Acetylcholinesterase from *Apis mellifera* head

Evidence for amphiphilic and hydrophilic forms characterized by Triton X-114 phase separation

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The polymorphism of bee acetylcholinesterase was studied by sucrose-gradient-sedimentation analysis and non-denaturing electrophoretic analysis of fresh extracts. Lubrol-containing extracts exhibited only one form, which sedimented at 5 S when analysed on high-salt Lubrol-containing gradients and 6 S when analysed on low-salt Lubrol-containing gradients. The 5 S/6 S form aggregated upon removal of the detergent when sedimented on detergent-free gradients and was recovered in the detergent phase after Triton X-114 phase separation. Thus the 5 S/6 S enzyme corresponds to an amphiphilic acetylcholinesterase form. In detergent-free extracts three forms, whose apparent sedimentation coefficients are 14 S, 11 S and 7 S, were observed when sedimentations were performed on detergent-free gradients. Sedimentation analyses on detergent-containing gradients showed only a 5 S peak in high-salt detergent-free extracts and a 6 S peak, with a shoulder at about 7 S, in low-salt detergent-free extracts. Electrophoretic analysis in the presence of detergent demonstrated that the 14 S and 11 S peaks corresponded to aggregates of the 5 S/6 S form, whereas the 7 S peak corresponded to a hydrophilic acetylcholinesterase form which was recovered in the aqueous phase following Triton X-114 phase separation. The 5 S/6 S amphiphilic form could be converted into a 7.1 S hydrophilic form by phosphatidylinositol-specific phospholipase C digestion.

INTRODUCTION

Acetylcholinesterase (AChE, EC 3.1.1.7) occurs in vertebrates in six distinct molecular forms which correspond to different quaternary associations of catalytic and non-catalytic subunits ([1,2] for reviews), including globular and collagen-tailed molecules [3,4].

In insects, although the presence of a tetrameric tailed enzyme has been described only in house fly [5], the biochemical polymorphism appears to be limited to globular molecules (see, e.g. [6,7]), with a major form common to the different species [8]. Some authors have described the existence of a single membrane AChE whose apparent sedimentation coefficient varies from 5.4 S to 5.7 S [9–11], whereas others have shown the presence of distinct biochemical variants [6,12–14]. Recent studies carried out in *Drosophila melanogaster* [15], *Tenebrio molitor* [16], *Pieris brassicae* [17,18] and on purified AChE from the housefly *Musca domestica* [19] have allowed the characterization of globular membrane forms with their hydrophilic counterparts.

In the present paper, we report advanced sedimentation and electrophoretic analysis of acetylcholinesterase from bee head, in which only a 7.3 S form solubilized by autolysis has been previously described [20]. The number of bee AChE forms and their properties were studied. The hydrophobic character of the bee enzyme was investigated by using the Triton X-114 phase-separation method [21] and the hydrodynamic behaviour upon removal of the detergent.

EXPERIMENTAL

Materials

Lubrol PX, Triton X-114, DTNB, iso-OMPA, bacitracin, benzamidin, leupeptin, aprotinin, pepstatin and acetylthiocholine iodide were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Triton X-100 was obtained from Merck (Darmstadt, Germany). PI-PLC purified from *Bacillus thuringiensis* was given by Dr. G. Rougon, Aix-Marseille II University, Marseille, France.

Solubilization of AChE

All steps were carried out between 0 and 4 °C. Adult bees were anaesthetized with CO₂ and the head was severed from the body. The heads were immediately homogenized, with a glass Potter homogenizer, in the appropriate buffer to make a 10% (w/v) extract. The homogenate was filtered through cheesecloth and centrifuged for 30 min at 10000 g (*r*_{av}, 5 cm). The post-mitochondrial 10000 g supernatant was kept and the pellet extracted and centrifuged twice as above. The 10000 g supernatants were pooled and centrifuged in a Beckman 50 Ti rotor (*r*_{av}, 5.9 cm) for 1 h at 100000 g_{av}. The 100000 g supernatant was kept and used for analysis. Different buffers were used for extraction procedures: high-salt detergent-containing buffer (high-salt Lubrol buffer), 1 M-NaCl/1% (w/v) Lubrol-PX/10 mM-Tris/HCl, pH 7.2; high-salt buffer, 1 M-NaCl/10 mM-Tris/HCl, pH 7.2; low-salt detergent-containing

Abbreviations used: AChE, acetylcholinesterase (EC 3.1.1.7); iso-OMPA, tetraisopropyl pyrophosphoramidate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PI-PLC, phosphatidylinositol-specific phospholipase C.

