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Acetylcholinesterase from *Drosophila melanogaster*

Identification of two subunits encoded by the same gene

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Purified acetylcholinesterase from *Drosophila melanogaster* is composed of a 55 kDa and a 16 kDa noncovalently associated subunit. Cleavage of disulfide bonds reveals that two 55 kDa polypeptides are linked together in native dimeric AChE. Western blots with two antibodies directed against the N- and C-termini of the predicted AChE primary sequence show that the 55 and 16 kDa polypeptides originate from proteolysis of the same precursor encoded by the *Ace* locus.

Acetylcholinesterase; Polypeptide; *(Drosophila)*

1. INTRODUCTION

Unlike vertebrate cholinesterases, which display distinct molecular forms [1], insects possess only one form of acetylcholinesterase (AChE) [2]. This unique form has been characterized as an amphiphilic dimeric protein of molecular mass 150 kDa (as determined by gel filtration, Fergusson plot and pore limit electrophoresis in the presence of anionic detergents) [3,4].

Analysis on SDS-PAGE gels under reducing conditions revealed several polypeptides. In the housefly, Steele and Smallman [5] found a major 59 kDa polypeptide along with a 82 kDa, a 20–23 kDa and traces of a 102 kDa polypeptide. A similar analysis in *Drosophila* revealed analogous bands of, respectively, 70, 55 and 16 kDa [6]. The 55–59 and 70–82 kDa polypeptides bind [^{3}H]diisopropyl fluorophosphate (DFP), suggesting that both polypeptides carry an active site [5,6]. In *Drosophila*, a glycolipid anchor has been identified on the 55 kDa polypeptide with an antibody specific to this structure [7].

In *D. melanogaster*, the gene coding for AChE was localized to a single locus (*Ace*) which has been cloned by chromosome walking [8,9]. Analysis of corresponding cDNA clones revealed an open reading frame encoding a putative protein of 70 kDa [10].

We have analyzed the disulfide bonds which link these different polypeptides to form AChE. By cloning particular DNA fragments derived from the *Ace* cDNA in a bacterial expression system, we have raised polyclonal antibodies directed against specific segments of the protein. Our results indicate that the 55 and 16 kDa polypeptides are the result of processing of the 70 kDa protein encoded by the *Ace* cDNA. However, the 70 kDa polypeptide observed on SDS-PAGE gels is probably a contaminant which co-purifies with AChE.

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Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; DFP, diisopropyl fluorophosphate

2. MATERIALS AND METHODS

AChE was purified from *Drosophila* heads by two affinity chromatographies on trimethylammonium and methylacridium resin as described [4]. AChE activity was monitored according

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to Ellman et al. [11]. Labeling with [^{3}H]DFP was performed at room temperature until no AChE activity was detected. Samples were electrophoresed on SDS-PAGE containing a 7–20% acrylamide gradient [12]. Gels were either stained with Coomassie blue and processed for fluorography, or electrophoretically transferred on nitrocellulose sheets. Protein blots were stained with Ponceau S and then incubated with antibodies. Protein-antibody complexes were revealed using peroxidase-protein A [13].

In order to raise antibodies directed against specific regions of the AChE protein, we have created two fusion proteins using the pEX bacterial expression system [14] (fig.1). The first fusion product (pEX-A1) was obtained by subcloning the *Ball-Bgl*II DNA fragment of the *Ace* cDNA [10] (numbered 997–1349 in the published sequence [10]) into the *Sma*I-*Bam*HI sites of the pEMBL18 cloning vector [15]. The insert was recovered by digestion with *Eco*RI and *Pst*I and ligated into the *Eco*RI-*Pst*I sites of the pEX2 plasmid. The second fusion product, pEX-A3 (fig.1), was obtained by subcloning the *Bam*HI-*Pst*I DNA fragment of the *Ace* cDNA [12] (position 2136–2597) into the *Bam*HI-*Pst*I sites of pEX3. In both cases, we obtained a fusion protein where *Ace* fragments were in translational reading frame with the *cro* β-galactosidase expressed by the vector. As already noted by Stanley and Luzio [14], the fusion proteins were highly insoluble. They were roughly separated from the soluble fraction of the bacterial clear lysates by centrifugation in 2 M urea. The pellets were sonicated and loaded on an SDS-PAGE preparative gel. The fusion proteins were injected into rabbits. Antisera were first depleted on an affigel 10 column (Biorad) coupled with the *cro* β-galactosidase alone. Specific antibodies were then recovered by immunopurification on columns coupled with the fusion proteins.

