THE ROLE OF TIGHTLY BOUND PHOSPHOLIPID IN THE ACTIVITY OF ERYTHROCYTE ACETYLCHOLINESTERASE

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SUMMARY

Acetylcholinesterase was extracted from bovine erythrocyte ghosts in a hypotonic (25 mOsm) phosphate buffer (pH 7.4) and solubilized with Lubrol WX (2 mg/ml). Solubilization resulted in a decrease of the partial specific volume of the acetylcholinesterase from 0.895 ml/g in the particulate fraction to 0.793 ml/g in the Lubrol WX solubilized fraction. Both the particulate fraction and the solubilized fractions gave Arrhenius plots with a discontinuity in the activation energy at 20°C. Phospholipase A_2 or C did not abolish the discontinuity. Treatment with 1.77 M NaCl resulted in a linear Arrhenius plot of intermediate activation energy. The high salt treatment removed phospholipid not previously extracted by chloroform/methanol. The extracted phospholipid corresponded to cardiolipin on one and two dimensional silica gel chromatography. Addition of cardiolipin to a high salt-treated preparation of acetylcholinesterase restored the discontinuity in the Arrhenius plot. It is concluded that the physical state of cardiolipin, tightly bound to acetylcholinesterase by ionic interactions, modulates the catalytic activity of acetylcholinesterase.

INTRODUCTION

Erythrocyte acetylcholinesterase (acetylcholine hydrolase EC 3.1.1.7), which catalyzes acetylcholine hydrolysis on the outer surface of the cell membrane, has been considered a "peripheral" protein (1, 2) as it can be solubilized from erythrocyte ghosts by mild ionic strength treatments (2, 3). It has been suggested recently that human erythrocyte acetylcholinesterase may be more firmly embedded in the membrane than previously thought, as it cannot be dissociated from intact human erythrocytes by high ionic strength (4). Furthermore, certain allosteric properties of the enzyme are sensitive to changes in the fluidity of lipids in the rat erythrocyte membrane (5, 6). Acetylcholinesterases released from bovine (3) and human (2) erythrocytes have densities characteristic of lipoproteins. Recently it was shown that

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human erythrocyte acetylcholinesterase activity was decreased by lipid depletion and was restored by addition of phospholipid (7). In the present study we report that tightly bound phospholipid modulates the temperature dependence of bovine erythrocyte acetylcholinesterase activity. The lipid was extracted by chloroform/methanol after 1 M NH_4OH or high NaCl treatment and migrates similarly to cardiolipin in one and two dimensional thin layer silica gel chromatography.

MATERIALS AND METHODS

Extraction of acetylcholinesterase

Packed cells from bovine blood obtained from a local slaughterhouse were washed twice in isotonic saline and hemolysed twice in 7 volumes of 8.93 mM (25 mOsm) sodium phosphate buffer (pH 7.4). Ghosts harvested at 20,000 x g for 10 min were incubated for 48 hours at 5°C in the same phosphate buffer. The activity in the supernatant from a 20,000 x g centrifugation was pelleted at 300,000 x g average hour. The pellet is referred to as the particulate fraction and contains over 90% of the extracted activity.

Lubrol WX solubilization

Particulate fractions of acetylcholinesterase were incubated with Lubrol WX (2 mg/ml) in 2 mM sodium phosphate buffer (pH 7.4) overnight at 5°C. (This concentration of Lubrol WX was shown to be the minimum required for solubilization of the enzyme with optimal retention of activity). Following 8000 x g centrifugation for 10 min (to remove large Lubrol micelles, devoid of activity) the supernatant was centrifuged at 300,000 x g average hour at 5°C. In a typical experiment 84% of the activity remained in the supernatant fraction. The supernatant was dialyzed against 2 mM sodium phosphate (pH 7.4) for 16 hours at 5°C. The enzyme was adsorbed to pre-swollen D.E.A.E.-Sephadex and the gel washed with 250 ml of cold distilled water on a scinterred glass funnel. The enzyme was eluted with 0.5 M NaCl (100 ml) in 2 mM phosphate buffer and concentrated to about 5 ml by ultrafiltration through an Amicon PM 10 membrane. 90% of the acetylcholinesterase activity was recovered during this process. This enzyme fraction was used for kinetic studies and the determination of physical parameters. For phospholipid analysis the procedure was modified for large scale purification. Following solubilization with Lubrol WX the extracted phospholipids and excess Lubrol WX were separated by layering the soluble enzyme fraction on a similar volume of 20% sucrose and centrifuging for 66 hours at 100,000 x g. The protein migrated into the sucrose and was separated from the bulk of the detergent and lipid. The procedure resulted in a 46.3% pure preparation, based on the turnover number reported by Berman (11).

