Biochem. J. (1985) 231, 237–240 (Printed in Great Britain)

Arrhenius plots of acetylcholinesterase activity in mammalian erythrocytes and in *Torpedo* electric organ

Effect of solubilization by proteinases and by a phosphatidylinositol-specific phospholipase C

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The temperature-dependence of the catalytic activity of acetylcholinesterase (AChE) from rat erythrocyte-ghost membranes and from *Torpedo* electric-organ membranes was examined. In the case of rat erythrocyte AChE, a non-linear Arrhenius plot was observed both before and after solubilization by a phosphatidylinositol-specific phospholipase C or by proteinase treatment. Similarly, no significant differences were observed in Arrhenius plots of *Torpedo* electric-organ AChE before or after solubilization. These results support our suggestion that the catalytic subunit of AChE does not penetrate deeply into the lipid bilayer of the plasma membrane and also suggest that care must be taken in ascribing break points in Arrhenius plots of membrane-bound enzymes to changes in their lipid environment.

INTRODUCTION

Numerous studies on membrane-bound enzymes have examined the temperature-dependence of their catalytic activity by using Arrhenius plots to obtain activation energies. Non-linear Arrhenius plots are often obtained and, in the case of membrane-bound enzymes, the break points are commonly ascribed to phase or order transitions in the membrane lipids which modulate the activity of the enzyme (Lenaz, 1974).

Acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7, AChE) from various sources occurs as a hydrophobic, detergent-soluble species (DSAChE) displaying the solubility characteristics typical of integral membrane proteins (Massoulié & Bon, 1982). Various groups have reported breaks in the Arrhenius plots of membrane-bound AChE from erythrocytes and other tissues and have taken these breaks as evidence for close association of the catalytic subunit with membrane lipids (Beauregard & Roufogalis, 1979; Mizobe & Livett, 1982; Austin *et al.*, 1983; Foot *et al.*, 1983; Meisami, 1984; Plummer *et al.*, 1984). We have, however, recently proposed that DSAChE may be anchored to the plasma membrane via a direct and specific interaction involving the head group of phosphatidylinositol (Futerman *et al.*, 1985*a*), and not via relatively non-specific interactions with the lipid bilayer, as postulated in the fluid-mosaic model (Singer & Nicolson, 1972). This suggestion is based on the observation that DSAChE from mammalian erythrocytes and *Torpedo* electric organ is selectively and specifically solubilized in the absence of detergent by a phosphatidylinositol-specific phospholipase C (monophosphatidylinositol phosphodieterase, EC 3.1.4.10, PIPLC) (Futerman *et al.*, 1983, 1985*b*), and on direct determination of the inositol content of purified AChE (Futerman *et al.*, 1985*c*).

In the model that we propose, the bulk of the catalytic subunit of DSAChE is external to the lipid bilayer and should not, therefore, be modulated by membrane lipids. We decide to compare the temperature-dependence of the catalytic activity of membrane-bound AChE with that of AChE solubilized by PIPLC or by proteinases, so as to evaluate the contribution of the membrane lipids to the activity of the enzyme.

EXPERIMENTAL

Materials

Torpedo californica electric organ (Pacific Biomarine, Venice, CA, U.S.A.) was stored at −80 °C. Male Sprague–Dawley rats (2 months old) were from the Weizmann Institute Breeding Centre. PIPLC, purified from *Staphylococcus aureus*, was a gift from Dr. M. G. Low, Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A. Proteinase K (proteinase type XI from the fungus *Tritirachium album*), papain [proteinase type III from papaya (*Arica papaya*) latex and phenylmethanesulphonyl fluoride were from Sigma. Sucrose, buffers and salts were of analytical grade. [^{3}H]Acetylcholine iodide (sp. radioactivity 90 mCi/mol) was from New England Nuclear Corp., Boston, MA, U.S.A.