3. RESULTS

The polypeptide components of the purified enzyme was determined by SDS-PAGE (fig.2). Under reducing conditions (lane a), two major bands of 55 and 16 kDa, and a minor band of 70 kDa were observed. In the absence of disulfide reduction (lane b), an additional band appeared at 110 kDa. In order to identify the origin of the 110 kDa polypeptide, we performed two-dimensional electrophoresis in which the first dimension was run under non-reducing conditions and the second in the presence of reducing agents. Our results reveal that the 110 kDa polypeptide is reduced only in the 55 kDa polypeptide (fig.2c).

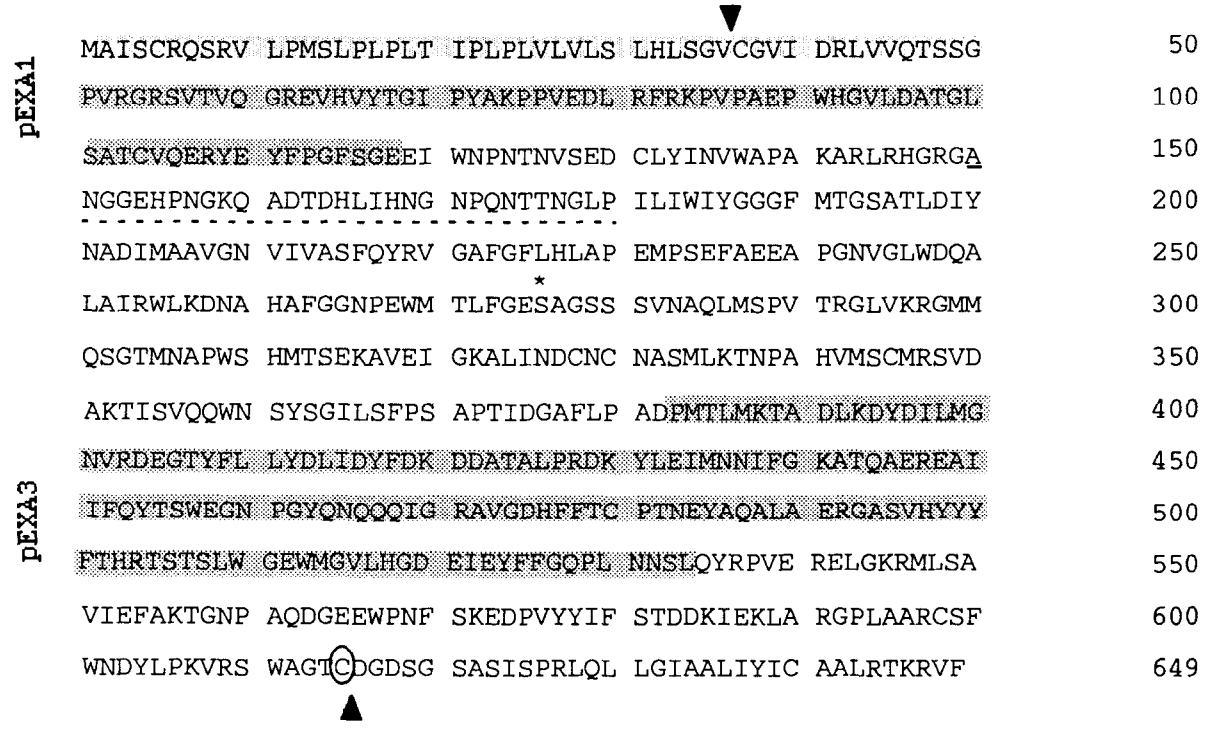

Fig.1. Primary structure of *Drosophila* AChE [10]. The two polypeptides used to raise antibodies are boxed and the 30 amino acids (nos 149–180) which are absent in *Torpedo* AChE or human BuChE are underlined. The cysteine supposedly involved in the inter-subunit disulfide linkage is encircled. The arrows indicate the presumed N- and C-terminal amino acids of the mature protein. The stars mark the serine residue of the active site, and the aspartic acid mutated in the inactive BuChE.

