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INVOLVEMENT OF CALCIUM IONS IN THE PROPERTIES OF CARDIOLIPIN-ASSOCIATED ERYTHROCYTE ACETYLCHOLINESTERASE

GUY BEAUREGARD and BASIL D. ROUFOGALIS

Laboratory of Molecular Pharmacology, Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

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Summary

Lipoprotein forms of acetylcholinesterase from bovine erythrocytes gave non-linear Arrhenius plots with a break at 20°C and contained cardiolipin. The break in the Arrhenius plot was abolished by incubation of the enzyme in high salt ($I = 1.8$), but only in Ca^{2+}-chelating conditions. At $I = 1.8$ neither NaCl alone, $CaCl_2$ nor sodium phosphate at acidic pH abolished the break. However, at this ionic strength either NaCl in 2 mM sodium phosphate (pH 7.4) or sodium phosphate, pH 8, or 1.0 M $Na_2CO_3/NaHCO_3$ (pH 8.5—10), were able to remove the break. The Arrhenius plot break was regenerated by the addition of Ca^{2+} to the high salt-treated enzyme with mild homogenization, but could not be regenerated in the presence of EDTA unless $CaCl_2$ was added in excess of the EDTA. Conditions which abolished the break enabled endogenous cardiolipin to be removed from the enzyme by chloroform/methanol extraction. Cardiolipin from acetylcholinesterase incubated in high salt in Ca^{2+}-chelating conditions was not accessible to digestion by phospholipase A_2, and was not separated from the enzyme by flotation in a sucrose density gradient or by Sephadex G-200 chromatography. Thus both Ca^{2+} and cardiolipin appear to be inaccessible, possibly by being tightly associated in the hydrophobic core of the enzyme by ionic and hydrophobic forces. Ca^{2+} may modulate the temperature dependence of acetylcholinesterase activity through a functionally linked ionic interaction with the enzyme-cardiolipin complex.

Introduction

A number of studies showed that acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was released from erythrocytes in a lipoprotein form

[1—4]. The bulk of the associated lipids in the bovine erythrocyte acetylcholinesterase were removed by Lubrol WX [5] and sodium deoxycholate removed essential lipids from the human erythrocyte enzyme [3]. A large particulate form of the enzyme was reconstituted by the addition of erythrocyte membrane lipids to soluble forms of bovine acetylcholinesterase [4,6]. In previous studies we showed that the particulate form of bovine erythrocyte acetylcholinesterase gave a non-linear Arrhenius plot, which was fitted by two straight lines with a break at around 20°C [5,6]. The non-linear Arrhenius plot was also obtained in a preparation solubilized from bovine erythrocytes by Lubrol WX and in a commercially purified preparation, both of which contained only a single phospholipid, cardiolipin [5]. A number of findings led us to conclude that the endogenous cardiolipin was associated with the break in the Arrhenius plots [5,6], by modulating the rate-limiting step in the hydrolysis sequence formation of the activated enzyme-substrate complex [7]. The break in the Arrhenius plot was abolished by treatment of the particulate and soluble forms with 1.8 M NaCl in 2 mM sodium phosphate, pH 7.4 [5,6]. In the latter conditions, or in the presence of 1 M NH_3, cardiolipin was extracted from the enzyme by chloroform/methanol [5,6]. In this study we report that the break in the Arrhenius plot of acetylcholinesterase activity is abolished by high-salt treatment of the enzyme in the presence of Ca^{2+}-chelating agents, and is recovered when Ca^{2+} is added back. Conditions which abolish the Arrhenius plot break do not cause release of cardiolipin from the enzyme.

