PHOSPHOLIPIDS IN 'NATIVE' *ELECTROPHORUS* ACETYLCHOLINESTERASE

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1. Introduction

Some time ago, our group showed that acetylcholinesterase, of *Electrophorus electricus* (electric eel) or *Torpedo marmorata* electric organs, exist in a number of molecular forms [1]. The three original, or 'native' forms: A, C, and D, have the physico–chemical properties of asymmetric particles [2]. Globular forms can be derived from these native forms by tryptic treatment [1, 3], sonication [2] or through the action of electric eel endogeneous proteases [4, 5]. A globular acetylcholinesterase, similar to that purified by other authors [6–8] can also be directly solubilized from the electroplax membrane by trypsin [9]. We have been able to obtain micrographs of both the globular and 'native' molecular forms we had described [10]: these pictures clearly show the asymmetric, or 'native' forms, as a combination of a cluster of subunits, which we called the 'head', and an elongated element, which we called the 'tail'. The active globular forms are tetramers (11.8 S) and dimers (7.7 S). We proposed a model in which the three native forms are composed of a tail and of, respectively, one, two, and three tetramers, for A (9 S), C (14.2 S) and D (18.5 S). We have indeed recently obtained micrographs of the D molecule, the 'head' of which is clearly made up of three clusters of four globules [11].

Assuming a great stability of tetrameric association, it is thus possible to understand the occurrence of all five molecular forms, and some of their properties. The hydrodynamic properties of the native asymmetric forms of acetylcholinesterase in solution are consistent with the idea that these molecules behave like rigid structures of the dimensions observed in electron micrographs [12]. Several authors have shown that acetylcholinesterase can be solubilized by proteases, but also by other enzymes, and some of these results seem to imply the occurrence of non-peptide linkages or non-protein residues in the structure.

It has, in fact, been concluded by Grafius et al. [1] – from lipase and phospholipase solubilization data – that acetylcholinesterase is imbedded in a lipoprotein matrix. We have therefore studied the sensitivity to lypolytic enzymes of the 'native' acetylcholinesterase.

2. Materials and methods

2.1. *Enzymes*

The following commercial preparations were used: Porcine pancreatic lipase (PL III 16A 45 U/mg, Worthington); phospholipase A (15 057 EPAR, 1 mg/ml in glycerol, Boehriger); phospholipase C (PHLC ODA from *Clostridium perfringens*, 2 U/mg, Worthington, and type I from *Clostridium welchii*, Sigma); phospholipase D (15315 EPAS, Boehringer).

We further purified the *Cl. perfringens* phospholipase C by chromatography on cellulose DE-52 (Whatman) (elution with a linear saline gradient of 0–1 M NaCl in 0.04 M $CaCl_2$ and 0.01 M Tris, pH 7). The purified fraction used contained 0.8 mg of protein/ml; 200 μl of this preparation hydrolysed 1 μM lecithin/hr, at 20°C (0.1 M Tris, pH 8, M $CaCl_2$). Pure pancreatic lipase was a gift from Dr. Charles (CNRS, Marseilles), phospholipase A from Dr. Bocquet (Institut Pasteur, Garches), and *Bacillus cereus* phospholipase C from Dr. R.F.A. Zwaal (Biochemisch Laboratorium der Rijksuniversiteit, Utrecht. This phospholipase preparation was maintained in 0.05 M Tris, pH 7.2, 0.005 M $CaCl_2$ and 50% glycerol.

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Table 1
Experimental conditions used for testing the activity of various enzymes on acetylcholinesterase.

Enzymes	Incubation medium (20°C)
Pancreatic lipase (glycerol ester hydrolase) EC 3.1.1.3	0.1 M Tris (pH 8); 0.05 M $CaCl_2$ Concentration (1 mg/ml)
Phospholipase A (phosphatide acyl hydrolase) EC 3.1.1.4	0.1 M sodium acetate (pH 5.6); 0.02 M $CaCl_2$; diethylether 40 μl/ml
Phospholipase D (phosphatidyl cholinephosphatido hydrolase) EC 3.1.4.4	Concentration: A, 0.1 mg/ml; D, 1 mg/ml (in this medium acetyl-cholinesterase is 50% inactivated)
Phospholipase C (phosphatidyl cholinephosphatido hydrolase) EC 3.1.4.3	0.1 M Tris (pH 8); 0.02 M $CaCl_2$ Concentration: 0.1 mg/ml

2.2. *Assays*

These enzymes were used under the experimental conditions described in table 1.