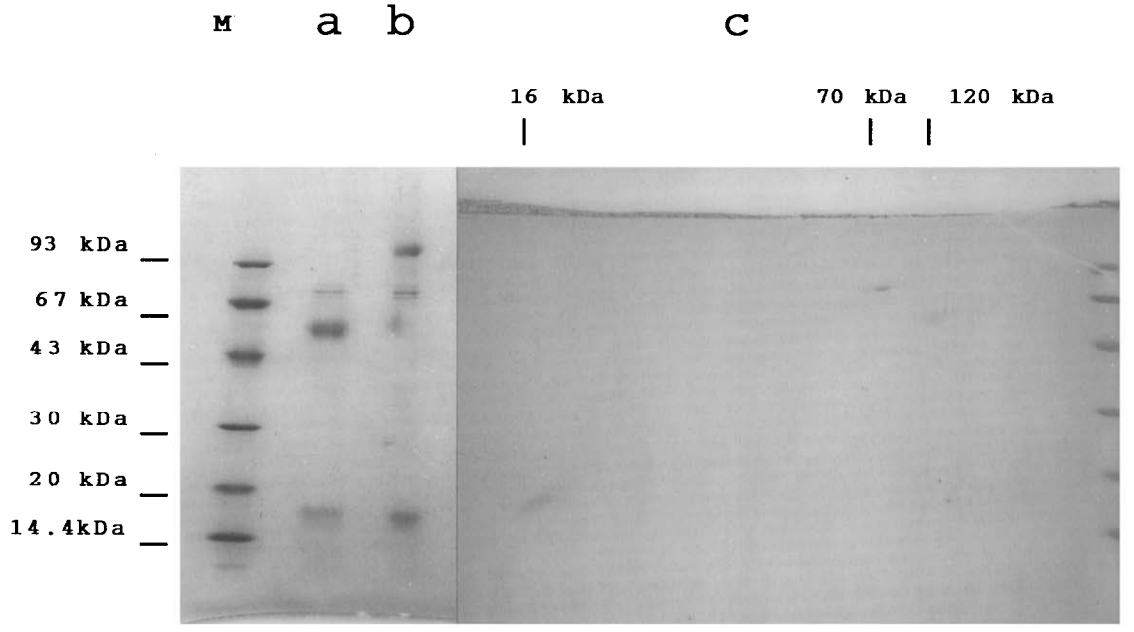

Fig.2. SDS-PAGE analysis of purified AChE from *Drosophila*. Purified AChE (300 U) was electrophoresed on slab gels and stained with Coomassie blue. (M) Pharmacia molecular mass standards, (a) reduced sample, (b) unreduced sample, (c) two-dimensional gel [first dimension was performed under non-reducing conditions, the gel was then incubated for 5 h in 5% β-mercaptoethanol, 10 mM Tris (pH 6.8) and loaded on the second-dimension gel].

The serine residues of the active site of the enzyme is localized on the 55 kDa polypeptide as monitored by binding of [^{3}H]DFP (fig.3). This polypeptide is labeled in purified extract (lane c) and in crude extracts (lane a). Labeling experiments under non-reducing conditions or on two-dimensional gels confirm that the 55 kDa band derives from the 110 kDa polypeptide