Phospholipid analysis

Lipids were extracted from intact bovine erythrocytes, particulate fraction and Lubrol WX solubilized fraction with chloroform/methanol according to Reed *et al.* (8). The extract was concentrated by vacuum rotary evaporation and chromatographed in one dimension on pre-coated Silica gel 60F-254 20 x 20 cm sheets (EM Labs. Inc., distributed by Brinkman Instruments) with chloroform 14, methanol 6, 30% ammonium hydroxide 1 (v/v). Phospholipid standards were

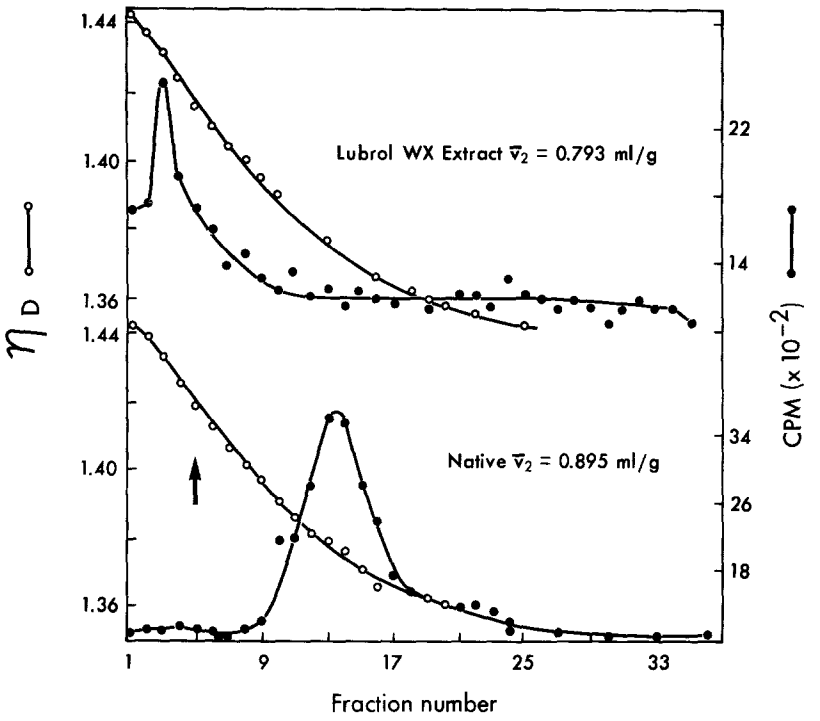

Figure 1. Equilibrium sedimentation of the particulate fraction (bottom) and the Lubrol WX extract (top). Acetylcholinesterase activity is shown on the right ordinate and the refractive index of the sucrose gradient on the left ordinate. The enzyme in both experiments was applied to the preformed sucrose gradient where indicated by the arrow.

run on the same sheet. Two dimensional thin-layer chromatography was performed on 20 x 20 cm Redi-Coat (Supelco, Inc.) glass plates pre-run in both dimensions with each solvent system and dried at 110°C before use. Two plates (one with standard cardiolipin and one with unknown phospholipid extract) were run simultaneously in the same chamber with chloroform 65, methanol 25, 30% ammonium hydroxide 5 (v/v) in the first dimension and chloroform 3, acetone 4, methanol 1, acetic acid 1, water 0.5 (v/v) in the second dimension. Spots were developed with Rhodamine 6G (Supelco, Inc.) and iodine.

Acetylcholinesterase activity

Acetylcholinesterase was assayed by a radiometric assay in the presence of 0.1 M sodium phosphate buffer (pH 7.4), as previously described (9). Blanks without enzyme were run at each temperature investigated. Enzyme concentrations were adjusted to give net radioactive counts (CPM) between 1800 and 6000 for maximum accuracy, and hence activities are reported as net CPM.

