A 13 kDa fragment is responsible for the hydrophobic aggregation of brain G₄ acetylcholinesterase

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Proteinase K treatment of the bovine brain acetylcholinesterase (AChE) releases a hydrophobic fragment of 13 kDa, which is entirely responsible for the aggregation of the G₄ AChE in the absence of detergent. This observation provides evidence that the 13 kDa fragment, which comes from a previously identified 20 kDa subunit, is directly involved in the attachment of the G₄ AChE to brain membranes. A model for the organization of the different sub-domains of the hydrophobic anchor of the G₄ AChE is presented.

INTRODUCTION

Several forms of acetylcholinesterase (AChE, EC 3.1.1.7) found in vertebrate tissues can be distinguished by their solubility characteristics, reflecting differences in their association with cellular structures and their hydrodynamic properties (Rosenberry, 1985; Silman & Futerman, 1988). Asymmetric forms correspond to one, two or three tetramers of catalytic subunits associated with a collagen-like tail which attaches the enzyme to the basal lamina (Dudai *et al.*, 1973; Inestrosa, 1984) by means of heparan sulphate proteoglycans (Brandan & Inestrosa, 1984; Brandan *et al.*, 1985). Globular (G) forms may be divided into 'soluble' molecules, which are extracted in low-ionic-strength buffer, and 'amphiphilic' forms, which require detergent for their solubilization (Rosenberry, 1985; Silman & Futerman, 1988). Amphiphilic G forms have hydrophobic domains that anchor the enzyme in the plasma membrane. The dimeric G₂ AChE forms possess a glycolipid anchor at the C-terminus of each subunit, which contains ethanolamine, glucosamine and inositol phospholipid (Low *et al.*, 1986; Roberts *et al.*, 1987; Inestrosa *et al.*, 1988). Although the tetrameric G₄ AChE forms are predominant in mammalian brain, little is known about their interaction with the plasma membrane.

We previously proposed that the brain G₄ form has a novel hydrophobic subunit of approx. 20 kDa, which is linked to the catalytic subunits by disulphide bonds (Inestrosa *et al.*, 1987). This subunit does not contain the characteristic components of the glycolipid anchor found in the G₂ forms (Inestrosa *et al.*, 1987; Fuentes & Inestrosa, 1988). In those studies, however, no direct proof was presented to indicate that the 20 kDa subunit was involved in hydrophobic interactions of native brain G₄ AChE.

With a G₄ AChE form labelled by 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine (¹²⁵I-TID), a reagent which selectively labelled the 20 kDa hydrophobic subunit of this enzyme (Inestrosa *et al.*, 1987), we show here that proteinase K treatment abolished the aggregation of

the ¹²⁵I-enzyme in the absence of detergent and generated a hydrophilic G₄ form lacking ¹²⁵I-TID. Proteinase K generated a 13 kDa fragment containing most of the ¹²⁵I-TID, suggesting that the previously identified 20 kDa membrane anchor of the G₄ form may be divided into at least three different sub-domains.

MATERIALS AND METHODS

Materials

The *N*-methylacridinium ligand coupled to Sepharose CL-4B was prepared as described by Rosenberry & Scoggin (1984). The photolabelling reagent ¹²⁵I-TID (10 Ci/mmol) was obtained from Amersham.

Purification of the G₄ AChE

The G₄ tetramer was purified from fresh bovine caudate nucleus by affinity chromatography on acridinium resin as previously described (Inestrosa *et al.*, 1987; Fuentes & Inestrosa, 1988). The specific activity of the purified enzyme was 5800 units/mg of protein, where 1 unit is 1 μmol of acetylthiocholine hydrolysed/min. Protein was measured by the method of Bradford (1976).

Radiolabelling of the G₄ AChE form

A 40 μg portion (240 units) of the purified enzyme was labelled in 1 ml of reaction mixture [20 mM-sodium phosphate, 5 mM-EDTA and 10 mM-*N*-ethylmaleimide (buffer A) plus 0.03% Triton X-100] with 200 μCi of ¹²⁵I-TID in a stoppered glass test tube. Photolysis was performed for 30 min at 350 nm in a Farrand Mark I spectrofluorimeter with the slits removed. The sample was extensively dialysed to remove the free ¹²⁵I-TID and then was re-purified by affinity chromatography.

Proteinase K digestion

A 100-unit sample of the ¹²⁵I-TID-labelled AChE (3500 c.p.m.) was incubated with proteinase K (50 μg/ml) in 250 μl of buffer A plus 0.1% Triton X-100 for

Abbreviations used: AChE, acetylcholinesterase; ¹²⁵I-TID, 3-(trifluoromethyl)-3-(*m*-[¹²⁵I]iodophenyl)diazirine.

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Fig. 1. Effect of proteinase K treatment on the aggregation of amphiphilic G_4 AChE

Samples of the untreated and of the proteinase K-treated enzyme were loaded in 5–20% sucrose gradients prepared as described in the Materials and methods section. (a) Untreated AChE loaded in a detergent-free gradient. (b) Untreated AChE loaded in a gradient containing 0.5% Triton X-100. (c) Proteinase K-treated AChE on a detergent-free gradient, ○, AChE activity; ●, ^{125}I (c.p.m.). Arrows indicated the sedimentation of the *Discopyge* electric-organ dimeric AChE, which sediments at 6.5 S (Fuentes & Inestrosa, 1988).