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buffer (low-salt Lubrol buffer), 10 mM-NaCl/1% Lubrol-PX/10 mM-Tris/HCl, pH 7.2; low-salt buffer, 10 mM-NaCl/10 mM-Tris/HCl, pH 7.2. All media were freshly made and sterile. Each buffer contained 1 mM-benzamidine, 5 μ g of leupeptin/ml and 45 units of aprotinin/ml as anti-proteolytic agents.

Enzyme assay

Acetylcholinesterase was assayed at 20 °C by the method of Ellman *et al.* [22], in 1 ml of medium consisting of 1.5 mM-DTNB, 0.3 mM-acetylthiocholine iodide and 80 mM-Tris/HCl, pH 8.0. The reaction rate was monitored spectrophotometrically by the increase in A_{412} . β -Galactosidase was assayed in 1 ml of medium consisting of 0.1 M-phosphate buffer, pH 7.0, containing 20 mM-*o*-nitrophenyl β -D-galactopyranoside, 10 mM-NaCl, 1 mM-MgCl₂ and 0.1 M- β -mercaptoethanol. The change in A_{410} was monitored. Alkaline phosphatase was assayed in 1 ml of medium consisting of 1 mM-sodium *p*-nitrophenyl phosphate and 0.1 M-Tris/HCl, pH 8.5. The change in A_{410} was monitored. Catalase was assayed by the spectrophotometric method of Beers & Sizer [23].

Sucrose-gradient-sedimentation analysis

Centrifugations were usually performed in 5–20% (w/v) linear sucrose gradients prepared in the homogenization buffers. Detergent or 1 M-NaCl was omitted or added as appropriate in order to study hydrophobic interactions and the sedimentation properties of the enzyme. Samples (200–300 μ l) of enzyme solution were layered on analytical gradients and of up to 500 μ l on preparative gradients. Centrifugations were performed at 4 °C on a Beckman SW 41 rotor for 14–16 h at 39000 rev./min (r_{av} , 10.95 cm; 185000 g_{av}). *Escherichia coli* β -galactosidase (EC 3.2.1.23) (16 S), ox liver catalase (EC 1.11.1.6) (11.3 S) and *E. coli* alkaline phosphatase (EC 3.1.3.1) (6.1 S) were used as internal sedimentation standards.

Non-denaturing gel electrophoresis

Electrophoreses were performed at 20 V/cm for 3.5 h, in the presence of 0.5% detergent, in a discontinuous anodic system with *NN'*-methylenebisacrylamide as cross-linker. The stacking gel consisted of 3.75% total acrylamide (T) and 2.6% cross-linker (C), 0.5% detergent and 62 mM-Tris/HCl, pH 6.8. The resolving gel consisted of 7% acrylamide (7% T, 2.6% C), 0.5% detergent and 0.375 M-Tris/HCl, pH 8.9. The running buffer was composed of 0.5% detergent and 10 mM-Tris/glycine, pH 8.3. The detergents used were Lubrol PX and Triton X-100. High-ionic-strength samples were dialysed against low-salt buffers and, before electrophoresis, all samples were equilibrated at pH 6.8. The final detergent concentration of the sample buffer was 0.5%. Electrophoreses were also performed at 10 V/cm for 2.75 h in a horizontal continuous system containing identical concentrations of polyacrylamide and cross-linker (7%T, 2.6%C), in the presence of 0.5% detergent. Gel and running buffer contained 50 mM-Tris/glycine, pH 8.9. The samples were equilibrated with the running buffer before electrophoresis.

