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BIOCHEMICAL CHARACTERIZATION OF *DROSOPHILA MELANOGASTER* ACETYLCHOLINESTERASE EXPRESSED BY RECOMBINANT BACULOVIRUSES

H. Chaabihi[4], D. Fournier[2], Y. Fedon [3], J. P. Bossy [1], M. Ravallec [1], G. Devauchelle [1], and M. Cérutti [1]

1. Unité de Biologie Cellulaire et Moléculaire, Laboratoire de Pathologie Comparée INRA-CNRS, 30380 Saint-Christol-Lez-Alès, FRANCE

2. Laboratoire d'Entomologie, Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, FRANCE

3. Laboratoire de Physiologie Animale, INRA, place Viala, 34060 Montpellier cedex, FRANCE

4. Protéine Performance, route d'Alès, 30380 Saint-Christol-Lèz-Alès, FRANCE

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Recombinant baculoviruses expressing full length and 3' truncated forms of c-DNA encoding the *Drosophila melanogaster* acetylcholinesterase (AChE) were constructed. Biochemical analyses showed that full length recombinant protein was enzymatically active and anchored to the cell membrane *via* a glycolipidic residue. DTT treatment dissociated the native form into monomers migrating as did the corresponding form of AChE extracted from drosophila heads. Finally, DFP labelling demonstrated that the specific proteolytic cleavage leading to the formation of 55 and 16 kDa subunits occurred in Sf9 cells. In contrast with the full-length enzyme, C-terminal-truncated forms were highly secreted, confirming the prominent role of the C-terminal hydrophobic peptide for the addition of the glycolipidic residue. Accumulation of inactive precursor was observed when recombinant proteins were overproduced using an improved baculovirus, suggesting a saturation of insect cell machineries.

Acetylcholinesterase (AChE, EC 3.1.1.7) plays a key role in the central nervous system of insects (1) by rapidly hydrolysing the neurotransmitter acetylcholine. The gene encoding *Drosophila melanogaster* AChE, localized to a single locus on chromosome 3 (2), has been cloned by Hall and Spierer (3). Analysis of c-DNA sequence revealed the presence of a large open reading frame encoding a precursor protein of 70 kDa. However, biochemical analyses showed that AChE of drosophila is an amphiphilic dimer anchored to the cell membrane *via* a glycosyl-phosphatidyl-inositol (GPI) residue (4)(5). The two subunits of 16 and 55 kDa respectively result from the proteolytic cleavage of the precursor polypeptide, the 16 kDa originating from the amino end and the 55 kDa from the carboxy end (6)(7). The 55 kDa subunit bears the active site and the glycolipidic residue. A signal peptide is also present at the N-terminal part of the precursor as expected for a secreted enzyme.

The baculovirus system has been largely used for the production of a wide range of proteins (8)(9). Foreign proteins expressed in insect cells with baculovirus vectors are, in most cases,

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enzymatically, antigenically, and functionally similar to their authentic counterparts, implying correct formation of disulphide bridges and proper protein folding. C-DNA encoding *Torpedo californica* acetylcholinesterase has already been expressed using baculovirus expression vectors (10). Native and mutated forms of *Torpedo* AChE were produced and purified allowing determination of catalytic parameters.

In this study, we have inserted the full-length and truncated fragments of c-DNA encoding the *D. melanogaster* AChE into baculovirus expression vectors and we have investigated post-translationnal modifications occurring on native and truncated form produced in insect cells. Biochemical analyses showed that insect cells were able to correctly achieve the complex processing of this enzyme ie. cleavage of the signal peptide, endoproteolytic cleavage of the precursor, formation of functional dimers and addition of a GPI residue. Overexpression of AChE cDNA in a highly efficient baculovirus vector led to the production and accumulation of a 70 kDa AChE precursor.

MATERIALS AND METHODS

Cell lines and viruses: *Spodoptera frugiperda* IPLB-*Sf*21 cells (11) and *Sf*9 cells were grown at 28°C in TC100 medium supplemented with 10% fetal calf serum. The fetal calf serum was incubated at 70°C for 1 hour for inactivation of endogenous AChE activity. To construct recombinant baculoviruses expressing AChE sequences, either the wild type virus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) clone 1.2 (12) or the AcSLP10 modified virus (13) were used. Infections, transfections, and isolation of recombinant baculoviruses were done as previously described (13).