30 min at room temperature. The reaction was stopped either with 2 mM-phenylmethanesulphonate or by dilution in the sucrose gradients.

Sedimentation analysis

Sucrose gradients (linear; 5–20%, w/v) were prepared in buffer A containing, when indicated, 0.5% Triton X-100 (Inestrosa & Ruiz, 1985). Gradients were run at 48000 rev./min for 8 h in a Beckman SW 65 rotor, at 4 °C. Fractions (30–35) were collected from the bottom of the tubes and assayed as described below.

SDS/polyacrylamide-gel electrophoresis and autoradiography

Samples were boiled for 5 min in the presence of 2.3% SDS and electrophoresed in a 5–15% gradient or 8% polyacrylamide slab gel as described by Laemmli (1970). 2-Mercaptoethanol (5%) was included before boiling in

reduced samples. Bands were made visible by silver staining (Wray *et al.*, 1981) or autoradiography on dried gels exposed to Kodak X-Omat XAR-5 film at –80 °C.

AChE assay

AChE activity was determined at 37 °C by the modified Ellman assay (Ellman *et al.*, 1961) in 1 ml of reaction mixture containing 100 mM-sodium phosphate (pH 7.0), 1% Triton X-100, 0.75 mM-acetylthiocholine iodide and 0.3 mM-5,5'-dithiobis-(2-nitrobenzoic acid).

RESULTS

Removal of non-ionic detergents from purified bovine caudate-nucleus G_4 AChE results in the formation of soluble aggregates that can be detected by sucrose-gradient centrifugation (Grassi *et al.*, 1982; Landauer *et al.*, 1983). Affinity-purified G_4 AChE previously labelled with ^{125}I -TID, a reagent that reacts with the hydrophobic domain of this enzyme (Inestrosa *et al.*, 1987), was subjected to velocity sedimentation under different conditions. The ^{125}I -AChE sediments as heterogeneous aggregates in the absence of detergent (Fig. 1a), but as a monodisperse species of approx. 11 S in the presence of Triton X-100 (Fig. 1b); note in both cases the precise superposition of ^{125}I radioactivity and enzyme activity. The hydrophobic domain of the bovine caudate-nucleus G_4 AChE is presumably responsible for aggregate formation, and proteinase K was used to cleave the hydrophilic and hydrophobic domains of the esterase; 85% of the AChE activity remained after the proteinase treatment (results not shown). When this activity was analysed by centrifugation in sucrose gradients in the absence of detergent, an enzymically active hydrophilic fragment was observed which sediments at 11 S in a very symmetrical peak (Fig. 1c). Concomitant with this conversion, the ^{125}I -TID label was clearly dissociated from the enzyme activity and sedimented close to the top of the sucrose gradient (Fig. 1c). SDS/polyacrylamide-gel electrophoresis of ^{125}I -TID-labelled brain AChE resulted in multiple labelled bands (Fig. 2c), and disulphide reduction released the 20 kDa subunit (Fig. 2a), as reported previously (Inestrosa *et al.*, 1987). Proteinase K treatment shifted the major ^{125}I -TID-labelled species to 13 kDa, after disulphide reduction (Fig. 2b). Comparison of Figs. 2(a) and 2(b) indicates that the 'broad' 20 kDa hydrophobic fragment previously identified (Inestrosa *et al.*, 1987) is formed by at least two bands, the major one of 20 kDa and the smaller one of approx. 13 kDa. This latter one corresponds to the major band generated by proteinase K. The proteolysed G_4 AChE also contained a minor band which migrated close to the front of the gel (5–8 kDa). This band probably represents the small fragment generated previously by extensive Pronase digestion (Inestrosa *et al.*, 1987). When ^{125}I -TID-treated AChE was submitted to such Pronase treatment, it yielded only the smaller band which migrated close to the front of the SDS/polyacrylamide gel (results not shown). The 13 kDa and 5–8 kDa ^{125}I -TID-labelled fragments generated by proteinase K also were observed without enzyme reduction (Fig. 2d), suggesting that most of the ^{125}I -TID-labelled material was located distal to the site where the disulphide bond(s) linked the 20 kDa fragment with the AChE catalytic subunits.

Fig. 3 shows the effect of proteinase K treatment on unlabelled AChE. Reduced control and treated samples

Fig. 2. SDS/polyacrylamide-gel electrophoresis of ¹²⁵I-TID-labelled AChE with (b, d) and without (a, c) proteinase K treatment

¹²⁵I-TID-labelled AChE was incubated with proteinase K until AChE activity had decreased to about 80% of the initial value and then subjected to SDS/polyacrylamide (5–15% gradient)-gel electrophoresis under reducing (a, b) and non-reducing (c, d) conditions before autoradiography.

showed no differences (Figs. 3a and 3b); however, there is an effect on the 90 kDa polypeptide. On the other hand, there are clear differences between non-reduced samples. High-molecular-mass polypeptides disappear after proteinase K treatment, most of the AChE becoming the 68 kDa monomer, as shown previously for the human brain enzyme (Gennari *et al.*, 1987). This effect was not observed on native samples run in sucrose gradients (Fig. 1c) in the absence of SDS.