Preparation of enzyme samples

Rat erythrocyte ghosts were prepared as described by Hanahan & Ekholm (1974). Heparinized whole blood (6 ml) from freshly killed rats was collected into glass Corex tubes (30 ml) and the whole blood immediately washed twice (1:15, v/v) in 0.14 M-NaCl/0.01 M-sodium phosphate, pH 7.4, by centrifugation (1000 *g*, 30 min, 4 °C) in a Sorvall SS-34 rotor. The second pellet was

Abbreviations used: AChE, acetylcholinesterase; DSAChE, detergent-soluble acetylcholinesterase; PIPLC, phosphatidylinositol-specific phospholipase C.

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resuspended (1:1, v/v) in 0.14 M-NaCl/0.01 M-sodium phosphate, pH 7.4, the cells haemolysed (1:15, v/v) in 10 mM-Tris/HCl, pH 7.6, and the resulting membranes pelleted immediately (20000 *g*, 40 min, 4 °C). The creamy-white ghost membranes thus obtained were washed three times in 10 mM-Tris/HCl, pH 7.6, by centrifugation (20000 *g*, 40 min, 4 °C) and finally reuspended in 6 ml of 10 mM-Tris, pH 7.6. Ghost membranes were incubated with PIPLC (8 μg/ml, 22 °C, 30 min) and then centrifuged (25000 *g*, 40 min, 4 °C). Of the erythrocyte membrane AChE, 95% was solubilized by this treatment. Papain (100 μg/ml, 22 °C, 30 min) was used for proteolytic solubilization of erythrocyte DSAChE (Rosenberry & Scoggin, 1984). Of the ghost membrane AChE, 80% was solubilized by papain.

Torpedo membrane fractions, enriched in DSAChE, were prepared on a flotation gradient (Viratelle & Bernhard, 1980). *Torpedo* electric-organ tissue (5 g) was chopped into small cubes, homogenized (1:3, w/v) in 10 mM-Tris, pH 8.0, in a Sorvall Omnimixer (max. speed, 2 × 90 s, 4 °C), filtered through eight layers of cheesecloth and centrifuged (24000 *g*, 90 min, 4 °C). The supernatant was discarded and the pellet rehomogenized in 11 ml of 10 mM-Tris, pH 8.0, and centrifuged as described above. The second pellet was rehomogenized in 8 ml of 10 mM-Tris, pH 8.0, and adjusted to > 40% (w/v) sucrose by addition of 9 ml of 80% (w/v) sucrose. This homogenate was placed at the bottom of a centrifuge tube, and 15 ml of 35% (w/v) sucrose and 3 ml of 15% (w/v) sucrose were successively layered above the homogenate. The tubes were centrifuged (Beckman L8-70 ultra-centrifuge, SW27 rotor, 78000 *g*, 5 h, 4 °C) and 1 ml fractions collected. By this procedure, DSAChE is obbtained as a low-density fraction near the top of the tube. Low-density *Torpedo* membrane fractions, diluted 1:10 (v/v) in 10 mM-Tris, pH 8.0, were incubated with PIPLC (10 μg/ml, 22 °C, 30 min) and then centrifuged in a Beckman Airfuge (100000 *g*, 10 min). DSAChE was quantitatively solubilized by PIPLC, whereas only 3% of the total activity was found in control supernatants, indicating that DSAChE was membrane-associated in the low-density membrane fractions employed. Similar low-density fractions were incubated undiluted with proteinase K (20 μg/ml, 22 °C). After 30 min, two successive additions of phenylmethanesulphonyl fluoride were made (final concentrations 1 mM and 2 mM, both for 30 min) to ensure complete inactivation of the proteinase K. Centrifugation (100000 *g*, 10 min) showed that 60% of the AChE activity was solubilized by proteinase K treatment under these conditions.

Assay of AChE

Enzyme activity was measured by a radiometric assay using [^{3}H]acetylcholine. The reaction mixture (100 μl) contained 40 mM-potassium phosphate buffer, pH 7.4, 120 mM-NaCl, 3 mM-[^{3}H]acetylcholine iodide and enzyme. Phosphate buffer was used, since its pH changes by only 0.15 unit over the temperature range employed. Incubation was for 15 min; the reaction was terminated and [^{3}H]acetate radioactivity counted in the organic phase of the scintillation fluid as described by Johnson & Russell (1975). Enzyme samples were diluted, before assay, in 40 mM-$MgCl_2$/100 mM-NaCl/10 mM-Tris, pH 8.0, containing 0.01% (w/v) gelatin in order to ensure that hydrolysis did not exceed 30% (30000 c.p.m.) of the total [^{3}H]acetylcholine. Under such conditions the reaction was linear throughout the temperature range employed.