Construction of plasmids: AChE sequences were inserted either in plasmid pGmAc3 (14) or in plasmid pGm8022 (13) (Fig.1). Plasmid pGmAc3 is a polyhedrin transfer vector carrying a unique SmaI site downstream of the polyhedrin promoter. Plasmid pGm8022 is a transfer vector allowing insertion of foreign sequences downstream of p10 promoter.

Expression of full length AChE protein: A 2195 bp FspI-SacI fragment containing the complete coding sequence of the gene was inserted in the SmaI site of pGmAc3 leading to the recombinant plasmid pGmAc3ache. The FspI site is located 14 nucleotides upstream of the ATG start codon of AChE gene and the SacI site is located 231 nucleotides downstream of the stop codon.

Expression of truncated forms of the AChE: To produce a C-terminal truncated AChE, the coding sequence was interrupted by insertion of a universal stop oligonucleotide (GCTTAATTAATTAAGC, Pharmacia) at the BstXI site located 52 nucleotides upstream of the AChE stop codon. The truncated sequence was inserted as a FspI-Sac I fragment into the SmaI site of pGmAc3, the resulting plasmid was designated pGmAc3acheB. A C-terminal truncated protein was also produced under the control of the p10 promoter. This was achieved by cloning a 1874 bp FspI-XmnI fragment at the BglII site located downstream of the p10 promoter of plasmid pGm8022. This construct resulted in a recombinant plasmid referred to as pGm8022acheX. In pGm8022acheX, the sequence encoding the AChE 28 C-terminal residues was replaced by a sequence coding for 8 new amino-acids originating from the 3' sequence of the p10 gene in frame with the AChE sequence.

***In vivo* radiolabelling of proteins:** *S. frugiperda* cells ($1.5X10^6$) were infected at a multiplicity of infection (m.o.i) of 10 PFU. At 66 hours post-infection (h p.i.) the medium was removed and cells were pulse labelled with 0.5 ml of methionine-free medium supplemented with 10 µCi of [^{35}S] methionine (1,000 Ci/mmol; Amersham). After 4 hours incubation, cells were lysed and proteins were separated by SDS-PAGE (15).

AChE assay: AChE activity was measured according to the method of Ellman (16).

Non-denaturing electrophoresis: Electrophoresis of native AChE forms was performed using 7.5% polyacrylamide gels containing 0.5% Triton X100 (17). AChE activity was detected according to the method of Karnovsky and Roots (18).

AChE purification: Recombinant AChE(s) were purified from infected cells as described in (19), using successive chromatographies on concanavalin A sepharose, trimethylammonium-6-

hexylamine (a gift from P. Masson CRESSA, Grenoble) and N-methylacridinium resins (Gift from J. Massoulié ENS, Paris).

Diisopropyl fluorophosphate (DFP) labelling: Labelling with [^{3}H] DFP was performed as previously described (7).

Phosphatidyl inositol-phospholipase C (PI-PL C) treatment: Suceptibility of expressed AChE to PIPL-C was monitored by incubation of samples with *Bacillus cereus* (Boehringer Mannheim) in 20 mM phosphate buffer pH 7.0 and 0.1% Triton X100 over 1hour at 30°C in the presence of protease inhibitors (Sigma): PMSF (100 µg/ml), Pepstatine A (5 µg/ml), Leupeptin (5 µg/ml), Aprotinin (5 µg/ml) and EDTA (0.5 mM). The samples were then cooled on ice and subjected to phase separation in Triton X114 (5). In order to test the reliability of the method, AChE from *Drosophila* heads was concomitantly digested.