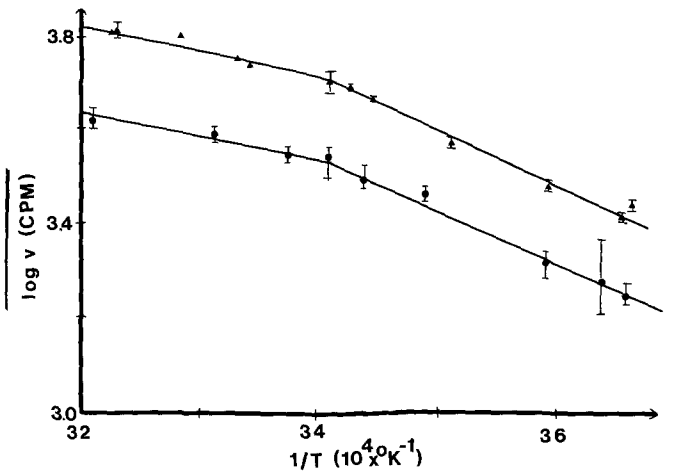

Figure 2. Arrhenius plots of the particulate fraction (●) and the Lubrol WX extract (▲) of acetylcholinesterase. In both cases the break in the Arrhenius plot occurred at 20°C. Energies of activation above and below the transition temperature were 2.2 and 5.1 kcal/mol for the particulate fraction and 2.5 and 5.4 kcal/mol for the Lubrol WX extract. The bars represent the range of two to four separate determinations.

Phospholipase digestion

Purified phospholipase C and A_2 were generously supplied by Drs. Roelofsen and Van Deenen, from Bacillus cereus and porcine pancreas, respectively (10). The Lubrol WX extract was incubated with phospholipase C (16 Units) and phospholipase A_2 (100 Units) for 2 hours at 37°C in the presence of 5% bovine serum albumin, 0.1 M KCl and 4 mM $CaCl_2$ in a histidine buffer (pH 7.4). Other experiments (not shown) were made with each of the phospholipases alone, and with sphingomyelinase also supplied by Dr. Roelofsen.

Reconstitution with phospholipid

Commercial bovine erythrocyte acetylcholinesterase (Sigma Chemical Co.) (.25 mg/ml) was treated with 1.77 M NaCl and dialyzed against water. Cardiolipin (1 mg) and solid $CaCl_2$ (2 mM) were added to salt-treated enzyme and the mixture homogenized ten times for 12 sec intervals (15 sec apart) at speed 8 in a Sorvall Omni-Mix homogenizer in ice. The mixture was kept at 4-6°C for 16 hours, then dialyzed against water and assayed.

Materials

Lubrol WX and bovine serum albumin were from Sigma Chemical Co. Bovine mitochondrial cardiolipin, bovinephosphatidylcholine, phosphatidylserine and phosphatidyl ethanolamine, plant phosphatidylinositol and DL-dipalmitoyl-phosphatidic acid were from Applied Science Res. Labs. Inorganic salts and solvents were Analytical Reagent grade.

RESULTS AND DISCUSSION

Equilibrium sedimentation of the particulate fraction of acetylcolinesterase on a sucrose gradient yielded a partial specific volume ($\bar{v}_2$) of 0.895 ml/g (Fig. 1). The enzyme activity was associated with large particles excluded from a Sepharose 6B column (not shown). Solubilization of the acetylcholinesterase activity with Lubrol WX (2 mg/ml) resulted in a decrease of the $\bar{v}_2$ to 0.793 ml/g (Fig. 1). Both the particulate fraction and the Lubrol WX solubilized extract exhibited non-linear Arrhenius plots, with a break in the activation energies occurring at around 20°C (Fig. 2). The finding that Lubrol WX extraction did not abolish the break in the Arrhenius plot suggests that some phospholipid remains associated with the solubilized enzyme. The higher $\bar{v}_2$ than expected from the amino acid composition of the enzyme (11) is consistent with this interpretation. Thermal transitions around 20°C in mammalian membrane bound enzymes have been widely interpreted to reflect changes in the fluidity of lipids modulating catalytic activity (12, 13). Our results showing a thermal transition with the Lubrol solubilized enzyme indicates that modulation of catalytic activity of acetylcholinesterase results from fluidity changes in immobilized lipids rather than bulk lipids (14).