DISCUSSION

This report establishes the involvement of a 13 kDa subunit fragment in the aggregation of bovine caudate-nucleus G₄ AChE in the absence of detergent. ¹²⁵I-TID is a reagent highly selective for the membrane-binding domains of several proteins, including the G₂ AChE (Stieger *et al.*, 1984; Roberts & Rosenberry, 1986). Previously (Inestrosa *et al.*, 1987) we have shown that ¹²⁵I-TID labelled a 'broad' 20 kDa non-catalytic subunit in the G₄ enzyme. This subunit appeared to be linked by disulphide bonds to the catalytic subunits of the G₄ form, since it was obtained only after reduction of the sample with dithiothreitol. Here we demonstrate that it is possible to obtain by proteinase K treatment a smaller hydrophobic ¹²⁵I-TID-labelled fragment which is responsible for the aggregation of the hydrophobic G₄

Fig. 3. Effect of proteinase K treatment on the unlabelled AChE

Purified AChE was submitted to proteinase K (PK) treatment until the activity was 60% of the control: a, c, control; b, d, proteinase K-treated samples; a, b, in the presence of reducing agent; c, d, non-reduced samples. Samples were electrophoresed on 8%-polyacrylamide gel and stained with AgNO₃.

AChE in the absence of detergent. This treatment generates a G₄ hydrophilic form of AChE which is conserved in its quaternary structure and catalytic properties, but which does not require detergent to remain disaggregated. Proteinase K treatment of membrane-bound G₂ AChE from *Torpedo* electric organ also released a small fragment concomitant with conversion of the enzyme into a catalytically active hydrophilic form. This fragment contained the glycopospholipid that serves as hydrophobic anchor to the dimeric AChE (Low *et al.*, 1986; Roberts & Rosenberry, 1986).

In the brain G₄ enzyme, the fragment released by proteinase K migrates as a 13 kDa polypeptide. This fragment both contains most of the ¹²⁵I-TID label and is responsible for the aggregation of intact AChE, a strong indication that this fragment contains the membrane-anchoring domain of the G₄ AChE.

The effect of proteinase K treatment on AChE was also analysed by SDS/polyacrylamide-gel electrophoresis of the unlabelled enzyme. In the absence of a reducing agent, the proteolysed enzyme migrates as a monomer, suggesting that proteinase K, in addition to removing the 13 kDa fragment, splits the enzyme close to the disulphide bonds, generating the 68 kDa monomer. The non-denatured enzyme remains as a tetramer, as shown by sucrose-gradient sedimentation of the proteinase K-

Fig. 4. Proposed structure of the hydrophobic anchor subunit of the brain G₄ AChE

(a) Scheme of the G₄ AChE showing the apparent masses of anchor subunit species under reducing conditions in SDS/polyacrylamide-gel electrophoresis: 20 kDa, intact enzyme; 13 kDa, proteinase K (PK)-treated enzyme; 7 kDa, Pronase-resistant fragment (Inestrosa *et al.*, 1987). (b) Three distinct anchor sub-domains: (a) the disulphide-linkage region, which connects the hydrophobic subunit with the catalytic subunits (molecular mass = 20–13 kDa, or about 7 kDa); (b) an intermediate domain limited by the cleavage site of proteinase K and the Pronase-resistant fragment (molecular mass = 13–7 kDa, or about 6 kDa); (c) the Pronase-resistant domain, which contains fatty acids and is involved directly in the anchorage of the G₄ form to the plasma membrane (molecular mass 7 kDa; Inestrosa *et al.*, 1987).

treated AChE. This is probably due to the existence of non-covalent interactions involved in the maintenance of the quaternary structure of the protein.

The observation that the 13 kDa fragment observed on reducing SDS/polyacrylamide-gel electrophoresis after proteinase K treatment appears to derive from the ¹²⁵I-labelled 'broad' 20 kDa subunit previously identified as the anchor domain of the brain G₄ AChE (Inestrosa

et al., 1987) suggests the existence of at least three different sub-domains in the 20 kDa hydrophobic sub-unit anchor of the G₄ AChE: (a) a proximal sub-domain containing the disulphide linkages involved in the binding of the hydrophobic subunit to the 68 kDa catalytic subunits; (b) an intermediate subunit sensitive to proteinase K; and (c) a third, Pronase-resistant, 7 kDa subdomain within the 13 kDa fragment which contains fatty acids, but not ethanolamine, glucosamine and phosphatidylinositol (Inestrosa *et al.*, 1987) and is involved directly in the binding of the G₄ AChE to the membrane bilayer. The location of the sub-domain c remains unresolved, but one arrangement proposed for clarity in Fig. 4 places this sub-domain at the end of the 20 kDa subunit distal to the catalytic-subunit linkages.

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