Scanning electron microscopy: Cells were grown on Cyclopore membrane (Falcon, Becton Dickinson) in 6-well plates. Forty eight h p.i., cells were prefixed by adding 1 volume of à 4% glutaraldehyde solution in 1 M cacodylate buffer, pH 7.4. After 10 min incubation at 35°C, the supernatant was discarded and replaced by glutaraldehyde 2% (in cacodylate buffer) for 50 min at room temperature. Cells were rinsed with distilled water. AChE activity was revealed by adding 1.5 ml per well of a solution containing 1 mg/ml acetylthiocholine, 6 mM tri-sodium citrate, 5 mM $CuSO_4$, 0.5 mM potassium ferricyanide, in phosphate buffer 0.1 M pH 6 (18). After 1 hour incubation at room temperature cells were rinsed rapidly with water and post-fixed for 30 min in 2% OsO_4 (made in cacodylate 0.1 M pH 7.4). Samples were dehydrated with acetone. Critic point was achieved by substituting acetone with CO_2, dessicated material was carbonated and subsequently observed in a Zeiss DSM 950 microscope using backscattered electrons mode.

RESULTS

Construction of recombinant baculoviruses:

The full length AChE c-DNA was inserted downstream of the baculovirus polyhedrin promoter in plasmid pGmAc3 (Fig.1) and transferred to the AcMNPV wild type baculovirus genome by homologous recombination. A polyhedra defective recombinant producing AChE in lieu of

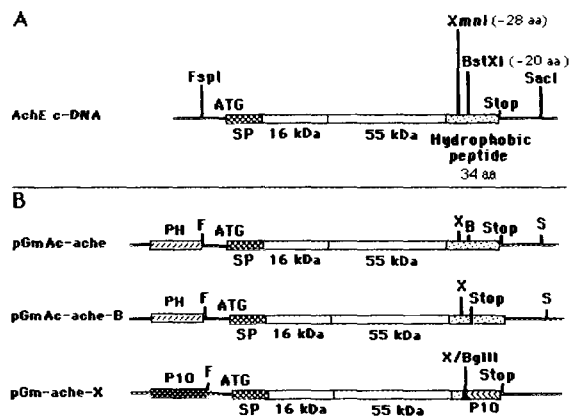

Figure 1. Construction of the baculovirus transfer vectors. (A) Major features present in AChE c-DNA sequence as reported by Hall and Spierer (3). White boxes represent DNA encoding the two enzyme subunits identified in mature form of AChE. Spotted boxes indicate (i) at 5' end, part of DNA encoding signal peptide (SP) (ii) at the 3' end, DNA encoding the hydrophobic peptide which is replaced by a glycolipid in the mature protein. (B) Diagrammatic representation of transfer vectors used to obtain recombinant baculoviruses. Polyhedrin (PH) and P10 promoters are represented as striped and black boxes, respectively. F: FspI, S: SacI, X: XmnI, B: BstXI.

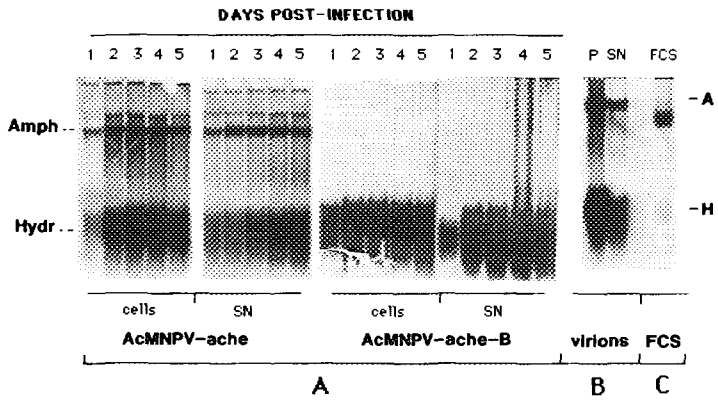

Figure 2. Acetylcholinesterase activity in insect cells infected with recombinant baculoviruses. (A) Time course production of recombinant proteins expressed in Sf9 infected cells. Cells were infected for 1 to 5 days with either AcMNPV ache or with AcMNPV-ache-B. Cells and cell culture supernatants (SN) were assayed for AChE activity. (B) Analysis of AChE activity found in the cell culture medium. Cells were discarded at low speed (3,000 rpm) for 5 min and supernatant was centrifuged at 18,000 rpm for 1 hour to sediment virions. Supernatant (SN) and pellet (P) fractions were assayed for AChE activity. (C) A AChE activity was present in fetal calf serum. 30µl of non-heat-treated fetal calf serum (FCS) was assayed for AChE activity.
In all cases, samples were run on a 7% non-denaturing polyacrylamide gel in the presence of 0.5% Triton X100. AChE activity was revealed according to (18). Amph. (A): amphiphilic form, Hydr. (H): hydrophilic form.