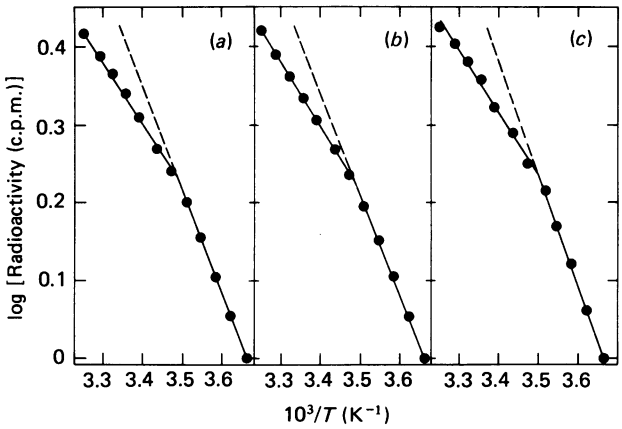

Fig. 1. Arrhenius plots of rate erythrocyte-ghost AChE activity

AchE activity was assayed over the temperature range 0–35 °C as described in the Experimental section. Each panel shows the results from three experiments averaged and normalized to 0 °C. (*a*) Membrane-bound AChE; (*b*) PIPLC-solubilized AChE; (*c*) papain-solubilized AChE.

The temperature-dependence of AChE activity was measured over the range 0–35 °C. Samples were assayed in triplicate at 3–4 °C intervals in a thermostatically controlled water bath. Temperatures below 22 °C were maintained by circulating ice-cold water through a copper coil placed in the bath. Enzyme samples and substrate were equilibrated at each temperature for 10 min before assay. Blanks without enzyme were run at each temperature so as to correct for spontaneous hydrolysis of [^{3}H]acetylcholine.

Treatment of results

The logarithm of the enzyme activity at each temperature was plotted against the reciprocal of absolute temperature (Arrhenius plot) for each experiment. Results from the two to five different experiments on each enzyme sample studied were normalized to 0 °C and averaged; the normalized means were then plotted against $1/T$. Statistical analysis was performed by making both one-line and two-line fits to each data set by minimizing χ^2: the two-line fit was chosen for the cases in which its χ^2 was substantially better than for the one-line fit.

RESULTS AND DISCUSSION

Arrhenius plots of rat erythrocyte-ghost membrane AChE (Fig. 1*a*) were non-linear. A transition temperature at 14 °C and activation energies of 25 kJ/mol and 15 kJ/mol below and above the break point respectively were obtained by statistical analysis. Upon solubilization with either PIPLC (Fig. 1*b*) or papain (Fig. 1*c*), the enzyme still exhibited a sharp discontinuity in its Arrhenius plot, with transition temperatures and activation energies resembling those found for the membrane-bound enzyme. Similar results were obtained with AChE from whole erythrocytes of rat or human blood before and after solubilization (P. L. Barton, A. H. Futerman & I. Silman, unpublished work).

A non-linear Arrhenius plot with a small discontinuity

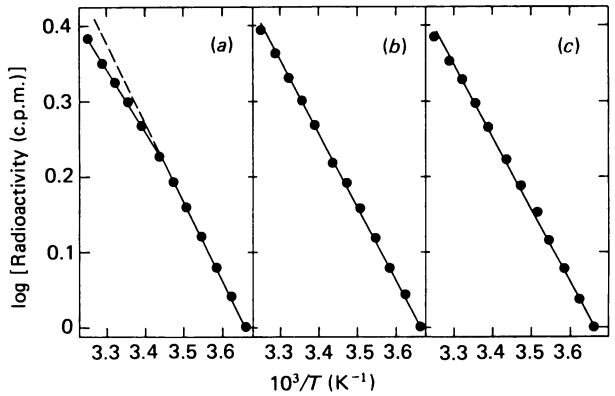

Fig. 2. Arrhenius plots of *Torpedo* AChE activity

Each panel shows results from five experiments averaged and normalized to 0 °C. (*a*) Membrane-bound AChE; (*b*) PIPLC-solubilized AChE; (*c*) proteinase K-solubilized AChE.