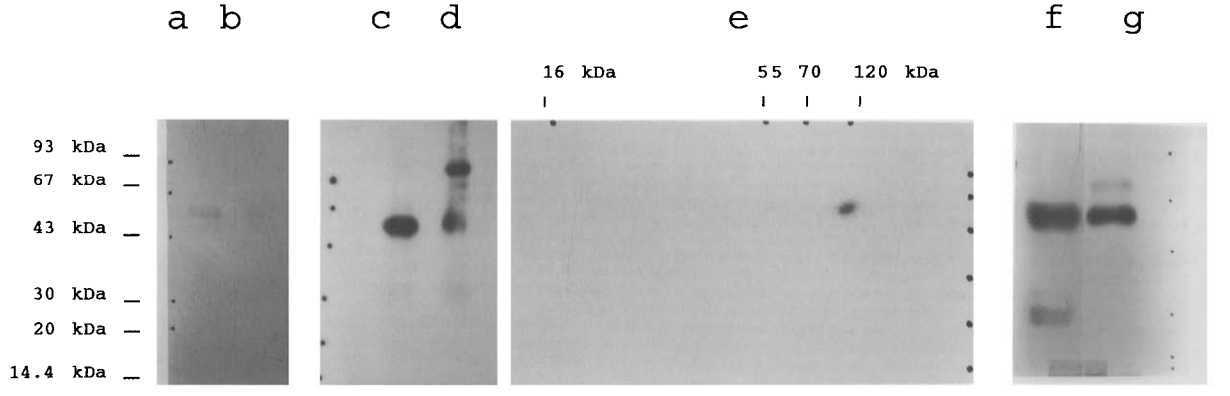

Fig.3. Fluorographic analysis of crude extract or purified AChE labeled with [^{3}H]DFP following SDS-PAGE. Extracts were labeled with [^{3}H]DFP at room temperature until inactivation of AChE was complete (5–10 min). (a) Reduced crude extract, (b) unreduced crude extract, (c) reduced purified extract, (d) unreduced purified extract, (e) two-dimensional gel [first dimension was performed under non-reducing conditions, the gel was then incubated for 5 h in 5% β-mercaptoethanol, 10 mM Tris (pH 6.8) and loaded on the second gel], (f,g) pattern of reduced purified extract showing additional band unusually labeled.

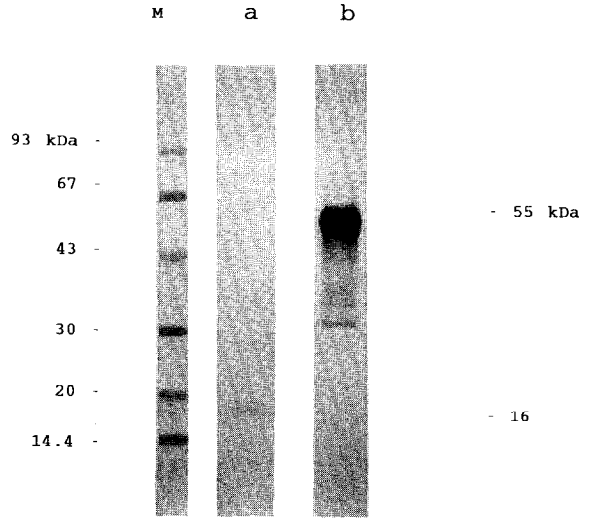

Fig.4. Detection of AChE polypeptides with antibodies raised against the products of various parts of the *Ace* cDNA (see fig.1). AChE was analyzed by SDS-PAGE, transferred to nitrocellulose and incubated with antibodies directed against the product of the 5′-end (a) or 3′-end of *Ace* cDNA (b).

(fig.3e). In addition, a weak radioactive band was sometimes observed at 30 and 70 kDa (fig.3f,g). The former was obtained with samples where proteolysis was suspected. Labeling of the 70 kDa polypeptide is not reproducible.

We raised antibodies against the pEX-A1 fusion protein to obtain a probe reacting with the N-terminal end of the protein encoded by the *Ace* cDNA [10] (see fig.1 and section 2). These antibodies reacted only with the 16 kDa band (fig.4a). Similarly, antibodies specific to the pEX-A3 fusion product were directed against a part of the C-terminal end of the precursor (fig.1). pEX-A3 antibodies reacted with the 55 and 30 kDa bands (fig.4b). We did not observe any reaction of these two antibodies with the 70 kDa polypeptide even when preparations contained large amounts of it.

4. DISCUSSION

In *Drosophila*, cloning of the locus containing the acetylcholinesterase gene (*Ace*) was performed by chromosomal walking [9]. Sequencing of corresponding cDNA clones made it possible to identify the structural gene because of a strong homology with *Torpedo* AChE [10]. The putative protein encoded by the *Ace* cDNA is of 70 kDa (see fig.1). We confirm here that this gene codes for AChE, since antibodies directed against segments of open reading frames of the cDNA react with purified AChE.