Characterization of AChE activity after electrophoresis

A modification of the method of Karnovsky & Roots [24] was used. The incubation solution consisted of 1 vol. of a solution of 5 mM-potassium ferricyanide mixed

immediately before use with 9 vol. of a freshly prepared 0.1 M-maleate buffer, pH 6.0, containing 5.5 mM-trisodium citrate, 3.3 mM-CuSO₄, 2 mM-acetylthiocholine iodide and 10 μ M-iso-OMPA as inhibitor of pseudocholinesterases. After electrophoresis, the gel was washed with distilled water and stained at room temperature with the incubation solution until AChE activities became visible. Quantitative densitometric scanning of gels were performed at 475 nm with a Shimadzu CS-930 spectrodensitometer. The proportions of molecular forms were estimated by integration of the areas of individual peaks.

Triton X-114 phase separation

The detergent Triton X-114 was used to study the hydrophobic character of AChE forms. Triton X-114 solutions were prepared as described by Bordier [21]. Detergent-free samples were mixed with $\frac{1}{3}$ vol. of the washed Triton X-114 solution at 4 °C and kept for 10 min at 0 °C with occasional stirring. The solution was then warmed to 30 °C for 10 min to allow phase separation. Triton X-114 was recovered by centrifugation at room temperature at 10000 g for 3 min. The lower Triton X-114 phase was kept, and the upper aqueous phase was subjected twice more to phase separation. AChE distribution in the lower detergent phase and in the upper aqueous phase was studied. Partial removal of the amphiphilic form from the extract may be performed by quick contact, at 4 °C, with a small amount of Triton X-114 solution.

Solubilization of AChE by PI-PLC

Bee heads were homogenized in low-salt buffer (10 mM-NaCl/10 mM-Tris/HCl, pH 7.2, containing 0.1 mg of bacitracin/ml) at 0 °C with a Sorvall Omni-Mixer to make a 10% (w/v) extract. The extract was filtered through cheesecloth and centrifuged at 4 °C for 10 min at 3000 g_{av} (r_{av} , 5 cm). The post-nuclear 3000 g supernatant was centrifuged at 4 °C in a Beckman 70.1 Ti rotor (r_{av} , 6.1 cm) for 20 min at 30000 g_{av} . The crude membrane pellet was washed and resuspended in $\frac{1}{8}$ extract vol. of low-salt buffer containing 0.1 mg of bacitracin/ml, 103 units of aprotinin/ml, 20 μ g of pepstatin/ml and 5 μ g of leupeptin/ml as anti-proteolytic agents. Electrophoretic analysis revealed that the membrane pellet contained exclusively the 5 S/6 S membrane-bound AChE. Phospholipase digestion was performed at 22 °C on the resuspended membrane pellet by adding PI-PLC to a final concentration of 5 units/ml. At different times, samples were removed and centrifuged with a Beckman Airfuge for 5 min at 30 lb/in² (210 kPa) (165000 g). The supernatant was kept and used for analysis. A part of the resuspended pellet was used as blank without PI-PLC. One PI-PLC unit corresponded to 1 μ mol of phosphatidylinositol hydrolysed/min.

RESULTS

Polymorphism of bee AChE

In these experiments a high degree of solubilization (98%) of AChE, achieved by high-salt Lubrol-PX-containing buffers, was of particular importance in order to characterize all native forms. Low-salt Lubrol buffer, high-salt buffer and low-salt buffer gave respectively 82%, 22% and 14% solubilization efficiencies. The polymorphism of bee AChE was studied by gradient

Fig. 1. Sedimentation analysis of AChE solubilized by different extraction procedures

AChE from bee heads was solubilized by using different buffers as described in the Experimental section. Extracts were sedimented in 5–20% (w/v) sucrose gradients made in the homogenization buffers in the presence (●) or in the absence (○) of 1% Lubrol PX. (a) High-salt Lubrol extract; (b) low-salt Lubrol extract; (c) high-salt detergent-free extract; (d) low-salt detergent-free extract. Activities are shown as changes in A_{412} and are plotted on relative scales. In (a) and (b) each graduation represents an absorbance variation of 0.5 per unit of time; in (c) and (d) each graduation represents an absorbance variation of 0.04 per unit of time. Fractions marked by square brackets were pooled and used for non-denaturing electrophoretic analysis in the presence of detergent.

sedimentation analysis and non-denaturing polyacrylamide-gel electrophoresis. Extraction procedures were carried out with a single buffer system instead of one with several buffers [17,25].