Materials and Methods

The source of bovine erythrocyte acetylcholinesterase was a partially purified preparation from Sigma Chemical Company (St. Louis, MO). The preparations contain 20 mg of acetylcholinesterase, 100 mg gelatin, 30.7 mg NaCl and 5 ml of sodium phosphate buffer, pH 7.6. Arrhenius plots were determined in enzyme preparations obtained by dissolving 0.25 mg of the acetylcholinesterase in 1 ml of the required medium (see figures) and dialyzing for 12—16 h against 2 mM sodium phosphate (pH 7.4) at 4°C. Incubation of the enzyme in high salt and Ca^{2+}-chelating conditions abolished the Arrhenius plot break (see Results). It was also found that preparation of a concentrated enzyme solution (100 mg/1 ml of water), followed by dilution to 0.25 mg/ml in water, abolished the break in the Arrhenius plot. These conditions are similar to the high salt and Ca^{2+}-chelating conditions described above because salt and phosphate are present in the Sigma enzyme preparation and their concentrations are high in the initial 100 mg/ml solution. Acetylcholinesterase activity was assayed radiometrically as described previously [5,8]. Aliquots (10 μl) were incubated in 0.2 ml of 0.1 M sodium phosphate (pH 7.4) in thick-walled glass test tubes at the required temperature, and the reaction was started by the addition of 0.02 ml of a mixture of acetyl[1-^{14}C]choline iodide (19 nCi) and acetylcholine perchlorate, to a final concentration of 1 mM acetylcholine. After 10 min the reaction was terminated with 0.2 ml ethanol (100%). Activities are reported as net cpm, after subtraction of non-enzymic hydrolysis blanks at each temperature.

A number of approaches were used in attempts to remove endogenous phos-

pholipids under non-denaturing conditions.

Flotation method. A solution of Sigma acetylcholinesterase (0.5 mg/ml) was prepared in 1.0 M $NaHCO_3$ (pH 8.0) and the required amount of sucrose was then added to it. Sucrose solutions were also prepared in 1.0 M $NaHCO_3$, pH 8.0. Sucrose gradients were prepared by layering the following solutions: 1.0 ml of 60% sucrose; 1.0 ml of the enzyme (0.5 mg) in 30% sucrose; 9.5 ml of 25% sucrose; and 1.0 ml of distilled water. The tubes were centrifuged for 66 h at 100 000 × *g* in a Beckman L2 65B ultracentrifuge in a SW41 Ti swinging bucket rotor. After centrifugation 0.29 ml fractions (ten drops) were collected upwards from the bottom of the tube with a peristaltic pump (Desaga 13190) and fraction collector (Gilson FC-80K).

Sephadex G-200 chromatography. Separation of protein and lipid was also attempted by Sephadex G-200 gel chromatography [9,10]. Sigma acetylcholinesterase was dissolved in 1.8 M NaCl and 2 mM sodium phosphate, pH 7.4 (0.5 mg/ml), and passed through a column of Sephadex G-200 (62 × 16 cm) equilibrated and eluted with 1.8 M NaCl and sodium phosphate (pH 7.4) by an ascending method. Fractions (3 ml) were collected (LKB Ultrorac fraction collector) and assayed for acetylcholinesterase activity. Fractions containing peak enzyme activities were collected and used for the determination of Arrhenius plots and partial specific volumes. Partial specific volumes ($\bar{v}$) were determined in preformed sucrose gradients in 2 mM sodium phosphate (pH 7.4) or, in the presence of 1.8 M NaCl and 2 mM sodium phosphate (pH 7.4), as previously described [5,6], or by layering the enzyme on 69% sucrose in 1.8 M NaCl and 2 mM sodium phosphate or 2 mM sodium phosphate alone, and centrifuging for 66 h at 100 000 × *g*. Densities were calculated from the refractive indices determined in a Fisher refractometer.

Phospholipase A_2 digestion. To Sigma acetylcholinesterase (150 mg) dissolved in 1.5 ml distilled water was added 100 units phospholipase A_2 (porcine pancreas from Sigma) and 0.75 ml each of 40 mM $MgCl_2$ and 0.2 M sodium phosphate, pH 7.4, and the mixture incubated for 1 h at 37°C [11]. In control experiments 1 mg cardiolipin (Supelco) was substituted for the acetylcholinesterase. The incubation mixtures also contained 50 μM calcium (acetylcholinesterase reaction) and 10 μM calcium (cardiolipin reaction), as determined by atomic absorption spectroscopy. The reaction was stopped with concentrated HCl (50 μl, 27.5%, w/v). After addition of 2.95 ml of distilled water lipids were extracted with 3.0 ml butanol [12]. The mixture from the enzyme digestion was filtered through a scinterred glass disc. The disc was washed with 1 ml butanol and the filtrate centrifuged for 6 min at 2000 × *g*. The cardiolipin extract was centrifuged without filtration. The upper butanol layers were evaporated in vacuo under nitrogen and the residues redissolved in chloroform. The samples were chromatographed on activated Silica Gel plates (60F-254, Brinkman) [13] with chloroform/methanol/5 N NH_3 (60/30/5, v/v/v). Spots were developed with rhodamine 6G.

