebrate species and may be distinguished on the basis of their specificity towards substrates and inhibitors. For example, acetylcholinesterases are defined by the fact that they hydrolyze acetylcholine faster than its analogs, propionylcholine and butyrylcholine, but its catalytic turnover varies considerably: 4.4, 1.64, 1.32 and 1.05 × 10^7 mol acetylthiocholine hydrolyzed/h per site at 28°C, and pH 7, respectively, in *Electrophorus, Torpedo,* rat and chicken [1].

The properties of butyrylcholinesterase appear even more variable among vertebrate species, since the preferred substrate of this enzyme may be butyrylcholine, as in man, propionylcholine, as in rat [2], or acetylcholine, as in *Torpedo* [3]. The only constant distinctive feature of this enzyme seems to be that BuChE hydrolyzes butyrylcholine at a rate at least similar to that of acetylcholine, while AChE does not hydrolyze this substrate, or only very poorly so.

Both AChE and BuChE present a multiplicity of molecular forms [3–6]. Each enzyme possesses both globular and collagen-tailed forms which appear to be catalytically equivalent [1,7]. A comparison of their macromolecular characteristics showed that the sets of molecular forms of all

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vertebrates cholinesterases are based on the same types of quaternary structures. For this reason, we have found it useful to propose a nomenclature that expresses this overall quaternary structure homology between the globular forms (G_1, G_2, G_4) and asymmetric, or collagen-tailed forms (A_4, A_8, A_{12}), although individual molecules may be very different from one enzyme to another. Within a given species, the sedimentation coefficients of homologous AChE and BuChE forms are generally slightly different. Large differences are observed between the sedimentation coefficient of a given form in different species: for example A_{12} AChE sediments at 16–17 S in mammals, 18.4 S in *Electrophorus* and 20 S in chicken.

There is extensive immunological cross-reactivity between AChE from different mammalian species but this does not extend to other vertebrates. No cross-reactivity has ever been demonstrated between AChE and BuChE, even in the case of *Torpedo*, although the catalytical characteristics of the two enzymes appear to be less different in this rather primitive cartilaginous fish than in other species [3].

The similarity of quaternary structures strongly suggests that all vertebrate AChE and BuChE derive from a common ancestor, and it is important to evaluate the degree of homology between the peptide sequences of these enzymes. This is necessary for example to assess the possibility that DNA probes obtained for one enzyme may hybridize with coding sequences for the other cholinesterase from the same organism, or cholinesterases of other species. It will also be essential, in an analysis of coding sequences, to determine the terminal peptide sequences of the enzymes catalytic subunits. We have therefore determined partial N-terminal sequences of AChE from *Torpedo* electric organ and from calf brain.

2. MATERIALS AND METHODS

2.1. *Purification of calf brain AChE*

AChE was purified by affinity chromatography as in [8] from a detergent extract of bovine caudate nucleus [9]. The preparation used for sequence analyses has been described in detail by Marsh and Massoulié [10].

2.2. *Solubilization of AChE from* Torpedo *electric ogans*

AChE was solubilized sequentially in a low-salt-soluble (LSS) fraction, detergent-soluble (DS) fraction and a high-salt-soluble (HSS) fraction [11]. The total AChE activity recovered varied between 4000 and 6000 Ellman units/g electric organ. Higher values were observed in males than in females. The LSS fraction contained 5–15% of the total activity, the DS fraction about 50% and the HSS fraction (asymmetric forms) 20–40%.

In practice, frozen tissue was stored at −80°C. It was cut into small dice (about 1 cm^3), and homogenized in the cold with 5 vols buffer A (40 mM $MgCl_2$, 10 mM Tris-HCl, pH 7) for 2 min at maximal speed in a Polytron homogenizer. The presence of 40 mM $MgCl_2$ prevented partial solubilization of asymmetric forms at this stage [11]. The homogenate was then centrifuged for 3 h at 17000 rpm in a Beckman R19 rotor. A total activity of about 450 U/g tissue was recovered in the supernatant (LSS fraction).

The pellet was extracted a second time in the same volume of buffer A, and centrifuged in the same manner. The second supernatant was discarded; its activity was about 1/10 that of the first LSS fraction. The pellet was resuspended again in the same volume of buffer A; Triton X-100 was then added to a concentration of 2% and mixed with the Polytron, at low speed to avoid excessive foaming. The mixture was maintained for 30 min at 4°C, and centrifuged for 1 h at 45000 rpm in a 50Ti rotor. The supernatant (fraction DS) contained an activity of about 300 U/g.

