THE EFFECT OF PHOSPHOLIPASE A ON HUMAN ERYTHROCYTE ACETYLCHOLINESTERASE

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1. Introduction

Erythrocyte acetylcholinesterase is insoluble in aqueous solvents because of its incorporation in the lipoprotein environment of the cell membrane. This has hampered the purification of the enzyme and investigations of its molecular properties. Non-ionic detergents have been used to disrupt the cell membrane and solubilise the acetylcholinesterase [1–5]. There is evidence that this soluble material may be associated with lipid or other protein [1,4,5]. Acetylcholinesterase has also been solubilised as a lipoprotein by washing erythrocytes with 1.2 M sodium chloride [6]. Accordingly, the effect of phospholipase A on the acetylcholinesterase solubilised by Triton X100 treatment of erythrocyte membranes has been investigated. The observed changes suggest a phospholipid dependent aggregation of the acetylcholinesterase.

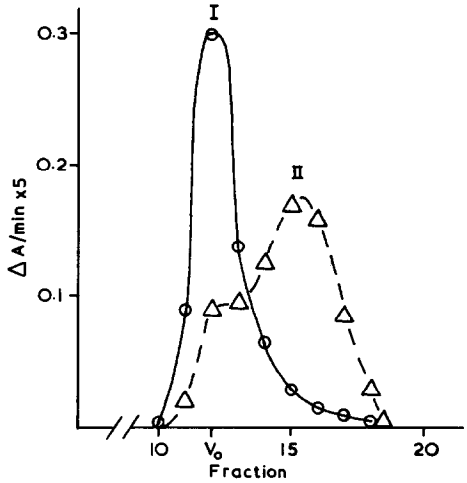

Fig.1. Gel filtration of Triton X100 solubilised acetylcholinesterase from human erythrocyte ghosts after treatment with phospholipase A for 20 min at 37°. 5 ml samples eluted with 2.8 mM Tris-HCl buffer pH 7.4. (o – o – o): Untreated enzyme (Δ – Δ – Δ): phospholipase digest. V_0 determined with blue dextran.

2. Materials and methods

Erythrocyte membranes were prepared from fresh human blood by the method of Dodge et al. [7] and solubilised in 2.8 mM Tris-HCl pH 7.4 by the addition of Triton X100 (2% v/v final concentration) according to Miller [8]. Excess Triton was removed by dialysis against Tris buffer.

The dialysed solution (1 ml) was treated with phospholipase A (25 λ, 1 mg protein/ml, Boehringer) and incubated at 37° for varying times. Control samples containing no phospholipase were incubated in parallel. After incubation both samples were cooled on ice and applied to a Sephadex G-200 column (35 cm long X 2.5 cm diameter) in a cold room at 5° and eluted with 2.8 mM Tris-HCl pH 7.4. The fractions were assayed spectrophotometrically at 412 nm for acetylcholinesterase activity [9]. The amount of enzymic activity was estimated by measuring the areas of the peaks of the elution diagrams shown in fig. 1.

Polyacrylamide gel slab electrophoresis was performed using a vertical apparatus described by Reid and Bieleski [10]. A continuous polyacrylamide gradient (3 – 12%, with constant 2% cross linking) was cast by upward displacement using a modification of the solutions employed by Thorun and Mehl [11].

The weak solution contained 1.25 g sucrose/100 ml, the gel buffer was 19 mM Tris/citrate pH 8.6 and the tank buffer was 0.3 M borate, pH 7.95. A current of 40 mA was applied for 80 min and the gels were stained for acetylcholinesterase [12].

Kinetic studies of the product after gel filtration of the phospholipid digest and the control were followed spectrophotometrically at 412 nm [9] using acetylthiocholine iodide as substrate at 25°. Michaelis constants were determined from Lineweaver-Burk plots.

3. Results

Chromatography of the Triton solubilised erythrocyte ghosts on Sephadex G-200 revealed a single peak of acetylcholinesterase activity eluting in the void volume and tailing into the column volume. This peak did not change for all incubation times in the absence of phospholipase A. Incubation with phospholipase A produced an additional peak in the column volume. The relative amount of enzyme in the second peak increased with time of incubation as shown in fig. 2. A typical phospholipase digest and control are shown in fig. 1. It is apparent that the volume occupied by peak I and its tail of the controls approximates that occupied by peaks I and II of the digests. In an attempt to reduce the tail of peak I control, the molarity of the eluting buffer was raised from 2.8 mM to 0.1 M. This did not affect the form of peak I but prevented the appearance of peak II in the phospholipase digests.

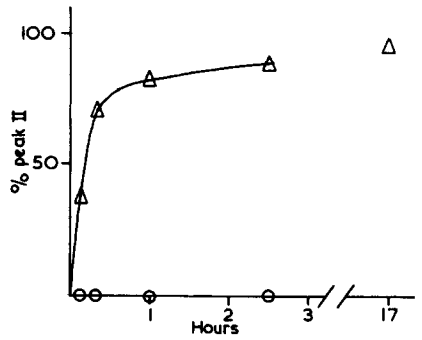

Fig. 2. Rate of appearance of modified enzyme represented by peak II (fig. 1) on treatment of Triton X100 solubilised human erythrocyte ghosts with phospholipase A at 37° expressed as percentage of total activity. Activities of individual peaks were estimated by measuring the area under the elution diagrams. (Δ – Δ – Δ): phospholipase digest, (0 – 0 – 0): untreated enzyme.