Cardiolipin determination after sucrose gradient. Sigma acetylcholinesterase (150 mg) was dissolved in 6 ml of 1.8 M NaCl and 2 mM sodium phosphate, pH 7.4. Aliquots (1 ml) were applied to each of six 5—20% isokinetic sucrose gradients (on a 60% sucrose plug) and centrifuged at 100 000 × *g* for 16 h in a SW41 Ti rotor. The top 1 ml fractions of each of the gradients, above the

acetylcholinesterase-containing fractions, were pooled and extracted with chloroform/methanol (2/1, v/v), and chromatographed on Silica gel plates as previously described [5].

Materials. Inorganic salts and solvents were Analytical-Reagent grade. Acetyl-[1-^{14}C]choline iodide (1—5 Ci/mol) was from New England Nuclear. Cation-exchange resin (Na^+ form, CGC 241, 200—400 mesh) was from J.T. Baker. Phospholipase A_2 (Porcine pancreas, Sigma Chemical Co.) was heated at 70°C for 10 min before use. Sephadex G-200 was from Pharmacia.

Results

The break in Arrhenius plot of acetylcholine hydrolysis was not abolished after treatment of acetylcholinesterase (0.25 mg/ml) with 1.8 M NaCl unless 2 mM sodium phosphate (pH 7.4) was also included (results not shown). (In our previous study [5] 2 mM sodium phosphate buffer (pH 7.4) was present in the high-salt treatment procedure). At the same ionic strength (I = 1.8) 0.7 M sodium phosphate (pH 7.6) abolished the Arrhenius plot discontinuity, but 1.7 M sodium phosphate (pH 5.2) was ineffective (Fig. 1). Since Ca^{2+} is complexed by phosphate only at alkaline pH, strong divalent cation chelation appeared essential for abolishing the break. Treatment of 0.5 mg acetylcholinesterase in 4 ml of 1 M Na_2CO_3/$NaHCO_3$ at pH 8.5, 9.5 and 10.0 (I = 1.8), followed by a 1 : 2.5 dilution in 0.1 M sodium phosphate (pH 7.4) resulted in the loss of the Arrhenius plot break (results not shown). However, incubation of acetylcholinesterase (0.25 mg/ml) in 1.8 M sodium chloride and 10 mM sodium or Tris/EDTA at pH 5.3 or 7.4 with or without dialysis for 12 h in distilled water did not abolish the break (results not shown). $CaCl_2$ (0.6 M) (I = 1.8) at pH 5.5 failed to abolish the Arrhenius plot break (Fig. 2). These results

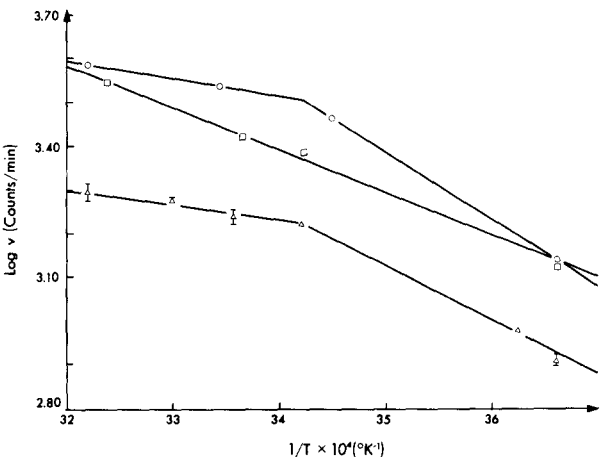

Fig. 1. Treatment of acetylcholinesterase with sodium phosphate at alkaline and acid pH. Without pretreatment (○) the energy of activation was 7.01 kcal/mol and 1.53 kcal/mol below and above the transition temperature (19.1°C). After pretreatment with 1.7 M sodium phosphate (pH 5.2, I = 1.8) (△) the energy of activation was 5.87 kcal/mol and 1.60 kcal/mol below and above the transition temperature (19.1°C). After pretreatment with 0.7 M sodium phosphate (pH 7.57, I = 1.8) (□) the energy of the activation was 4.5 kcal/mol. The bars represent the range of duplicate experiments.