Lipase was assayed by nephelometric monitoring (at 650 nm) of hydrolysis of an emulsion of tributyrin (30 μg of pure tributyrin emulsified in 10 ml of buffer with an MSE ultra-sound generator).

Proteolytic activity contaminating the enzyme preparations was estimated by measuring the 15% TCA soluble products of azocasein hydrolysis (0.5% casein solution, 440 nm, Schwarz/Mann), or of [^{125}I] casein (a gift of Dr. Ph. Regnier, IBPC, Paris).

The assays used for acetylcholinesterase, as well as the methods of preparation, gradient centrifugation, molecular sieve chromatography, gel electrophoresis, have all been described in earlier publications [1–5].

3. Results

When purified pancreatic lipase was used, no effect was observed on acetylcholinesterase (fig. 1), and we were able to show that the conversions caused by commercial preparations can be attributed to proteolytic

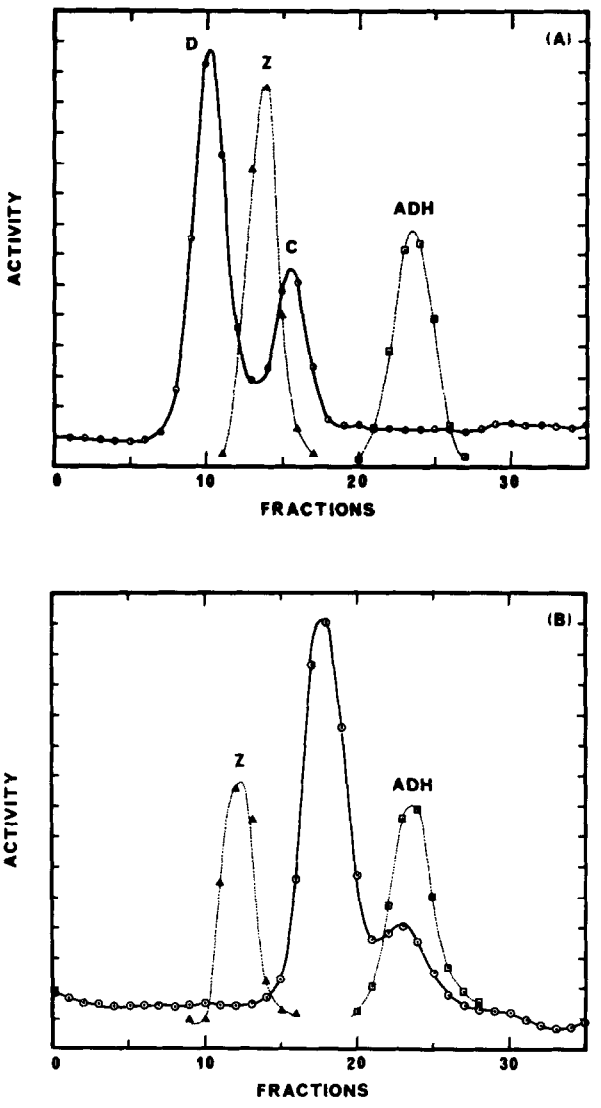

Fig. 1. Transformation induced by a commercial lipase preparation. The variation of sedimentation constants is determined by saccharose gradient centrifugation (1 M NaCl; 0.05 M $MgCl_2$; 0.01 M Tris, pH 7.5): A) Control, acetylcholinesterase: C and D forms; B) Sedimentation pattern of acetylcholinesterase activity after incubation with lipase (cf. table 2). The observed transformation is similar to that observed after proteolytic attack and is not obtained with pure pancreatic lipase. ⊙, acetylcholinesterase; △, β-galactosidase; ⊡, alcohol dehydrogenase; ordinates, enzymatic activities on an arbitrary scale.

* ADH, yeast alcohol dehydrogenase; Z, *E. coli*, β-galactosidase.

Table 2
Proteolytic activity, transforming activity and action of some lipolytic enzymes on the activity of acetylcholinesterase C and D forms.

Enzymes	Contaminant proteolytic activity	Transforming activity	Acetylcholinesterase activity after incubation 1H	2H	28H
Pancreatic lipase					
Commercial	250	+++ (tetramer)	96	65	1
Pure	0	–	100	100	100
Phospholipase A					
Commercial	20	+(?) (tetramer)	100	95	22
Pure	0	–	100	100	100
Phospholipase C (from *Cl. perfringens* or from *B. cereus*)	0	heavy non-aggregating forms	100	110	110
Phospholipase D	10		100	100	80

The pure preparations of pancreatic lipase and phospholipase A have approximately twice the activity of the commercial preparation. Proteolytic activity is determined from azocasein hydrolysis: 100 is an arbitrary value corresponding to that of a 0.01 mg/ml tryptic solution which induces a transformation of D into G in about 24 hr (+++) without any loss of acetylcholinesterase activity. The acetylcholinesterase activities, determined after incubation (modified method of Ellman) are compared to non-incubated samples (100%).

contaminants (table 2). Phospholipase A (commercial preparation) induced a very limited degradation which may also be related to the presence of a protease: pure phospholipase A does not cause this transformation.