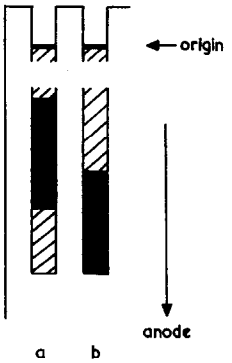

Fig. 3. Part of a polyacrylamide gel slab stained for acetylcholinesterase showing: a) Triton X100 solubilised human erythrocyte ghosts, incubated at 37° for 20 min. b) Triton X100 solubilised human erythrocyte ghosts incubated at 37° for 20 min with phopholipase A (1 ml: 25 λ). 40 mA current passed for 80 min. Intensity of shading indicates amount of enzymic activity.

Polyacrylamide gel electrophoresis (fig. 3) did not reveal discrete banding of the enzyme. A greatly intensified area in the middle of the control streak was observed. In the digests, however, this area showed an increased mobility. In both cases a small amount of activity remained at the origin. No visual differences in electrophoretic pattern was observed in samples that had been incubated with phospholipase for 10 min to 3 hr. This could be due to the inevitable delays in setting up the gels at room temp.

The enzymes obtained from peak I control and from peak II digest were found to have different substrate

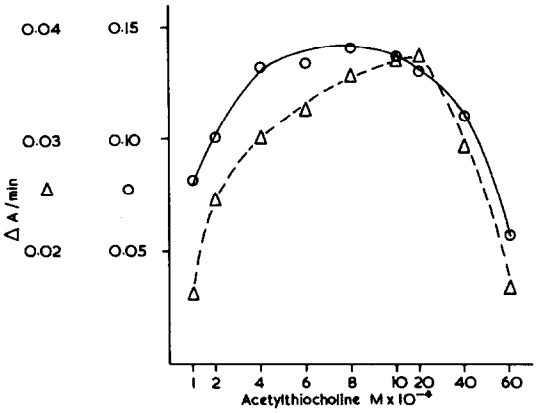

Fig. 4. The effect of acetylthiocholine iodide concentration on the activity of phospholipase treated (Δ – Δ – Δ), and untreated (0 – 0 – 0), Triton X100 solubilised human erythrocyte acetylcholinesterase. The activities for treated enzyme are shown on an expanded scale to facilitate comparison with those for the untreated enzyme.

optima (fig. 4). Untreated enzyme had an optimum substrate concentration of about 7×10^{-4} M whereas the phospholipase digest from peak II had maximum activity at 2×10^{-3} M substrate. The Michaelis constants also differed from 9.5×10^{-5} for untreated enzyme to 1.6×10^{-4} for phospholipase digest.

4. Discussion

Gel filtration and electrophoresis indicates that the acetylcholinesterase liberated from erythrocyte membranes by Triton X100, under our conditions, is a lipoprotein complex. Phospholipase A appears to decrease the molecular weight of the enzyme.

The "tailing" of peak I (fig. 1) and streaking of the electrophoretic pattern (fig. 3) suggest that the control is probably an equilibrium with a very small amount of the enzyme of peak II present. The action of phospholipase A could be a displacement of this equilibrium to about 95% of peak II enzyme (fig. 2).

Phospholipases have been used by other workers studying the erythrocyte membranes. Roelofsen et al. [13] report that pancreatic phospholipase A_2 effects the "complete" hydrolysis of erythrocyte ghost lecithins to lysolecithins and fatty acids. *Ringhals cobra* phospholipase A caused complete hydrolysis of erythrocyte ghost lecithins whereas the phospholipase A from *Vipera palestinae* requires the presence of an activator such as Ca^{2+} or the direct lytic factor for complete hydrolysis of ghost lecithins [14]. It has been shown that as much as 95% of the lysolecithin produced by *Naja naja* venom phospholipase A_2 remained bound to the cell membrane [15]. Other workers [16,17] have found that the optical changes occurring in the erythrocyte ghosts produced by snake venom phospholipase A and lysolecithin are similar but Wallach [17] observes that the effect of the two reagents on membrane bound ATPase differs. This he attributes to the production of membrane bound lysolecithin by phospholipase A. Gordon et al. [16] concluded that membrane architecture is dependent on lipid–protein interactions and/or lipid dependent protein–protein interactions. In our case the lysolecithin produced by phospholipase A on Triton solubilised acetylcholinesterase probably disturbs such an association which could be an aggregation of acetylcholinesterase molecules or an association of the enzyme with other membrane proteins. The lysolecithin may remain bound in situ within the enzyme complex possibly resulting in some dissociation by a reduction of hydrophobic bond strength. Alternatively the lysolecithin, freed from the complex, could exert an additional detergent action on the acetylcholinesterase aggregate causing some dissociation.

Simpkins et al. [15] have reported an increase in a low molecular weight protein of phospholipase A treated erythrocyte ghosts solubilised in S.D.S. when examined by disc electrophoresis. An apparent decrease in molecular weight of acetylcholinesterase was observed in the present study.

The different K_m and optimum substrate values for the enzymes of the two peaks I and II indicate that the lipid associated complex (peak I) is more active at low substrate concentrations than the digest (peak II). In the intact membrane, the membrane probably exhibits such associations and these could considerably modify its catalytic properties and provide a regulatory mechanism.

Aggregation of acetylcholinesterase from *Electrophorus electricus* [18,19] and *Torpedo marmorata* [20] have been reported. These aggregations are dependent on ionic strength, low ionic strength (below about 0.3) favouring aggregation. As reported above, raising the molarity of the eluting buffer for the G-200 column prevents the appearance of peak II and in our case, high ionic strength appears to favour aggregation. The relationship of this ionic effect to the lipid–protein aggregation is not clear and more experimental evidence is required.

5. Acknowledgements

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