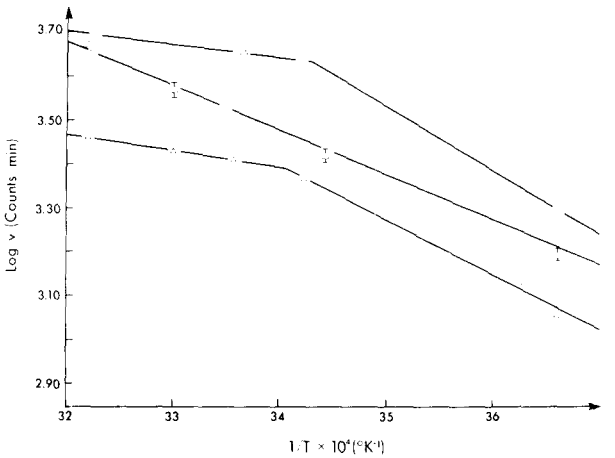

Fig. 2. Effect of high salt in phosphate and $CaCl_2$ on acetylcholinesterase. Without pretreatment (○) the energy of activation was 6.28 kcal/mol and 1.46 kcal/mol below and above the transition temperature (19° C). After dissolving the enzyme preparation at 100 mg/ml and then diluting with water to 0.25 mg/ml before assay (□) the energy of activation was 4.6 kcal/mol. After pretreatment of the enzyme (0.25 mg/ml) in 0.6 M $CaCl_2$ (pH 5.5, i = 1.8) (△), an aliquot (10 µl) was assayed in 0.05 M sodium barbital and 0.16 M NaCl, pH 7.4. The energy of activation was 5.56 kcal/mol and 1.63 kcal/mol below and above the transition temperature (21° C). The bars represent the range of duplicate determinations.

suggested that high ionic strength alone was insufficient for abolishing the break, but divalent cation chelation was also necessary.

Further evidence for the role of Ca^{2+} in the functional cardiolipin-acetylcholinesterase complex was obtained in experiments on the reconstitution of the Arrhenius plot break. Acetylcholinesterase (100 mg/ml) which had lost the break in the Arrhenius plot (Fig. 2) was diluted to 0.25 mg/ml in either distilled water, 2 mM $CaCl_2$ or 10 mM EDTA at pH 7.8, 7.6 and 6.0, respectively. To promote reconstitution, presumably by reassociation of the endogenous cardiolipin to the acetylcholinesterase, the solutions were homogenized ten times for 12-s periods (at 20-s intervals) at speed 8 in a Sorvall Omni-Mix homogenizer. This procedure resulted in the partial reconstitution of the break in the Arrhenius plot in distilled water, and to a greater extent in 2 mM $CaCl_2$ (Fig. 3). However, reconstitution did not occur in the presence of 10 mM EDTA (Fig. 3), unless additional $CaCl_2$ (22 mM) was added to the mixture (result not shown). Thus Ca^{2+} supported the functional reconstitution of the break in the Arrhenius plot of acetylcholinesterase.

Attempts were made to determine if treatment of the enzyme with high salt in Ca^{2+}-chelating conditions caused the release of endogenous cardiolipin from acetylcholinesterase. Treatment of acetylcholinesterase with phospholipase A_2 under conditions where the break in the Arrhenius plot was abolished (150 mg enzyme/1.5 ml of water, see Fig. 2) did not result in an increase in the accessibility of the endogenous cardiolipin to the added phospholipase, as only undigested cardiolipin was recovered from the enzyme preparation. Under similar extraction conditions the R_F of a standard sample of cardiolipin was 0.74 ± 0.09 in the absence of phospholipase A_2 and two spots were found after phospholipase A_2 treatment (R_F = 0.59 ± 0.01 and 0.37 ± 0.01, duplicate determinations). (We also confirmed in a separate experiment that the presence of