Sedimentation analysis of detergent-containing extracts gives relatively simple patterns. The sedimentation of high-salt Lubrol extracts on detergent-containing gradients revealed a single peak of activity whose apparent sedimentation constant was 5 S (Fig. 1a). For low-salt Lubrol extracts a single peak was still observed, but the apparent sedimentation coefficient was 6.1 S (Fig. 1b). No shoulder was observed in either case. High- and low-salt Lubrol extracts sedimented in detergent-free conditions resulted in the formation of polydisperse aggregates (Figs. 1a and 1b).

Sedimentation analysis of detergent-free extracts exhibited more complicated patterns than those observed with detergent extracts. Three well-resolved peaks of activity, sedimenting at 13.6 S, 11.3 S and 7.2 S, were observed when high-salt extracts are sedimented on detergent-free gradients, but the 14 S peak (13.6 S peak) was not always found in low-salt extracts (Figs. 1c and 1d). The presence of larger aggregates (over 14 S) varies from one homogenate to another, but the 14 S and the 11 S peaks were observed in all high-salt extracts analysed if great care was taken to avoid lipid resuspension, after

the 100 000 *g* centrifugation, which resulted in the formation of polydisperse AChE activity along the gradient (Figs. 1c and 1d). Sedimentation analysis of high-salt extracts performed in the presence of detergent exhibited only one major peak, corresponding to the 5 S form previously characterized (Fig. 1c), whereas with low-salt extract a peak sedimenting at 6.1 S with a shoulder at about 7 S was found (Fig. 1d).

The major form sedimented at 5 S in high-salt detergent conditions and 6 S in low-salt detergent conditions. The variation of the apparent sedimentation coefficient could be due to the density of the sedimentation medium, or the 5 S form could correspond to the 6 S form from which a subunit was dissociated at high ionic strength. To check the validity of these alternative hypothesis, the reversibility of the sedimentation shift was first tested. The 6 S form recovered from low-salt detergent gradient was re-centrifuged on a high-salt Lubrol gradient and, as expected, the characteristic 5 S form was observed (Fig. 2). In order to test the 5 S to 6 S shift, the 5 S form recovered from high-salt Lubrol gradient was dialysed for 36 h against low-salt Lubrol buffer and sedimented on a low-salt Lubrol gradient. Then the 6 S form was observed (Fig. 2). Furthermore, electrophoretic analysis of the 5 S and 6 S forms (isolated from gradients in high- and low-salt conditions respectively) then revealed no

Fig. 2. Identity between the 5 S and the 6 S forms recovered from high-salt and low-salt Lubrol-containing gradients

The 6 S form recovered from a low-salt Lubrol gradient was re-sedimented on a high-salt Lubrol gradient (●). The 5 S form recovered from a high-salt Lubrol gradient was dialysed for 36 h at 4 °C against the low-salt Lubrol buffer and then re-sedimented on a low-salt Lubrol gradient (○).

Fig. 3. Non-denaturing electrophoresis, in the presence of 0.5% Triton X-100, of AChE activities recovered from sucrose gradients

Lane (a) *Drosophila melanogaster* AChE molecular forms. From the top to the bottom: dimeric amphiphilic form, monomeric amphiphilic form, dimeric hydrophilic form, monomeric hydrophilic form (according to Toutant *et al.* [15]). Lane (b), 5 S form. Lane (c), 6 S form. Lane (d), 5 S/6 S form from the microsomal pellet. Lanes (e)–(i), fractions corresponding to aggregates from the gradients of Fig. 1, fractions E–I respectively.

difference in their migration (Fig. 3). Thus the sedimentation shift was not due to the dissociation of a lighter putative subunit.