polyhedrin was plaque purified and designated as AcMNPV-ache. In a similar way, the two 3' truncated sequences of AChE interrupted at BstXI or XmnI sites were inserted in transfer vectors and recombined with wild type or AcSLP10 viral DNA, respectively. The corresponding recombinant viruses are referred to as AcMNPV-acheB and AcSLP10-acheX.

Characterization of the recombinant AChE produced in baculovirus system:

To assess AChE activity in *S. frugiperda* cells infected with the recombinant baculovirus AcMNPV-ache, both cells and cell culture supernatants were investigated as described in Materials and Methods. Figure 2 shows that an active AChE was produced. The expression rate was estimated at approximately 1mg/liter (10^9 cells). Although associated with the cell fraction (60% of total activity), AChE activity was also found in the cell culture supernatant. One explanation could be that during the budding of the virus particles at the cell surface, occurring between 18 and 24 hours post-infection, some AChE molecules are integrated into the virus membrane. Such an hypothesis was supported by the detection of a high AChE activity associated with virus pellets obtained after high speed centrifugation of cell culture supernatant (18,000 rpm for 1 hour, rotor SW 27.1 Beckman) (Fig. 2). The presence of residual activity in high speed supernatant suggested that a part of AChE molecules produced are (i) associated with very small cellular and/or viral membrane pieces and/or (ii) present as soluble protein complexes.

AChE activity was localized using electron microscopy. Cells were prefixed with glutaraldehyde solution and activity was subsequently revealed by adding acetylthiocholine as described by Karnovsky and Roots (18). Cells infected with virus expressing the full length AChE appeared

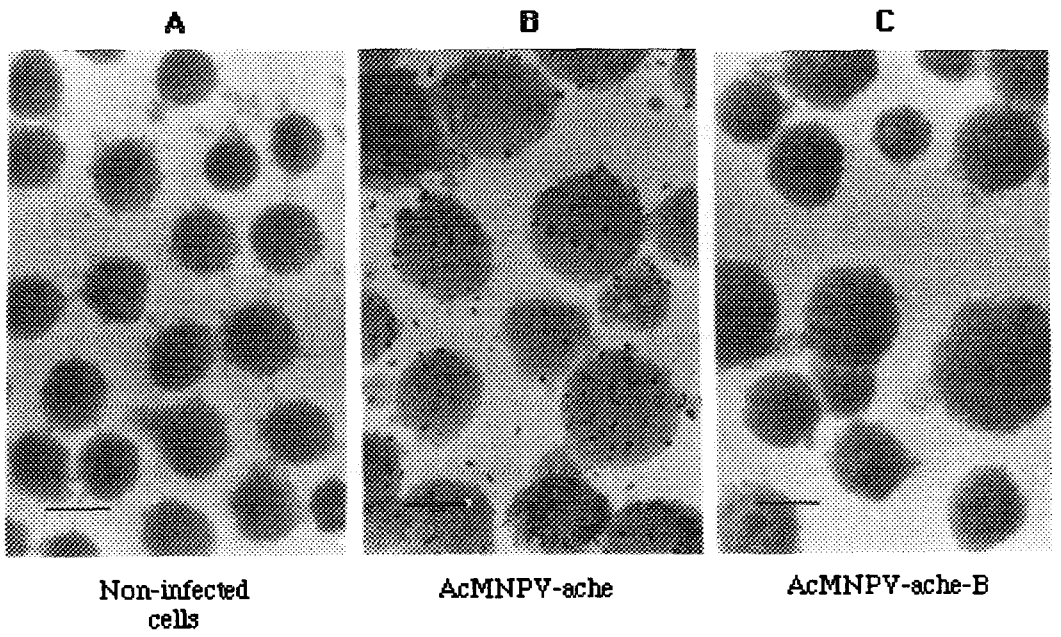

Figure 3. Localization of AChE activity using electron microscopy. Cells were prefixed with glutaraldehyde 3 days post-infection, and AChE activity was revealed after addition of acetylthiocholine according to (18). (A) non-infected cells (B) cells infected with AcMNPV-ache (C) cells infected with AcMNPV-ache-B. (Bar = 10 μm).