The pellet was homogenized yet another time in buffer A, in order to eliminate the remaining detergent-soluble G_2 AChE, but without adding Triton X-100. After centrifugation, the supernatant, which contained about 500 U/g, was discarded. The pellet could be stored frozen. It was reextracted in a high salt buffer (buffer B: 2 M $MgCl_2$, 10 mM Tris-HCl, pH 7). The supernatant recovered after centrifugation for 1 h at 45000 rpm, in a 50Ti rotor, contained about 2000 U/g. The solubilized A forms were found to inactivate in buffer B. This fraction was therefore dialyzed in several steps against 0.8 M NaCl, 10 mM Tris-HCl, pH 7. A flocculate formed during dialysis and contained some of the enzyme; it was removed by centrifugation at 17000 rpm in an R19 rotor and discarded.

2.3. *Affinity chromatography of AChE on N-methylacridinium-Sepharose*

N-Methylacridinium-Sepharose (N-MAC-Sepharose) was prepared as described in [8] at a ligand concentration of 0.25–0.5 μmol/ml gel. For routine purification of *Torpedo* AChE, we used 200-ml columns washed in 0.5 M guanidine-HCl, 10 mM Tris, pH 7, before equilibration. Both LSS and DS fractions (containing G_2 AChE) were adjusted to 10 mM Tris-HCl, pH 7, and 0.5% Triton X-100. The HSS fraction was dialyzed against 0.8 M NaCl and diluted to bring the salt concentration down to 0.2 M NaCl.

A volume of extract containing about 7000 U/ml gel was loaded on the column, at a rate of 1 column vol./h. Under these conditions, approx. 70% of the activity was retained (a higher fraction could be adsorbed at a slower flow rate). In the case of the LSS or DS fraction the column was then washed with 3 vols of buffer containing 1% Triton X-100, 1 vol. of buffer without detergent, 3 vols of buffer with 0.4 M NaCl. Essentially no activity was lost during washing. The enzyme was eluted in the presence of 1% Triton X-100, 10 mM decamethonium bromide and 0.4 M tetraethylammonium bromide, at a rate of less than 0.1 column vol./h. In these conditions, about 50% of the bound activity (i.e. about 2500 U/ml gel) was eluted in about 1 column vol. The asymmetric forms (HSS fraction) were chromatographed in the same manner, except that the fixation buffer contained 0.2 M NaCl, 50 mM $MgCl_2$ and that the elution buffer contained 0.6 M NaCl, 1% Triton X-100, 0.4 M triethylammonium bromide and 10 mM decamethonium bromide.

The purified fractions were usually subjected to a second chromatography according to the same procedure, after extensive dialysis to remove decamethonium and tetraethylammonium. In that case, the totality of the enzyme was bound to the column, and about 50–60% was eluted by the specific ligands.

After chromatography, we performed an isopycnic equilibration in a CsCl gradient, to remove the bulk of Triton X-100, and to concentrate the enzyme. We dissolved 35 g CsCl in 100 ml enzyme solution, and centrifuged for 5 days at 47000 rpm in a 50Ti rotor, at 10°C. The gradients were collected, and after assaying AChE activity, the fractions corresponding to the peak of activity were pooled, and centrifuged again for 80 h at 47000 rpm in an SW 60 rotor, at 10°C.

The concentrated enzyme fractions were stored at −80°C. Before use, they were dialyzed to remove CsCl and remaining traces of decamethonium and tetraethylammonium. We found that hydrophobic aggregates formed during the isopycnic equilibration in CsCl. To analyze the purified molecular forms or to isolate pure G_2 AChE in sucrose gradients, we therefore added 3% Triton X-100 to the samples before centrifugation.

From LSS and DS extracts, we obtained exclusively G_2 AChE, and about 70% A_{12} + 30% A_8 from HSS extracts.