was obtained for membrane-bound AChE in *Torpedo* (Fig. 2*a*) with a transition temperature at 16 °C and activation energies of 20 kJ/mol and 15 kJ/mol. Linear plots were found for enzyme solubilized with either PIPLC (Fig. 2*b*) or proteinase K (Fig. 2*c*). Both of the solubilized enzymes had activation energies of about 18 kJ/mol, a value intermediate between those obtained for the membrane-bound enzyme. Similarly, a linear Arrhenius plot was obtained for AChE affinity-purified after proteinase K solubilization and after cholate solubilization (Futerman *et al.*, 1985*a*), with activation energies of about 20 kJ/mol (P. L. Barton, A. H. Futerman & I. Silman, unpublished work).

Comparison of our results with those in the recent literature, which have in general been confined to membrane-bound and detergent-solubilized forms of AChE, shows that the activation energies which we report are of the same order (i.e. 15–25 kJ/mol) as reported by others (i.e. 16–30 kJ/mol, Mizobe & Livett, 1982; 19 kJ/mol, Meisami, 1984). However, higher transition temperatures have been reported, ranging from 16–21 °C for rat brain and spinal cord (Nemat-Gorgani & Meisami, 1979; Meisami, 1984) to 28–29 °C for human erythrocytes (Austin *et al.*, 1983).

Although the mathematical basis for the Arrhenius plot is unequivocal (Dixon & Webb, 1964), its empirical nature should be stressed. Break points might well be fitted to smooth curves, and are usually assigned to the middle of the temperature range studied, since break points in this region can be detected with most accuracy. Particular care must be taken to ensure that intrinsic kinetic parameters, or extrinsic factors which might affect these parameters, do not vary over the temperature range studied, thus creating 'artefactual' discontinuities (cf. Silvius *et al.*, 1978). In the present study we selected a phosphate buffer, the pH of which has a low temperature-dependence. In order to ensure linear reaction rates we selected a substrate concentration (3 mM) much larger than the apparent K_m (0.04 mM) for rat AChE, which is not, furthermore, temperature-dependent (Foot *et al.*, 1983).

As mentioned above, the Arrhenius-plot treatment is empirical, and few attempts have been made to understand its physicochemical basis (see, e.g., Han, 1972; Sandermann, 1978). Our results, particularly those for the membrane-bound and enzymically solubilized rat erythrocyte AChE, rule out the possibility that the discontinuity is due to a membrane lipid transition. It must, therefore, be ascribed to a conformational change in the enzyme protein. Such Arrhenius-plot breaks have indeed been reported for a number of soluble enzymes [see Massey *et al.*, (1966) and references therein]. Furthermore, a recent report (Taguchi *et al.*, 1984) shows that bovine erythrocyte AChE, whether solubilized with Triton X-100 or with PIPLC from *Bacillus thuringiensis*, also displays break points in Arrhenius plots, with activation energies similar to those which we report.

Our results are in support of the model that we have proposed for the anchoring of membrane-bound AChE via a phosphatidylinositol moiety, since it postulates that the bulk of the catalytic subunit is external to the lipid bilayer (Futerman *et al.*, 1985*a*). Less-marked kinetic changes resulting from solubilization would be expected than for proteins that penetrate more deeply into the lipid bilayer (cf. Houslay, 1979). Our data also imply that, for AChE and for other membrane-bound enzymes, care must be taken in ascribing breaks in Arrhenius plots to changes in lipid environment.

A.H.F. was supported by a studentship from the Science and Engineering Research Council (U.K.). A grant from the Muscular Dystrophy Association of America is gratefully acknowledged. We thank Mr. P. Jacobs of the Department of Nuclear Physics of this Institute for writing the computer program used to analyse the data.

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Received 21 June 1985; accepted 21 July 1985