Treatment of the particulate fraction with 1.77 M NaCl resulted in a linear Arrhenius plot with an activation energy (4.9 kcal/mol) intermediate between the high and low energies obtained before treatment (Fig. 3). The linear Arrhenius plot suggests that the specific protein-lipid coupling responsible for modulation of catalytic activity was altered by this treatment. This was confirmed by phospholipid analysis. The particulate fraction contained all of the phospholipids identifiable in the bovine erythrocyte membrane following chloroform/methanol extraction and one dimensional chromatography, namely, phophatidylethanolamine, phosphatidylcholine, sphingomyelin, phosphatidylserine plus phosphatidylinositol (Table 1). Chloroform/methanol extraction of the Lubrol WX solubilized fraction of acetylcholinesterase

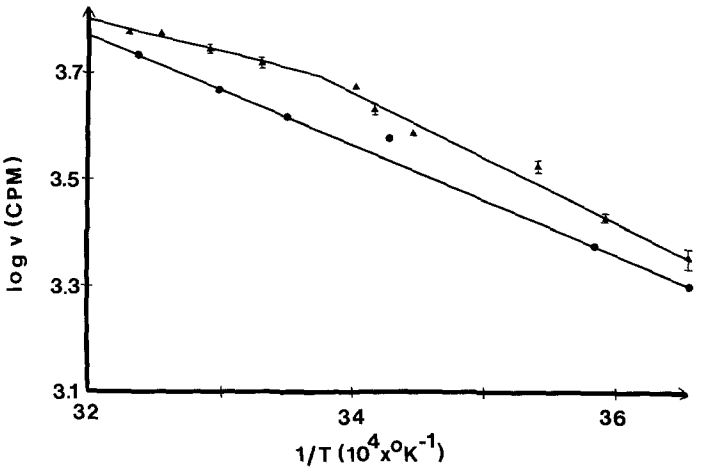

Figure 3. Arrhenius plots of the Lubrol WX extract after treatment with phospholipases A_2 + C (▲) and the particulate fraction treated with 1.77 M NaCl (●) (see Methods). Energies of activation for the Lubrol WX treated enzyme were 2.7 and 5.1 kcal/mol above and below the transition temperature (22.7°C) and 4.9 kcal/mol for the NaCl treated particulate fraction. The bars represent the range of two determinations in the phospholipase A_2 + C treated extract. The NaCl treated enzyme represents a typical single experiment.

prepared on a large scale to detect smaller quantities of minor phospholipids resulted in removal of phospholipid only when 1 M NH_4OH (Table 1) or >1 M NaCl (not shown) is present. The phospholipid removed under these conditions had an R_f corresponding to that of cardiolipin. This was confirmed by two dimensional thin layer chromatography. Phospholipid extracted from a commercial preparation of bovine erythrocyte acetylcholinesterase by chloroform 1, methanol 1 (v/v) in the presence of 2.0 M NaCl (following three initial extractions with chloroform/methanol in the absence of salt) in duplicate experiments gave an R_f of 0.48, 0.62 in the first dimension and 0.74, 0.66 in the second dimension. This coincides with the R_f found for cardiolipin standards, namely 0.52, 0.55 and 0.73, 0.73, respectively, especially considering the difference in acyl fatty acid chains of the cardiolipin from the different sources.

Treatment of acetylcholinesterase with both phospholipase C and A_2 did

Table 1.

Phospholipid composition of various fractions of bovine erythrocyte acetylcholinesterase.