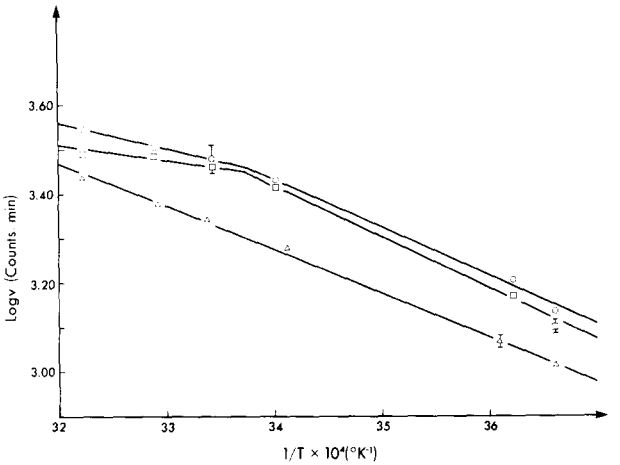

Fig. 3. Effect of $CaCl_2$ and EDTA on the reconstitution of the Arrhenius plot discontinuity. A 100 mg/ml solution of acetylcholinesterase was diluted to 0.25 mg/ml and homogenized in distilled water (○), 2 mM $CaCl_2$ (□) and 10 mM EDTA (△). Assays were performed 16 h after homogenization. Below the transition temperature (23°C) the energy of activation was 4.95 kcal/mol and 5.35 kcal/mol for the distilled water and $CaCl_2$-treated enzyme, respectively, and 2.16 kcal/mol and 1.52 kcal/mol above the transition temperature. In the EDTA-treated preparation the energy of activation was 4.54 kcal/mol. The bars indicate the range of duplicate determinations.

1.8 M NaCl and 2 mM sodium phosphate allowed a similar digestion of cardiolipin with R_F values of 0.65, 0.40 and 0.26.) The digestion products corresponded to triacyl-lysocardiolipin and diacyl-lysocardiolipin [13]. Only a single spot was obtained from acetylcholinesterase both in the absence of phospholipase A_2 ($R_F = 0.67$) or in its presence ($R_F = 0.71$). Thus the high-salt treatment did not appear to dissociate the cardiolipin from the enzyme sufficiently to alter its accessibility to phospholipase A_2 digestion.

Experiments were also made to see if cardiolipin could be separated from acetylcholinesterase incubated in high-salt and Ca^{2+}-chelating conditions by flotation in a sucrose gradient. No cardiolipin was detected in fractions at the

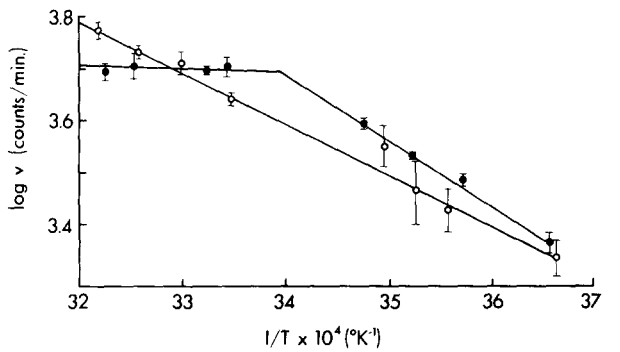

Fig. 4. Reversal of the Arrhenius plot break with time after treatment of acetylcholinesterase (0.5 mg) with 1 M $NaHCO_3$, pH 8. The $NaHCO_3$-treated enzyme was centrifuged in a sucrose gradient (see Materials and Methods) and assayed after 24 h (○) or 8 days (●). The bars represent the range of duplicate determinations (○) and S.D. (●). The energy of activation of the $NaHCO_3$-treated enzyme 24 h after centrifugation is 4.4 kcal/mol.