2.4. *Treatment with endoglycosidase F*

The purified enzymes were incubated for 7 h at 37°C with 25 U/ml endoglycosidase F (NEN) in 100 mM Na acetate, pH 6.2, 50 mM EDTA, 1% Nonidet NP40, 0.1% SDS, 1% 2-mercaptoethanol. Control samples were incubated under identical conditions in the same medium. Control and treated samples were analyzed after complete denaturation and reduction at 90°C for 10 min (1% SDS, 80 mM DTT) by polyacrylamide gel electrophoresis according to Weber and Osborn [12] or Laemmli [13], in an LKB vertical slab gel apparatus. M_r standards were obtained from LKB.

2.5. *Amino acid sequence analyses*

Automated Edman degradation was performed in a gas-phase sequenator (Applied Biosystems model 470A, Foster City, CA) [14,15]. We used a standard degradation program and phenylthiohydantoin derivatives of amino acids were identified by HPLC on-line by absorption at 270 nm (Applied Biosystems model 120A).

3. RESULTS AND DISCUSSION

3.1. *Characterization of the catalytic subunits of* Torpedo *asymmetric and globular forms; effect of deglycosylation*

When characterized by sucrose gradient sedimentation, AChE purified from the LSS and DS fractions from *Torpedo* electric organs was found to correspond exclusively to the amphiphilic G_2 form, sedimenting at 6 S in the presence of Triton X-100. The enzyme purified from the HSS fraction contained about 70% A_{12} form and 30% A_8 form.

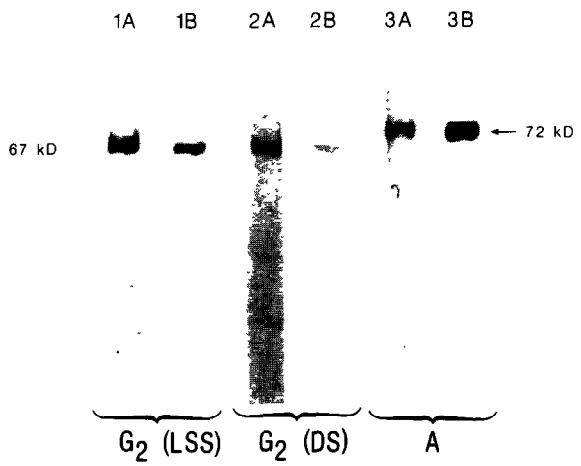

Fig.1. Comparison of the catalytic subunits of globular and asymmetric forms of *Torpedo* AChE. The catalytic subunits were labelled with [^{3}H]DFP before electrophoresis. The gels were stained with Coomassie brilliant blue (A) and exposed for autoradiography (B). Lanes: 1A,1B, soluble G_2 form (LSS fraction); 2A,2B, detergent-soluble G_2 form (DS fraction); 3A,3B, asymmetric forms (HSS fraction).

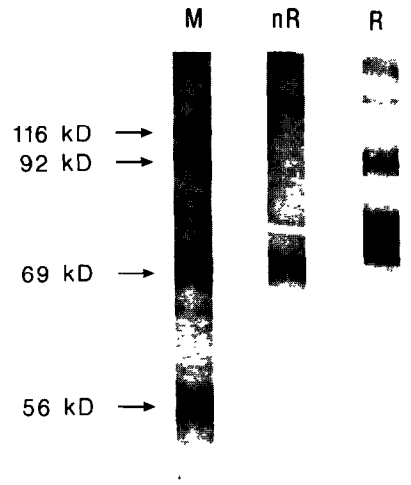

Fig.2. Effect of reduction on the electrophoretic mobility of the catalytic subunit of asymmetric AChE from *Torpedo*. Lane M: molecular mass standards *β*-galactosidase (116 kDa), phosphorylase *b* (92 kDa), bovine serum albumin (69 kDa), catalase (56 kDa). The preparation of asymmetric AChE was analyzed without reduction (nR) or after complete reduction (R), as indicated in the text; the protein bands were stained with Coomassie brilliant blue. In the reduced sample, most of the protein corresponds to the monomeric catalytic subunit, with some '100 kDa' subunit, dimer and heavier components; the tail peptides are barely detectable. In the absence of reduction, a small proportion of catalytic monomer and dimer is visible, while most of the protein did not enter the gel.