The nature of the aggregates obtained from high- and low-salt Lubrol extracts sedimented in detergent-free conditions was investigated by electrophoretic analysis, which is more sensitive than re-centrifugation analysis. Fractions collected from the top to the bottom of the gradients were subjected to electrophoresis in the

Fig. 4. Non-denaturing electrophoresis, in the presence of 0.5% detergent, of the AChE activity from the 14 S, 11 S and 7 S peaks recovered from high-salt detergent-free gradients

Because the activity of AChE was low, the sensitive detection of electromorphs was carried out by spectrodensitometric scanning of electrophoretic gels at 475 nm. In (a)–(c) electrophoresis was performed in the presence of 0.5% Triton X-100, whereas in (d) it was performed in the presence of 0.5% Lubrol PX. (a) AChE activity from the 7 S peak. (b) AChE activity from the 11 S peak. (c) AChE activity from the 14 S peak. (d) Activity from the 7 S peak in the presence of 0.5% Lubrol PX, and 5 S/6 S marker. Relative migration corresponds to the ratio migration distance of the band of activity/migration distance of the 5 S/6 S band.

presence of detergent (0.5%). The results obtained (Fig. 3) showed the presence of the 5 S/6 S form in all aggregates analysed. The presence of this form at the top of the detergent-free gradient is noteworthy, likely complexed with lipids. Thus it appears that the solubilization of the 5 S/6 S form was very dependent on the presence of amphiphilic compounds, which allow the dissociation of aggregates.

The 14 S, 11 S and 7 S forms were also subjected to electrophoresis in the presence of detergent. The presence of the 5 S form in the 14 S and 11 S peaks was unambiguously demonstrated, but, for the 7 S peak, only a very weak 5 S activity was observed when electrophoresis was performed in the presence of Triton X-100. However, electrophoresis of the 7 S activity recovered from preparative gradients, in the presence of Lubrol PX, showed an additional fast-migrating form (Fig. 4). Thus this 7 S form appears to be inhibited by the detergent Triton X-100.

The AChE activity remaining in the microsomal pellet was investigated. After solubilization, the activity in the microsomal pellet accounted for about 34 % and 20 % of the total non-solubilized activity when high-salt buffer

Fig. 5. Sedimentation analysis of AChE remaining in the microsomal pellet solubilized by low-salt Lubrol buffer

Bee heads were homogenized either in high-salt buffer (1 M-NaCl/10 mM-Tris/HCl, pH 7.2) or in low-salt buffer (10 mM-NaCl/10 mM-Tris/HCl, pH 7.2), and the homogenates were centrifuged at 4 °C for 30 min at 10000 $g_{av.}$. The post-mitochondrial supernatants were centrifuged at 4 °C for 1 h at 100000 $g_{av.}$. The crude microsomal pellets were washed and solubilized by the low-salt Lubrol buffer (1% Lubrol PX/10 mM-NaCl/10 mM-Tris/HCl, pH 7.2), and then centrifuged at 4 °C for 1 h at 100000 $g_{av.}$ to obtain soluble fractions. The soluble fractions were sedimented in low-salt gradients (5–20% sucrose in 1% Lubrol PX/10 mM-NaCl/10 mM-Tris/HCl, pH 7.2). ▲, Soluble fraction from high-salt microsomal pellet; ●, soluble fraction from low-salt microsomal pellet; ○, centrifugation in the absence of detergent.

and low-salt buffer, respectively, were used. The acetylcholinesterase activity in the microsomal pellets, recovered after the high- and low-salt extractions, was completely solubilized in low-salt Lubrol buffer. Sedimentation and electrophoretic analysis revealed in both cases a form identical with the 5 S/6 S one, which aggregates upon removal of detergent (Figs. 3 and 5). The same results were obtained for the mitochondrial pellets.

All of the extracts studied (low- and high-salt detergent-free or detergent-containing), subjected to electrophoretic analysis, exhibited the fast-migrating minor form corresponding to the 7 S peak. The amount of the 7 S form was assessed by two different methods: the comparison of the 7 S and 5 S/6 S activities obtained from gradient centrifugation and the spectrophotometric analysis of electrophoretic gels. Accordingly, it was found that the 7 S form represented 3–6% of the total AChE activity present in high-salt Lubrol extracts.