Phospholipid	R_f					
	Standards[a]	Particulate Fraction	Standards	Lubrol WX Extract ($-NH_4OH$)	Standards	Lubrol WX Extract $+NH_4OH$ (1M)[b]
Phosphatidyl-inositol		0.100	0.081	-[d]		-
Phosphatidyl-serine	0.181	0.181	0.132	-		-
Sphingomyelin	0.259	0.253		-		-
Phosphatidyl-choline	0.464	0.458	0.331	-		-
Phosphatidyl-ethanolamine[c]	0.560	0.554	0.438	-	0.500	-
Phosphatidic acid[c]		-	0.472	-		-
Cardiolipin		-	0.566	-	0.596	0.578

a. Phospholipid standards were applied separately on the same thin layer chromatogram for each of the three experiments shown.

b. The Lubrol WX extract was treated with 1M NH_4OH prior to $CHCl_3$/MeOH extractio of lipids (see Methods).

c. Not resolvable with this chromatographic system.

d. Indicates none detected.

not abolish the temperature-dependent lipid modulation of catalytic activity (Fig. 3). This could be due to the inaccessibility of the lipids tightly bound to the enzyme, as cardiolipin is a substrate for both enzymes (personal communication from Dr. Roelofsen). Sphingomyelinase was also ineffective (not shown). A commercial preparation of bovine erythrocyte acetylcholinesterase also showed a discontinuous temperature dependence which became

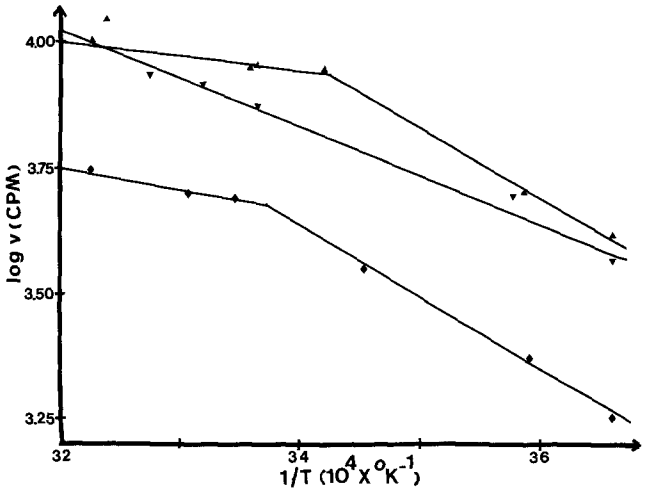

Figure 4. Reconstitution of the thermal discontinuity by addition of cardiolipin to high salt-treated acetylcholinesterase. Sigma acetylcholinesterase (0.25 mg/ml) was assayed without treatment (▲), after treatment with 1.77 M NaCl (▼) and following reconstitution of the salt-treated enzyme with cardiolipin and $CaCl_2$ (◆) (see Methods). Energies of activation for the untreated enzyme were 1.5 and 6.3 kcal/mol above and below the transition temperature (19°C), 4.2 kcal/mol for the salt treated enzyme and 2.0 and 6.8 kcal/mol (23.3°C) after addition of cardiolipin.

linear after high salt treatment of the enzyme preparation (Fig. 4). Addition of cardiolipin to the salt treated preparation restored the discontinuity in the Arrhenius plot (Fig. 4). (Dialysis alone following high salt treatment did not affect the linear Arrhenius plot). This result is consistent with the suggestion that cardiolipin is the phospholipid endogenously associated with erythrocyte acetylcholinesterase and modulates the thermal discontinuity in the enzyme activity. The slightly higher transition temperature (23.3°C) may reflect differences in the acyl chain composition between the endogenous and applied cardiolipin.

We conclude that lipid, probably cardiolipin, is tightly bound to bovine erythrocyte acetylcholinesterase by predominantly ionic forces. Changes in fluidity of this bound lipid appear to modulate the catalytic activity of the enzyme. From the decrease in molecular weight following

1.8 M NaCl treatment we concluded that 30 molecules of cardiolipin are associated with the acetylcholinesterase dimer (unpublished observation). Immobilized phospholipid that requires ammonium hydroxide for its extraction has also been described for cytochrome oxidase (14). A minimum number of "annulus" lipids is required for the activity of the (Mg^{2+} + Ca^{2+})-ATPase Ca^{2+} transport protein (15, 16). Phosphatidylserine has been found to activate lipid-depleted human erythrocyte acetylcholinesterase, but the activity of other phospholipids has not been reported (7). We have found that a variety of lipids tested can substitute for cardiolipin to regenerate the break (unpublished observation). We are at present investigating the influence of cardiolipin in acetylcholinesterase activity and function.

Acknowledgement

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