As shown in fig.1, the reduced catalytic subunits of AChE purified from the LSS, DS and HSS fractions were analyzed by SDS-polyacrylamide gel electrophoresis. They were stained by Coomassie brilliant blue and also identified by autoradiography after labeling with [^{3}H]DFP, or by immunolabelling with a monoclonal antibody (mAb ME8) directed against *Torpedo* AChE (Musset et al., in preparation). The apparent masses of the G_2 forms from the LSS and DS fractions were indistinguishable (67 kDa), but clearly different from that of the asymmetric forms (72 kDa). This difference between the catalytic subunits of globular and asymmetric forms is in good agreement with previous observations on *T. californica* [16,17], *T. marmorata* [18–20] and *Narke japonica* [21,22].

In the absence of reduction, we observed mostly dimers (about 140 kDa) for the G_2 form. In the case of the A forms, we observed dimers and heavier components which did not penetrate the separation gel. We also found a small proportion of monomers. The apparent molecular mass of these non-reduced monomers was similar to that of bovine serum albumin. After complete reduction the apparent molecular mass of G_2 monomers remained at 67 kDa but that of A monomers was increased to 72 kDa. This difference between the reduced and non-reduced asymmetric subunits (fig.2) is probably due to intra-subunit disulfide bonds, which maintain a compact structure in the absence of reduction, as observed for a number of proteins such as serum albumin.

After reduction, it is possible to detect minor polypeptide components in the asymmetric forms, around 100 and 40 kDa. They clearly correspond to the structural subunit that has been described in the case of *T. californica* [22,23] and to the collagenic tail peptides. They were not labeled by [^{3}H]DFP. The proportions of these structural subunits relative to the catalytic subunits were variable in different preparations, possibly because of their high sensitivity to proteolytic degradation

[23]. The relative abundance of the 100 kDa subunit also appears to vary between different Torpedinid species, as observed by Sakai and his colleagues [24] in the case of *T. californica* and *N. japonica*.

Under denaturing conditions, endoglycosidase F cleaves the *N*-linked polysaccharides of both complex and high mannose types, leaving only the internal *N*-acetylglucosamine attached to asparagine [25]. After such treatment, the apparent molecular masses were decreased by approx. 8 kDa for both the globular and asymmetric subunits, so that their difference was maintained (fig.3).

3.2. *N-terminal sequences*

Bovine AChE, purified from caudate nuclei, was found to be homogeneous, by polyacrylamide gel electrophoresis, with a subunit of 70 kDa [10]. We also found a major type of subunit in the case of G_2 AChE from *Torpedo*. In the case of the A forms, the structural 100 kDa and tail subunits were minor components. For sequence analyses, we used either the purified enzyme as such, or we performed a separation of polypeptides by HPLC (in 75 × 600 mm TSK G4000SW + TSK G3000SW columns), in denaturing conditions. The results were identical in several runs.

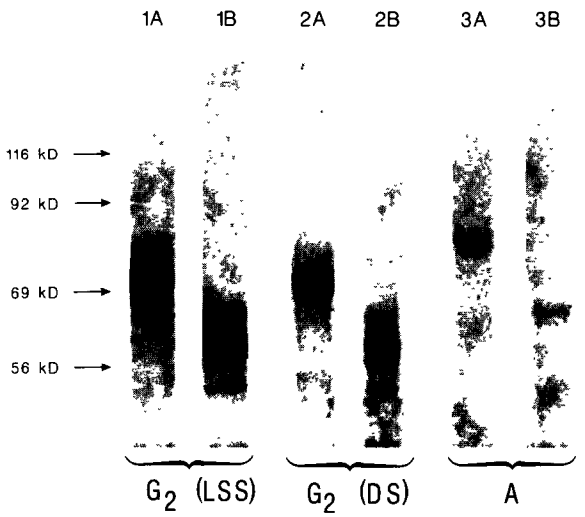

Fig.3. Effect of deglycosylation by endoglycosidase F on the apparent molecular mass of AChE catalytic subunits from *Torpedo*. The samples were incubated with or without endoglycosidase F (see section 2) before electrophoresis. (A) Controls, (B) deglycosylated. Lanes: 1A,1B, soluble G_2 form (LSS fraction); 2A,2B, detergent-soluble G_2 form (DS fraction); 3A,3B, asymmetric forms (HSS fraction). The gels were stained with Coomassie brilliant blue.

The N-terminal sequences obtained are given in fig.4, together with similar data for *T. californica* AChE [26] and human butyrylcholinesterase [27].