Hydrophobicity of bee AChE

The hydrophobic character of the two AChE forms was studied by electrophoresis in the presence of detergent, by the Triton X-114 phase-separation method. Phase separation of Triton X-114 solutions occurs at

Fig. 6. Electrophoresis, in the presence of 0.5% Lubrol PX, of AChE from low-salt crude extract after Triton X-114 phase separation

Lanes: (a) 5 S/6 S form isolated from gradient; (b), AChE from crude low-salt extract; (c), AChE recovered in aqueous phase after Triton X-114 phase separation; (d), AChE recovered in detergent phase after Triton X-114 phase separation.

about 20 °C, resulting in the appearance of two phases respectively depleted and enriched in detergent. Amphiphilic integral membrane proteins interact with detergent by the formation of micelles and are recovered in the Triton X-114 phase, whereas hydrophilic proteins are recovered in the aqueous phase [21]. Such a property was applied to detergent-free extracts of bee heads. The results (Fig. 6) clearly showed that the 5 S/6 S form was recovered in the Triton X-114 phase after phase separation, as the 7 S form remained in the aqueous phase even after four runs of phase separation. Thus the amphiphilic character of the 5 S/6 S AChE was confirmed, whereas the 7 S AChE appeared to be essentially hydrophilic.

Solubilization of AChE by PI-PLC

The ability of PI-PLC to solubilize bee AChE was studied on the 5 S/6 S form from a crude 30000 g membrane fraction. Electrophoretic analysis revealed that this fraction contained only the 5 S/6 S membrane-bound AChE and was devoid of the hydrophilic form. Results shown in Fig. 7(a) showed that PI-PLC from *B. thuringiensis* at a concentration of 5 units/ml was able to release bee AChE directly from membranes. The initial solubilization rate was 0.58% of the membrane-bound activity solubilized/min per unit of PI-PLC. After incubation for 4 h, no significant release of AChE from membranes occurred, and the amount of solubilized AChE became stable at about 60% of the initial membrane-bound activity. After digestion for 5.5 h, the membrane fraction was pelleted and washed twice, and finally resuspended in a fresh digestion medium containing 15 units of PI-PLC/ml. A 3-fold concentration of PI-PLC did not enhance significantly the initial release rate, which was about 240 times lower than that of the preceding digestion (2.4×10^{-3} % of the membrane-bound activity released/min per unit of PI-PLC) (Fig. 7a).

Fig. 7. Solubilization of membrane AChE by PI-PLC

Membrane-bound AChE from a 30000 *g* membrane pellet resuspended in low-salt buffer containing 0.1 mg of bacitracin/ml, 103 units of aprotinin/ml, 20 μ g of pepstatin/ml and 5 μ g of leupeptin/ml was submitted to digestion by PI-PLC (5 units/ml) at 22 °C. (a) At different times of the digestion, samples were removed and centrifuged at 165000 *g* for 5 min. The activity of the supernatant was assayed and corrected by the activity of the blank without PI-PLC (●---●). AChE remaining bound on the membrane fraction was expressed as a percentage of the initial membrane-bound activity (○---○). After 5.5 h, the digestion mixture was centrifuged for 20 min at 30000 *g*, and the pellet washed, resuspended and centrifuged again. The pellet was resuspended in the low-salt buffer containing the anti-proteolytic agents and supplemented with PI-PLC at 15 units/ml. At different times samples were processed as above and the activity in the supernatant was assayed and corrected by the blank activity (●---●); (○---○), percentage of the initial membrane-bound activity. (b) After 5.5 h hydrolysis by PI-PLC, the remaining digestion mixture was centrifuged at 30000 *g* for 20 min and the supernatant was subjected three times to Triton X-114 phase separation. The aqueous phase was sedimented on a sucrose gradient made in low-salt buffer in the presence (●) or in the absence (○) of 1% Lubrol PX. The membrane-bound AChE was assayed by measuring the activity in the digestion mixture before and after centrifugation.