On the contrary, we find specific effects with phospholipase C. We used two different phospholipase C preparations: a commercial preparation from *Cl. perfringens*, which we chromatographed again, and a purified preparation from *B. cereus* [14]. These preparations contained a negligible amount of proteolytic contamination (equivalent to less than 0.01 mg of trypsin per ml, as detected by [^{125}I]casein hydrolysis); they were still active after 3 min heating at 90°C, and required divalent cations (the transforming activity is inhibited by 0.01 M EGTA*).

After incubation of acetylcholinesterase D and C with *Bacillus* phospholipase, we did not find any significant change in their sedimentation constants. The molecular forms obtained, however, no longer aggregate in low salt solutions. As a result, standard polyacrylamide gel electrophoresis becomes possible.

The *Clostridium* phospholipase converts acetylcholinesterase D into a slightly faster sedimenting (apparent sedimentation constant 20 S), non-aggregating molecular form, without loss of activity (see table 2, and fig. 2). We shall call this molecular form 'D'. Acetylcholinesterase C is converted into a non-aggregating 15.5 S molecular form. With more concentrated (40-fold) phospholipase C besides 'D' (20 S), one finds a 17 S and a 15.5 S peak, and prolonged incubation eventually leads to an 11.8 S form: it is not clear whether these effects are due to the phospholipase activity or to contaminants.

We have determined the Stokes radius R_e of the transformed 'D' molecular form, obtained in the conditions described (table 1), with the molecular sieve method. Using the same calculations as for the native molecular forms [12], we obtain a mean Stokes radius of 13.7 nm (three determinations gave 13.6, 13.6 and 13.9 nm). From H_2O- and ^2H_2O-sucrose gradient centrifugations [12], we find that its $\bar{\nu}$ is equal to that of the other acetylcholinesterase forms. It is thus possible, from the apparent sedimentation constant, to evaluate the sedimentation constant in standard conditions: $S_{20,w}$ = 19.7. From $S_{20,w}$ and $\bar{\nu}$, we may now estimate the molecular mass of 'D': we find it identical

* EGTA; Ethylene glycol-bis-(2 aminoethylether)-*N,N'*-tetra-acetic acid.