Purified *Drosophila* AChE is mainly composed of two polypeptides of 16 and 55 kDa. We have raised antibodies directed against two peptides, at the N- and C-terminus, respectively, of the coding region of *Ace* cDNA. The N-terminus antibody reacts with the 16 kDa polypeptide, the C-terminus antibody reacting with the 55 kDa polypeptide. We tentatively conclude that the protein is translated into a precursor of 70 kDa which is then processed into two polypeptides of 16 kDa at the N-terminus and 55 kDa at the C-terminus. Proteolysis occurring during the purification procedure appears unlikely, since [^{3}H]DFP also labeled the 55 and 110 kDa peptides in crude extracts.

Purified *Drosophila* AChE also contains a 70 kDa polypeptide which was previously interpreted as being AChE because it comigrates with AChE from human erythrocytes and is also labeled by [^{3}H]DFP [6]. Three lines of evidence lead us to conclude that this polypeptide is probably a contaminant of the purified AChE fraction. Firstly, the 70 kDa polypeptide did not react with any of our antibodies. Secondly, it is not covalently linked to the 55 or 16 kDa polypeptide. Finally, labeling with [^{3}H]DFP is weak and not reproducible.

The processing of a single AChE precursor into two polypeptides is not observed in vertebrates. When compared to vertebrates, the putative *Drosophila* AChE contains a 30 amino acid insertion in position 150–180 [10]. Perhaps this additional segment contains the proteolysis site(s). Furthermore, as it was impossible to sequence the N-terminal end of the 55 kDa polypeptide and as the 55 and 16 kDa polypeptides sometimes appear to be composed of several bands (see fig.3a), it is likely that there are several cutting sites in that region.

The association of the 16 and 55 kDa polypeptides, appears to be essential for AChE activity. First, the degree of amino acid sequence conservation at the N-terminus, i.e. in the 16 kDa polypeptide, is very high between *Drosophila* and

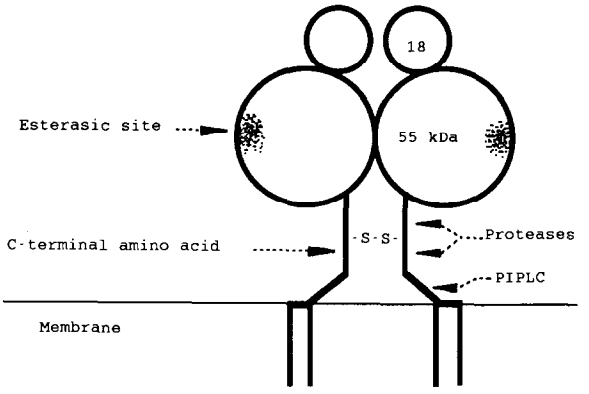

Fig.5. A model for *Drosophila* AChE structure. AChE has been characterized as a dimeric protein composed of two active units covalently associated. Each is composed of two polypeptides (55 and 16 kDa) noncovalently associated. AChE is an amphiphilic protein linked to the membrane of the neuronal cholinergic synapses via a glycolipid anchor located at the C-terminal end of the 55 kDa polypeptide.

vertebrate AChE. Second, a mutation on aspartic acid in position 130 of human BuChE (contained in the 16 kDa polypeptide of *Drosophila*) leads to inactivation of the protein [16].

Our results are in agreement with the location of disulfide bridges in other cholinesterases. It is likely that the residues Cys 104 and Cys 131, belonging to the 16 kDa polypeptide, form an internal disulfide bridge as in *Torpedo* AChE [17] and human BuChE [18]. The 55 kDa polypeptide has a sulfhydryl group involved in the inter-subunit linkage, located near the C-terminus. As moderate proteolysis of native forms converts G2 into G1 [4], it is likely that Cys 615 is involved in this inter-subunit linkage.

Our results are also in agreement with the location of the glycolipid anchor, which is responsible for the attachment of AChE to the membrane. Such anchors are localized at the C-termini of proteins [19] and in *Drosophila* AChE we localized it on the 55 kDa polypeptide [7].

On the basis of our results, we propose a model for the structure of *Drosophila* AChE (see fig.5). The active unit is composed of the non-covalently linked 55 and 16 kDa polypeptides. Two active units are linked together by a disulfide bond at the C-terminal end of the 55 kDa polypeptide leading to the G2 form which is anchored to the membrane via a glycolipid anchor.

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