covered with crystals indicating that AChE activity was present at the cell surface (Fig.3B). This localization was confirmed by observation of ultrathin sections of infected cells (data not shown). Thus, as expected, enzyme active sites were sticking out from the cell membrane. As shown in figure 2, AChE produced in Sf9 cells appeared as amphiphilic and hydrophilic forms migrating as did the corresponding forms of AChE extracted from drosophila (Fig.4A). To confirm the dimeric structure, purified recombinant AChE was treated with increasing amounts of DTT and run on a polyacrylamide gel in non-denaturing conditions (Fig.4A). In the presence of 10 mM DTT, some dimers were dissociated providing a form with mobility corresponding to that of monomers. All dimeric form was dissociated with 100 mM DTT, leading to a significant loss of activity as a consequence of the intra-chain disulfide bonds reduction (Fig.4A). This experiment demonstrated that the recombinant AChE processed in insect cells formed active dimers by disulfide bridging (7).

Specific proteolytic cleavage leading to the formation of the 55 and 16 kDa subunits was analyzed after labelling purified recombinant AChE with [^{3}H] DFP. DFP specifically binds to the active site of AChE allowing a specific labelling of subunits bearing it. After denaturation with SDS and ß-mercaptoethanol, electrophoresis and autoradiography, a polypeptide of about 55 kDa was revealed (Fig.4B). Thus, a specific proteolytic cleavage occurred in *S. frugiperda* infected cells.

Figure 4C shows that when cells were infected with AcMNPV-ache, about 50% of the active

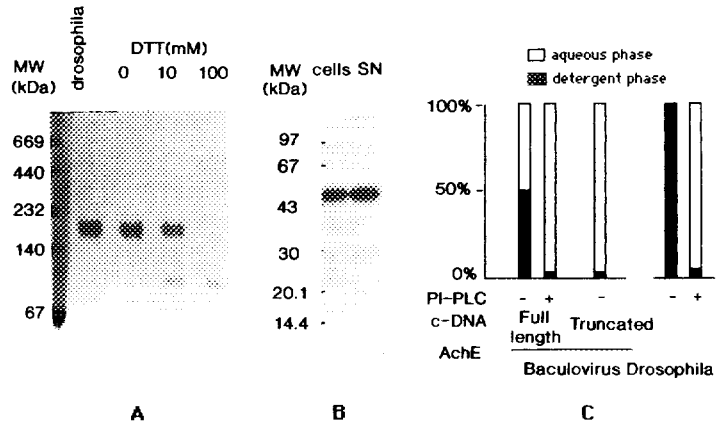

Figure 4. Biochemical characterization of full length AChE purified from cells infected with AcMNPV-ache. (A) gradient gel electrophoresis containing 1% NP40 shows the effect of DTT on purified AChE. Enzyme preparation was treated with 1, 10, and 100 mM DTT for one hour at 37°C. Activity was revealed according to Karnovsky and Roots (18). (B) Autoradiography of purified AChE labelled with [^{3}H] DFP and run on a SDS polyacrylamide gel. (C) Conversion of membrane associated AChE (detergent phase) to soluble AChE (aqueous phase) after digestion with PI-PLC.