PI-PLC converted the amphiphilic AChE into a hydrophilic form, since no loss of activity was observed in the aqueous phase after three runs of Triton X-114 phase separation. Sedimentation analysis of the aqueous phase showed that the hydrolysis product had a hydrodynamic behaviour very close to that of the 7 S form previously characterized. It sedimented at 7.1 S either in the presence or in the absence of detergent (Fig. 7b).

DISCUSSION

Accurate analysis of bee head AChE has allowed the characterization of at least two distinct molecular forms. The major membrane form interacts with detergents and corresponds to 94–97% of the total activity. It sediments at 5 S in high-salt detergent conditions and 6 S in low-salt detergent conditions (Fig. 1). The sedimentation shift found between low-salt and high-salt detergent conditions appears only to be the consequence of the interaction with the detergent, whose micelles enhance the molecular volume, resulting in a change in the apparent density of the enzyme [1,12,26]. The increase in the molecular volume by interaction with detergent is not negligible, since an average of 106 molecules of detergent aggregate per micelle of Lubrol PX, which corresponds to an absolute molecular mass of 64000 Da [27].

The second, minor, form is hydrophilic and corresponds to $4.5 \pm 1.5\%$ of the total activity. It is well characterized in non-denaturing electrophoresis and is not observed in sedimentation analysis in detergent conditions, because it is low in quantity when compared with the 5 S/6 S form and because its sedimentation coefficient is close to that of the 5 S/6 S form. The hydrophilic AChE of *Pieris brassicae* is a 7.3 S enzyme and accounts for 20–40% of the total activity [17]. Solubilization by autolysis has been found to produce a 7.3 S or 7.4 S detergent-insensitive enzyme in bee heads [20], house-fly heads [28,29] or thoraces [30], which have the same molecular mass as the major fraction of the native soluble form [31]. Thus the hydrophilic minor form characterized here corresponds very probably to the 7.3 S AChE solubilized by autolysis which has been characterized by Krysan & Kruckeberg [20]. The hydrodynamic behaviour of the 7.1 S form released by PI-PLC may suggest that the minor hydrophilic AChE might be a digestion product of endogenous phospholipase. At the present time, it is not possible to know whether the hydrophilic form results from biosynthetic or secretory events, or from phospholipase or proteinase digestions.

The presence of a light form is not excluded, since in some low-salt extracts a small peak of activity at about 4.3 S is seen (Fig. 1d), but no additional study has been carried out.

Proteins associated with membranes can be mainly classified into two categories: extrinsic or peripheral proteins, presumably bound to membranes by polar interactions, and integral or intrinsic membrane proteins, which appear to have an amphiphilic structure. The amphiphilic character depends on the existence of two domains in the same molecule: one, mainly hydrophilic, that contains the catalytic site in the case of AChE, and a second, mainly hydrophobic, that may interact with detergent molecules, phospholipids, hydrophobic molecules or itself [27,32–34]. The hydrophobic domain ensures the anchoring of the enzyme in the plasma

membrane. According to this principle, the 5 S/6 S form may be classified as an amphiphilic enzyme, like integral membrane proteins, on the basis of its aggregation upon removal of detergent and on its recovery in the detergent phase after Triton X-114 phase separation. On the contrary, the 7 S minor form does not interact with Triton X-114 and remains in the aqueous phase, and may be classified as a hydrophilic enzyme, like fully soluble proteins [21,32,33]. Similar results have been obtained in *Drosophila melanogaster* and *Pieris brassicae* [15,17] by using the charge-shift electrophoresis method [35].