TABLE I

PARTIAL SPECIFIC VOLUME OF SIGMA ACETYLCHOLINESTERASE BEFORE AND AFTER TREATMENT WITH HIGH SALT IN PHOSPHATE

The number of determinations is shown in brackets. Method A: A partially preformed sucrose gradient in 2 mM sodium phosphate was made by layering 1.3 ml (measured by weight) of each of the following sucrose concentrations (M): 2.696, 1.604, 0.978, 0.624, 0.408, 0.276, 0.195, 0.140, 0.105, 0.083. The enzyme was layered on top (first result) or in layer 9 from the top (second result). Method B: The enzyme was layered on 2.696 M sucrose (approx. 69%) in 2 mM sodium phosphate (pH 7.4) and centrifuged for 66 h at 100 000 × *g*. The gradient was formed in the upper part of the tube. Method C: The enzyme was layered on 69% sucrose in water and centrifuged for 66 h at 100 000 × *g*. Method D: The gradient was formed as in method A but in 1.8 M sodium chloride in 2 mM sodium phosphate. The enzyme was placed in layer 9 from the top.

Enzyme treatment	Partial specific volume	
	Method	$\bar{v}$ (ml/g)
Untreated	A	0.81 (3) *
	B	0.78 (2)
		0.80 (2) **
	C	0.82 (1)
1.8 M NaCl + 2 mM sodium phosphate (pH 7.4)	A	0.79 (1)
	C	0.78 (1)
	C	0.81 (1)
	C	0.82 (1) ***
	D	0.83 (2)
	D	0.83 (1) ***

* In one experiment the sucrose gradient was prepared in the presence of 0.05 M NaCl and 2 mM sodium phosphate while the other two experiments were in 2 mM sodium phosphate.

** The Sigma enzyme was eluted from a Sepharose 6B column with 0.2 M NaCl and 2 mM sodium phosphate, pH 7.4.

*** The Sigma enzyme was eluted from a Sephadex G-200 column equilibrated with 1.8 M NaCl and 2 mM sodium phosphate, pH 7.4.

top of the sucrose gradient, suggesting that the cardiolipin had penetrated the gradient in association with the acetylcholinesterase.

Enzyme pretreated with 1.0 M $NaHCO_3$ (pH 8) and centrifuged in a sucrose gradient designed to allow the flotation of any released phospholipid towards the top of the tube showed a distinct break in the Arrhenius plot when assayed 5—8 days following its recovery from the gradient. A typical result is shown in Fig. 4. Between one and four days Arrhenius plots tended to be scattered, probably reflecting heterogeneous protein-lipid interactions during this period. It appeared that the initial disruption of the complex responsible for the Arrhenius plot break was slowly reversible even after the flotation procedure on the sucrose gradient. A similar variability in the Arrhenius plots was found after Sephadex G-200 chromatography, but a break in the Arrhenius plots was obtained on standing of the eluted enzyme. After various treatments the $\bar{v}$ of the enzyme incubated in high-salt and Ca^{2+}-chelating conditions was essentially unaltered when determined by four procedures. The $\bar{v}$ values 0.78—0.83 ml/g) (Table I) suggest that cardiolipin remained attached to the enzyme after high-salt and phosphate treatment, despite the apparent protein-lipid reorganization that seemed to occur under these conditions.

Discussion

Cardiolipin is the only phospholipid common to various lipoprotein forms of acetylcholinesterase which show a break in the Arrhenius plot at around 20°C. Conditions which abolished the break in the Arrhenius plot, namely high-salt treatment in sodium phosphate buffer, also allowed the extraction of cardiolipin from the enzyme by chloroform/methanol, whereas cardiolipin was not extracted before the high-salt treatment [5,6]. We have now investigated the conditions which abolish the Arrhenius plot break and the changes in protein-lipid interaction accompanying the disruption of the break.

The break in the Arrhenius plot could only be abolished in high salt (I = 1.8) when Ca^{2+}-chelating conditions were also present. Thus at I = 1.8 neither NaCl alone, $CaCl_2$ (pH 5.5) nor sodium phosphate at acidic pH disrupted the break. On the other hand at I = 1.8 NaCl in 2 mM sodium phosphate at pH 7.4, sodium phosphate (pH 8) or 1 M Na_2CO_3/$NaHCO_3$ (pH 8.5—10) effectively removed the break. When $CaCl_2$ was added back to a high-salt and phosphate-treated preparation with mild homogenization in the presence of endogenous cardiolipin, the Arrhenius plot break was regenerated, while EDTA prevented the regeneration. Taken together, the experiments showing the ability of Ca^{2+} to regenerate the break, failure of $CaCl_2$ at high ionic strength (I = 1.8) to disrupt the break and the ability of known Ca^{2+}-chelating conditions to disrupt the break, provided evidence that Ca^{2+} was involved in the modulation of the acetylcholinesterase-cardiolipin complex. The failure of EDTA to abolish the break in the presence of high salt was surprising, and may be due either to an inaccessibility of EDTA to the Ca^{2+} site due to its polarity or to its inability to remove Ca^{2+} irreversibly.