AChE was present as an amphiphilic form interacting with detergents. As for the natural AChE from drosophila after treatment with PI-PLC, most of the enzyme was recovered in the aqueous phase, indicating that PI-PLC allowed the formation of a hydrophilic AChE. Non specific proteolytic cleavages and the specific cleavage resulting from PI-PLC digestion could be discriminated using antibodies directed against the cross reacting determinant (anti CRD) of the variant surface glycoprotein (VSG) of *Bacillus cereus* (20). Hence, the carbohydrate moiety appearing during PI-PLC digestion can be specifically revealed with these antibodies. The hydrophilic form generated during incubation with PI-PLC cross-reacted with anti-CRD antibodies (a gift from M. Fergusson, University of Dundee) confirming the presence of a glycolipidic residue in the native amphiphilic recombinant protein (Data not shown).

Production of an AChE truncated form:

A truncated AChE c-DNA interrupted at the BstXI site located within the sequence coding for the C-terminal hydrophobic peptide was inserted in the baculovirus genome giving a recombinant virus named AcMNPV-acheB (Fig.1). In contrast with the full-length enzyme, the truncated AChE was exclusively found as a hydrophilic form, both in cells and cell culture supernatant (Fig.2), and could not be detected on the cell membrane (Fig.3). These observations confirmed that the deletion of 19 C-terminal amino-acids of AChE prevents addition of the GPI residue leading to the production of a secreted form (90% of total activity were found into the cell culture medium). Moreover, the total AChE activity was about 5 to 10 times higher (5 to 10 mg/ 10^9 cells) when compared to that measured for the full length sequence expression.

Overexpression of a truncated form of AChE:

In order to increase the yield of soluble AChE produced in *S. frugiperda* cells, a 3' truncated sequence was expressed using an improved baculovirus (AcSLP10) (13). In this recombinant

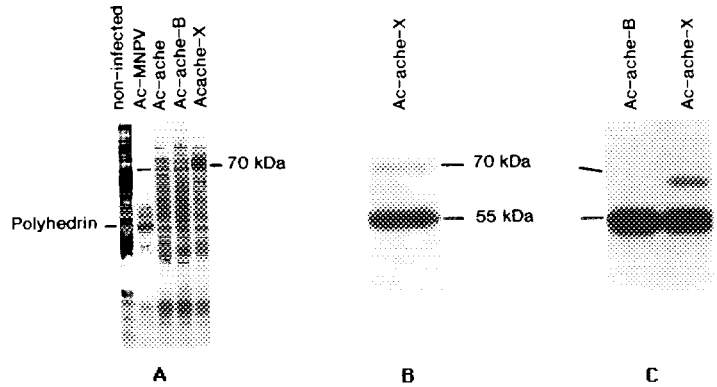

Figure 5. Biochemical characterization of the 70 kDa protein expressed in cells infected with AcSLP10-ache-X. (A) [^{35}S] methionine labelling of proteins produced during infection. Sixty six hours post-infection, cells were pulse-labelled for 4 hours with 10µCi/ml of [^{35}S] methionine. Total cell extracts were analysed by SDS-PAGE and polypeptides were revealed by autoradiography. (B) Detection of AChE polypeptides with antibodies raised against 55 kDa subunits. Purified AChE was run on a SDS polyacrylamide gel, transferred onto nitrocellulose membrane and incubated with antibodies. The antigen-antibody complexes were revealed by reaction with anti-rabbit IgG conjugated with horse-radish peroxydase and visualized with 4-choro-naphtol. (C) Autoradiography of purified AChE labelled with [^{3}H] DFP and run on a SDS polyacrylamide gel.