The involvement of phosphatidylinositol in the anchoring mode of various membrane proteins is now well established (for reviews, see [36–38]). Globular AChE from different vertebrates have been solubilized mainly by PI-PLC from *Staphylococcus aureus* or alternatively by PI-PLC from *Bacillus thuringiensis*, *Bacillus cereus* and *Trypanosoma brucei* (for review, see [39]). Recently, it has been reported that the amphiphilic AChE dimer from *Drosophila melanogaster* and *Musca domestica* is susceptible to the action of PI-PLC from *B. cereus* and can be fully converted into a hydrophilic form [40]. In the present study, about 60% of bee AChE is easily solubilized by PI-PLC from *B. thuringiensis*. The conversion of the 5 S/6 S amphiphilic form into a 7 S hydrophilic one demonstrates that the releasing effect of PI-PLC is not due to a disruptive effect on membrane structure, such as microvesiculation [38]. By contrast, about 40% of the membrane AChE exhibits a very low susceptibility to PI-PLC. Re-digestion experiments show that this weak solubilizing effect is not due to enzyme inactivation, or to inhibition by the hydrolysis products. The existence of a PI-PLC-resistant fraction is not restricted to insects, since only 5–10% of AChE from human or mouse erythrocyte is solubilized by *S. aureus* PI-PLC [41,42]. Moreover, various brain regions of different mammalian species exhibit totally resistant AChE [42]. The partial solubilization of AChE and the presence of a PI-PLC-resistant fraction might suggest the involvement of an alternative anchoring mode, such as a transmembrane polypeptide. In fact, these data must be interpreted with care, because little is known on interactions of AChE with other membrane proteins and on accessibility problems that could result. It has been suggested that an apparent PI-PLC-resistance might also be due to the binding of the released AChE to a putative glycosylinositol phosphate 'receptor' [38]. In a recent study, evidence is given that structural modifications in the glycolipid anchor of erythrocyte AChE and DAF (Decay-Accelerating Factor) could induce a resistance to PI-PLC [43]. Similarly, the differential susceptibility of human and bovine erythrocyte AChE to PI-PLC arises from structural differences in their glycolipid anchor [44]. Hence, PI-PLC-sensitive AChE could co-exist with forms linked to a PI-PLC-resistant phosphatidylinositol anchor, and thus would explain the 40% PI-PLC-resistant fraction observed in the bee.

The bee head membrane AChE solubilized in the absence of detergent occurs in two types of aggregates: (i) non-specific aggregates, which probably correspond to the association with different membrane proteins and which are characterized mainly by their fast-sedimenting AChE activity; (ii) specific aggregates, which are responsible for the apparent polymorphism observed in detergent-free extracts. These specific aggregates are found in

well-resolved peaks of AChE activity (14 S and 11 S), like other membrane proteins [34], whereas non-specific aggregates exhibit a polydisperse distribution in sedimentation analysis and are scattered along the gradient (Figs. 1c and d). The formation of polydisperse aggregates at pH 6.8 which are dissociated at pH 8.0 has been pointed out in bees by Krysan & Kruckeberg [20]. This aggregation phenomenon rather seems to depend on the charge of the molecules, whereas in the present case both types of aggregates are dissociated in the presence of detergent, but not at high ionic strength (Fig. 1c), thus showing that they are mediated by detergent-sensitive hydrophobic interactions.

The analyses do not provide any information on the nature of the specific aggregates, which might be isologous or heterologous. In *Drosophila melanogaster*, electrophoretic analysis of crude extracts, in the absence of detergent, showed artifactual forms whose number depended on the association of AChE with other proteins [45]. On the other hand, Silver & Prescott [10] reported that AChE from *Manduca sexta* aggregated in the presence and in the absence of detergent, and proposed the hypothesis that the enzyme might exist in the membrane as aggregates of high molecular mass. More recently, Rotundo & Carbonetto [46] have shown that neurons contain clusters of AChE along the neurites. In a similar manner, aggregation phenomena that occur during extraction procedures might account for molecular interactions at the membrane level.

Much evidence tends to show that insect AChE may exist in hydrophilic and amphiphilic (membrane-bound) states. So far, this has been reported for Coleoptera [16], Lepidoptera [17,18] and Diptera [15,19], and the proportions of both states vary from species to species. Studies carried out in the present work, especially by use of the Triton X-114 phase-separation method, confirm that this is also the case in a Hymenopteran species. However, the possible function of the hydrophilic form remains obscure in all cases. The hydrophilic variant might result from lytic as well as secretory phenomena. It is very likely that both these cases correspond to a part of the reality, and that very subtle differences occur within the hydrophilic components.

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