It is unclear from these studies whether both Ca^{2+} and cardiolipin cooperatively regulate the temperature-dependent modulation of acetylcholinesterase, or if Ca^{2+} alone is sufficient for the modulation process. The second alternative is less likely, unless the ability of high salt in Ca^{2+}-chelating conditions to increase the extractability of cardiolipin from the enzyme by chloroform/methanol [5,6] is coincidental and unrelated to the modulation mechanism. A number of studies have shown a direct involvement of phospholipid in Arrhenius plot breaks of membrane-bound enzymes [14—16], but phospholipid-independent protein conformation changes have also been observed [17, 18]. In the present case with erythrocyte acetylcholinesterase, if the break requires both Ca^{2+} and cardiolipin, it appears that cardiolipin is buried or otherwise inaccessible to phospholipase A_2 and C [5,6]. The Ca^{2+} may form a salt bridge between anionic sites of the enzyme and the phosphate groups of cardiolipin, or it may be required to maintain the cardiolipin in a particular conformation compatible with optimal enzyme activity [19—21]. Changes in temperature may modulate a local transition between conformations of a Ca^{2+}-cardiolipin complex or the extent of the protein-lipid interaction. The resulting changes in the protein conformation may be reflected by changes in the activation energy of the enzyme activity. The exact nature of the Ca^{2+}-dependent cardiolipin-enzyme changes that occur at the transition temperature at 20°C is unknown at present. Acyl chains of cardiolipin do not appear to undergo fluidity changes between 4°C and 40°C when cardiolipin is in the hexagonal

H_{II} phase [19], although the influence of adjacent protein or other lipids [22] is unknown. Cardiolipin undergoes cluster formation around 20°C [20], but there is no evidence on the biological significance of this transition. We showed previously that from 19 to 50 mol of cardiolipin are associated with 1 mol of acetylcholinesterase [6]. We have calculated that an average value of 31 molecules of cardiolipin can form a cylinder of length 3.36 nm, which could overlap with 80% of the length of the enzyme determined by hydrodynamic measurements [6].

Cardiolipin was not released from the enzyme in conditions where the Arrhenius plot was abolished. It was considered that if high-salt and Ca^{2+}-chelating conditions released cardiolipin from the enzyme it should become accessible to phospholipase A_2. However, the failure of phospholipase A_2 to digest endogenous cardiolipin in high-salt conditions, despite its ability to digest cardiolipin in control experiments in those conditions, suggest that cardiolipin remained bound to the enzyme. Cardiolipin was not separated from the enzyme after high salt treatment in Ca^{2+}-chelating conditions by flotation in sucrose gradients or by gel chromatography on Sephadex G-200. No cardiolipin could be detected at the top of the sucrose gradient. Furthermore, the break in the Arrhenius plot determined for the enzyme recovered from the sucrose gradients was gradually regenerated when the enzyme was allowed to stand for a few days, suggesting that the initial disruption of the Ca^{2+}-cardiolipin-acetylcholinesterase interaction was reversible. The values of the partial specific volume ($\bar{v}$) determined after attempts to separate the enzyme from cardiolipin in a variety of sucrose gradients and by gel chromatography were also characteristic of a lipoprotein, being higher than the values of 0.725 ml/g predicted from the amino acid composition of the delipidated form of the enzyme [23]. Thus after high-salt and Ca^{2+}-chelating conditions the cardiolipin appears to remain attached to the enzyme in a functionally altered form, possibly through hydrophobic interactions, allowing the ionic interactions to be regenerated with time. We are at present continuing attempts to investigate the effect of complete delipidation of acetylcholinesterase under non-denaturing conditions on its activity and kinetic properties.

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