named AcSLP10-acheX, the 3' truncated AChE sequence was expressed under the control of the p10 promoter (Fig.1). Non denaturing gel analysis of extracts prepared from cells infected with AcSLP10-acheX recombinant showed the presence of a hydrophilic form secreted into the culture medium (Data not shown). However, the use of an improved p10 vector did not increase significantly the production of active AChE. Further analyses using [^{35}S] methionine, DFP or antibodies labelling of the recombinant AChE were done. Cells infected with wild type AcMNPV, or with any of herein described recombinants were pulse labelled with [^{35}S] methionine between 66 and 70 h p.i. Total extracts were run on a SDS-PAGE and polypeptides were detected by autoradiography (Fig.5A). At this time, polypeptides corresponding to the 55 and 16 kDa AChE subunits could not be clearly identified. However, a large band of approximately 70 kDa was seen in cells infected with AcSLP10-acheX (Fig. 5A). This polypeptide was not found in the cell culture supernatant (not shown). Antibodies directed against the 55 kDa AChE subunit revealed the 70 kDa polypeptide, indicating that it was probably an AChE precursor (Fig.5B). This was confirmed by DFP labelling experiments performed with AChE purified from cells infected with AcSLP10-acheX. Indeed, in contrast with observations reported in figure 4B, two polypeptides were labelled: a 70 and 55 kDa corresponding respectively to the putative precursor and the mature catalytic subunit (Fig. 5C). While methionine labelling gave a very significant incorporation in the precursor polypeptide, antibody and DFP labelling led to a very faint detection, suggesting that an important amount of this protein was lost during the purification process. Since the first step consisted in a concanavalin A chromatography, one explanation could be the presence of a large amount of non-glycosylated forms of the precursor, suggesting a saturation of secretion machinery.

Subsequent steps were affinity chromatographies based on specific interactions between resins and catalytic sites. Unproper folding could result in a protein unable to interact with such specific resins. Thus, overexpression of AChE led to the accumulation of an unprocessed precursor which is probably for the most part inactive. The first step of AChE maturation is a dimerisation of precursor molecules followed by glycosylation, proteolytic cleavage and secretion (21). However, since previously reported non-glycosylated monomeric or dimeric forms of the precursor were active (21), the lack of activity of the precursor described in this study suggests that folding of the enzyme was probably the limiting step.

DISCUSSION

Native purified AChE extracted from drosophila heads presents three principal forms which can be identified after separation by non denaturing gel electrophoresis (19): (i) a globular amphiphilic membrane-associated dimer which is the main native form (ii) a hydrophilic dimer which originated from the amphiphilic dimer being cleaved by an endogenous cellular PI-PLC activity (5), though this form is found in the presence of protease inhibitors (22) (iii) a hydrophilic monomer probably resulting from the dimeric form partial reduction of disulfide bridges. We have expressed, in the baculovirus-insect cells system, the full length and two truncated forms of c-DNA encoding *D. melanogaster* AChE. Non denaturing gel electrophoresis of extracts prepared from cells expressing the full length c-DNA, showed that two active forms were produced (i) a dimeric amphiphilic form interacting with detergent and (ii) a soluble dimeric form. PI-PLC digestion demonstrated that amphiphilic form was anchored to the cell membrane *via* a glycolipid residue (GPI) sensible to PIPL-C. The presence of a large amount of hydrophilic form could result from degradation of the native amphiphilic form. This hydrophilic enzyme appeared very early in infection (24 h p.i.), at this time cell integrity is well maintained and in most reported cases no protein degradation was noticed. However, non-glycolipidated dimeric form shows a higher apparent molecular weight both in denaturing and non denaturing conditions (23). It seems more likely that these hydrophilic forms correspond to limited proteolytic cleavage of the dimeric form, occurring during sample preparation.

A hydrophilic AChE was also produced when C-terminal hydrophobic peptide was deleted, thus confirming the prominent role of this C-terminal region for addition of the GPI residue. Indeed, no amphiphilic AChE could be detected in cells infected with these recombinant viruses. Moreover, the hydrophilic enzyme was efficiently secreted into the culture medium thus demonstrating that membrane anchorage was no longer possible.

Using electron microscopy, we have localized enzyme activity in cells infected with all recombinants. While a very strong activity was observed both into the cell and along the cell membrane for cells expressing the full length c-DNA, activity was only detected into the cells when truncated forms were produced.

A 5 to 10 times higher activity was obtained when secreted forms were expressed. One explanation could be the saturation of the cell membrane by the native amphiphilic AChE thus limiting protein expression. In addition, our improved vector failed to increase synthesis of the

soluble form. No significant production increase of active AChE was observed and accumulation of an inactive precursor occurred.

This work confirms that the baculovirus/Sf9 cells system is very efficient for expression and production of very complex proteins. However, high yield production of such proteins remains troublesome because of the limited cell capacity to fully achieve all post-translational modifications for all nascent polypeptides synthesised.

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