Although analyses of proteolytic peptides suggested differences in the primary sequence of catalytic subunits from amphiphilic globular and from asymmetric forms [28,29], MacPhee-Quigley et al. [26] obtained an identical N-terminal sequence for the soluble G_2 form and for the lytic G_4 form derived from asymmetric AChE, in the case of *T. californica*. We find that this identity is in fact valid for the soluble and detergent-soluble G_2 forms and for the native asymmetric forms of *T. marmorata*. It is interesting that the two species differ at positions 3 (His/Asp) and 20 (Val/Ile).

There is a clear homology between *Torpedo* AChE, bovine AChE, human AChE and BuChE, and even *Drosophila* AChE, particularly when taking into account conservative amino acid substitutions. In particular, there is a complete coincidence between the 5–8 sequence of *Torpedo* AChE and the 7–10 sequence of bovine AChE and a similar peptide sequence is also present in the N-terminal sequence of human serum BuChE [27].

A six amino acid sequence that contains the active site serine, Phe-Gly-Glu-Ser-Ala-Gly, seems to be entirely conserved in all cholinesterases: it appears at position 197–202 in *Torpedo* AChE [26,30] and 195–200 in human serum BuChE [31,32]. It is identical in BuChE from horse [33] as well as AChE from *Drosophila* (Hall and Spierer, in preparation). This sequence is clearly exposed to a strong conservative pressure because it is directly involved in the catalytic activity of the enzyme. There is no obvious reason however to expect an equivalent restriction on variations of the N-terminal sequences. The homology that we observed suggests therefore very strongly that all cholinesterases are closely related proteins, originating from a single ancestor. Considering their lack of immunological cross-reactivity, it is extremely likely that, in a given organism, AChE and BuChE are encoded by distinct genes. However, the question remains as to whether the multiple molecular forms of each enzyme correspond to a set of closely similar genes, or are

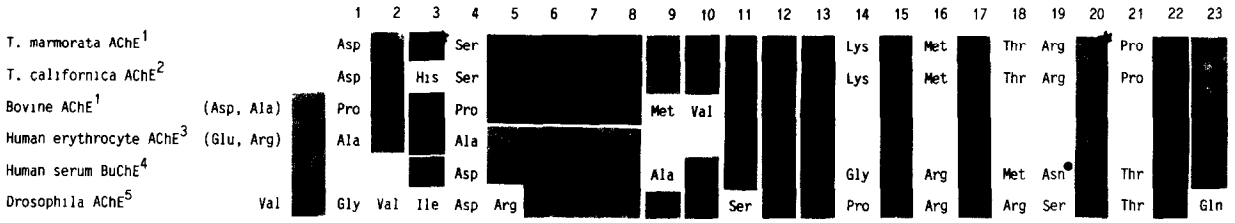

Fig.4. N-terminal sequence of the catalytic subunit of AChE from *T. marmorata* electric organs and bovine caudate nucleus; comparison with other cholinesterases. The different sequences are arranged so as to bring homologous amino acids in register. The amino acids which belong to the same category (acidic, basic, uncharged polar or apolar) in at least three different sequences are shown in bold type, in light shaded areas for conservative changes and dark shaded areas for identical amino acids. The asterisks at positions 3 and 20 of *Torpedo* AChE indicate differences between *T. marmorata* and *T. californica*. The bold-face dot indicates a glycosylated Asn residue in human BuChE. (1) Sequences obtained in this study; we obtained identical sequences for the soluble and detergent-soluble fractions of G_2 forms and for the asymmetric forms. We could not resolve the first amino acid of bovine AChE. (2) Sequence obtained by MacPhee-Quigley et al. [26] for the soluble G_2 form and for the lytic G_4 form (derived from asymmetric forms) of *T. californica*, and from cDNA analysis by Schumacher et al. [30]. (3) From [34], human erythrocyte AChE was found to contain two N-terminal amino acids with stoichiometries of 0.66 (Glu) and 0.34 (Arg). (4) From [27,31]. (5) From Hall and Spierer (in preparation). This sequence was determined from cDNA analysis and is shown here from position 32 after the presumed initiation codon.

generated from a single gene, through the operations of differential splicing and post-transcriptional modifications. Recent results obtained in our laboratory by Southern blot analysis of a genomic *Torpedo* library with an AChE cDNA probe argue in favour of the second hypothesis (Sikorav et al., in preparation).

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