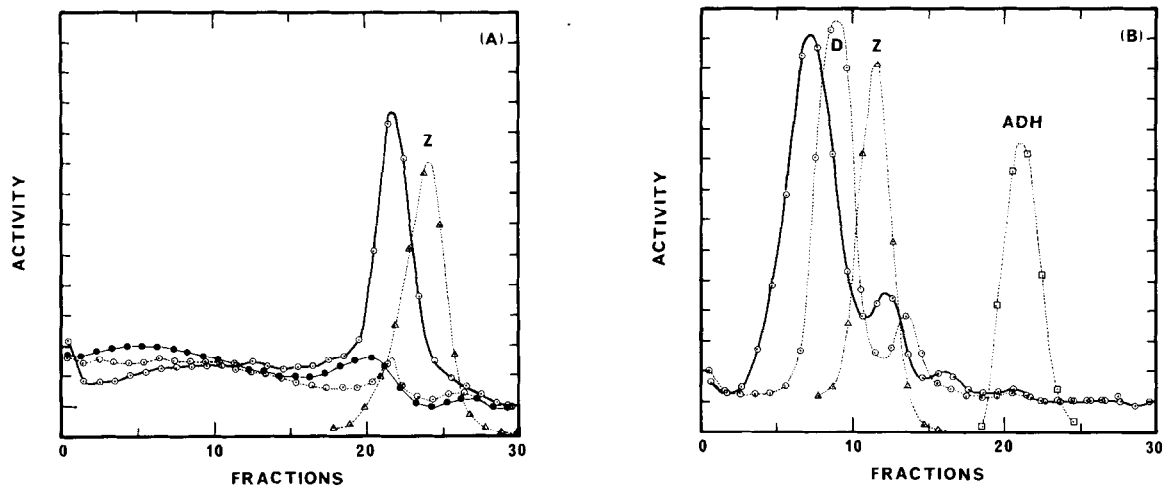

Fig. 2. Effect of *Clostridium perfringens* phospholipase C or acetylcholinesterase D: A) Low salt gradient centrifugation, (5%–20% saccharose gradient, containing 0.01 M NaCl; 0.05 M $MgCl_2$; 0.01 M Tris, pH 7. Centrifugation in Spinco SW 56 rotor – 1600 rpm – 15 hr at 2°C. (o––o––o) Control: untreated acetylcholinesterase D (containing a minor C contaminant) aggregates: little activity is found in the gradient. We usually find two small polydisperse peaks at about 25–30 S and 60–75 S. Acetylcholinesterase (activity corresponding to an absorption variation of 180D min/min in 1 ml of Ellman's reagent [9] is incubated for 90 min at 24°C in a small volume (10 µl) with 1 µl of phospholipase C (sufficient to hydrolyse 510^{-3} nm lecithin/hr). (•—•—•) Without $CaCl_2$, in the presence of 0.015 M EGTA. The sedimentation is very similar to the control. (o—o—o) In the presence of 0.001 M $CaCl_2$: most of the activity no longer aggregates. B) High salt gradient centrifugation. (5%–20%) saccharose gradient, containing 1 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, pH 7. Centrifugation in Spinco SW56 rotor, 32 000 rpm, 15 hr at 2°C. Control; acetylcholinesterase incubated with phospholipase C as above. The apparent sedimentation constant of the major peak has shifted from 18.4 S to 20 S.

* ADH, yeast alcohol dehydrogenase; Z, *E. coli*, β-galactosidase.

to that of D (1 100 000), since the products $S_{20,w} \times R_e$, as well as $\tilde{\nu}$, are equal for both molecular forms (D: 18.1 X15.9; 'D': 19.7 X13.7).

4. Discussion

Although lipase and phospholipases C and D were recently reported to dissociate heavy aggregates (70 S) of acetylcholinesterase [13] into a 'slow acetylcholinesterase' (11.5 S) we could explain the activity of the same commercial enzymes on acetylcholinesterase solely on the basis of their proteolytic contaminations. Our observations, therefore, do not support the idea that acetylcholinesterase is normally inbedded in a lipoprotein matrix from which it can be liberated as a tetramer [13].

However, we do observe a specific action of phospholipase C on the native forms D and C. These are rapidly converted, without losing acetylcholinesterase activity, into non-aggregating molecular forms. This transformation is basically different from that observed with proteases, which only yield the globular tetrameric and dimeric forms [1, 3, 16]. The minor differences we observe between the *Clostridium* and *Bacillus* enzymes, are probably related to their substrate specificity. The action of phospholipase C seems to imply that some phospholipid molecules, although perhaps very few, are involved in the peculiar structure of 'native' acetylcholinesterase. Burger et al. [15] have already suggested that erythrocyte acetylcholinesterase is associated with phospholipids.

We find that acetylcholinesterase D, upon treatment with *Clostridium* phospholipase C, undergoes a conformational change, in which the molecule seems to become more flexible or less asymmetric (R_e is decreased

while S increases) without any significant change in mass.

The loss of low salt aggregation properties without conversion to tetramers or dimers, may apparently be achieved by other primary alterations of the 'native' forms: we have found that it is produced by collagenase [16], which had been shown to solubilize rat diaphragm [17] or frog muscle [18] acetylcholinesterase. It may be therefore that the native forms possess collagen-like sequences, perhaps in their tail moiety. On the other hand, we have shown [19] that acetylcholinesterase is a glycoprotein (this has also been found for erythrocyte acetylcholinesterase [20]. Splitting off of the terminal sialic acids by *Clostridium perfringens* neuraminidase modifies the binding of specific antiserum to acetylcholinesterase, [21] and leads to a non-aggregating acetylcholinesterase [16].

It thus appears that these native forms readily change into non-aggregating derivatives, since presumably very different primary alterations can bring about similar conformational transformations. Conformational transitions of native acetylcholinesterase might prove extremely significant, especially since structural changes might occur upon solubilization of these complex molecules. The inter-relationship of the different subunits composing the 'native' forms of acetylcholinesterase certainly pre-exist in the membrane-bound state. However, the actual shape of the molecules in situ might be quite different from that obtained in solution and revealed by electron micrographs (molecules 'en grappe' with a rigid tail).

A more detailed knowledge of the chemical nature of non proteic residues, phospholipids and carbohydrates, and of their binding to the polypeptide chains, will certainly be necessary in order to understand the particular structure of 'native' acetylcholinesterase forms and their significance.

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