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EFFECT OF LIGANDS ON A DISCONTINUOUS TEMPERATURE DEPENDENCE OF THE DECARBAMYLATION REACTION OF ERYTHROCYTE ACETYLCHOLINESTERASE

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SUMMARY

1. The temperature dependence of the decarbamylation of dimethylcarbamylated bovine erythrocyte acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) shows discontinuities when this reaction is studied in the absence of added ions or other ligands.

2. It is suggested that the carbamylated enzyme can exist in at least two temperature-dependent states, a low temperature state below 20 °C (characterized by an activation enthalpy of 13–18 kcal/mole and an activation entropy of −34 to −16 e.u.) and a high temperature state above 20 °C (7.2 kcal/mole and −54 e.u.).

3. The transition between the two states could occur through an intermediate state of low catalytic activity at around 15 °C.

4. Ligands such as tetramethylammonium and possibly acetylcholine probably stabilize a high temperature state of the enzyme, whereas gallamine and $MgCl_2$ probably stabilize a low temperature state of the enzyme, as indicated by linear Arrhenius plots in the presence of these ligands.

5. At concentrations which bind predominantly at allosteric sites, tetraethylammonium, NaCl, KCl and $CaCl_2$ probably bind to multiple forms of the carbamylated enzyme, as indicated by discontinuous Arrhenius plots in their presence.

6. The discontinuous temperature dependence of the decarbamylation reaction does not appear to be related either to gross changes in state of enzyme aggregation, the presence of isoenzymes or to changes in state of phospholipids in the preparation.

INTRODUCTION

Deacylation reactions of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from a variety of sources are accelerated by nondepolarizing neuromuscular blocking quaternary ammonium compounds[1,2] and inorganic cations[2–4],

Abbreviations: TMA, tetramethylammonium iodide; TEA, tetraethylammonium iodide.

but not by depolarizing agents[1-4]. The molecular mechanisms of activation and inhibition of the enzyme are of particular interest since they may shed light on the mechanism of their pharmacodynamic properties on cholinergic receptors[5]. Previous studies suggested that activators may bind to allosteric sites on acetylcholinesterase[1,2,6]. This was confirmed in a recent study from our laboratory by the use of a water-soluble carbodiimide to irreversibly abolish activation of the enzyme without affecting acetylcholine hydrolysis[7].

In the present report the effect of temperature on the decarbamylation of dimethylcarbamylated acetylcholinesterase, and on the activation of this reaction by ligands, is described. Discontinuities in the temperature dependence of the decarbamylation reaction have been observed, and the effect of ligands on these discontinuities is reported. The existence of different temperature and ligand dependent states of the enzyme has been suggested to account for the results. An effect of temperature on the relative concentrations of multiple forms of erythrocyte and serum cholinesterase has also been suggested by Main[8].

EXPERIMENTAL

Materials

The source of acetylcholinesterase was a partially purified bovine erythrocyte preparation from Nutritional Biochemicals. At low ionic strength the enzyme had a specific activity of 150 μmoles of acetylcholine hydrolyzed/h per mg of protein, and a K_m value of 27 μM.

o-Nitrophenoldimethylcarbamate was prepared as previously described[4]. Inorganic ions were of analytical reagent grade. Tetramethylammonium (TMA) iodide and tetraethylammonium (TEA) iodide (Baker) and acetylcholine perchlorate (British Drug Houses) were used as received. Gallamine triethiodide was a gift from Poulenc, Ltd. Phospholipase C was obtained from Sigma and partially purified phospholipase A was a gift from Dr D. Godin, University of British Columbia. Acetyl-1-[^{14}C]choline iodide (New England Nuclear) had a specific activity of 4.53 Ci/mole. Cation exchange resin CGC 241, Na^+ form, 200–400 mesh (reagent grade) was from Baker.

Decarbamylation measurements

The procedure for determining decarbamylation rates has been described previously[4], but was modified slightly in this study in order to provide an internal control. Essentially 0.04 ml of ethanol (0.05%) was added to the enzyme (4 or 8 mg) and the mixture was incubated for 1 h at 25 °C. An aliquot (10 μl) was diluted to 20 ml in glass-distilled water or a solution of the ligand under investigation. The pH was maintained at 7.40 $\pm$ 0.05 in a jacketed vessel at the required temperature, with mild stirring and under a blanket of nitrogen. Aliquots (2.0 ml) were removed and assayed titrimetrically for enzyme activity toward 1.0 mM acetylcholine in a second jacketed vessel, as previously described[4,6]. This procedure gave the control activity of the enzyme. To the remaining enzyme reaction mixture (30 μl) was added 30 μl of a solution of *o*-nitrophenoldimethylcarbamate in 0.05% ethanol to give a final carbamate concentration of 0.163 mM or in some cases, 0.082 mM. This reaction mixture was incubated for 1 h at 25 °C. An aliquot (50 μl) was diluted to 50 ml with

the required reactivation medium and the procedure described above was repeated. The 1000-fold dilution of the free carbamate effectively terminated its inhibitory action. Aliquots were removed at various time intervals and were assayed titrimetrically for remaining acetylcholinesterase activity, until at least 50% reactivation of the dimethylcarbamylated enzyme was achieved. Rates of decarbamylation were studied over a temperature range 5 to 30 °C.

Acrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed essentially as described by Raymond[9], in the E–C continuous vertical gel slab electrophoresis system, with a 5% running gel and 5 mM Tris–glycine buffer (pH 8.6). 20 μg of protein was applied and run for 60 min at 40 mA. The required temperature in the gel, measured by a thermistor probe embedded in the area of the gel where the enzyme was known to run, was maintained by water circulating at a constant temperature. Runs were made at 15 and 20 °C. General protein staining was achieved with Naphthol Blue Black reagent. Acetylcholinesterase activity was determined by slicing frozen gel strips with a Mickle gel slicer in 1-mm sections. These sections were placed in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) at 0 °C for 12 h. Aliquots (50 μl) were assayed for acetylcholinesterase activity by a modification of the radiometric assay of Berg and Maickel[10] (see Fig. 3).

Phospholipase digestion

After dialysis against 0.1 M Tris–HCl buffer (pH 7.8), 5 mg of acetylcholinesterase preparation was incubated with 0.5 mg of phospholipase A at 37 °C or 0.2 mg of phospholipase C at 25 °C for 2 h in the presence of bovine serum albumin (0.2%), $CaCl_2$ (5 mM) and 50 mM Tris–HCl buffer (pH 7.8), in a final volume of 2.0 ml. The reaction was stopped by the addition of EDTA to a final concentration of 50 mM, followed by dialysis for 16 h against 0.1 M Tris–HCl buffer. The samples were then tested for phosphate content by the method of Bartlett[11], for acetylcholinesterase activity by the modified radiometric assay (Fig. 3) and for electrophoretic mobility on acrylamide as described above.

RESULTS

The decarbamylation of dimethylcarbamylacetylcholinesterase followed apparent first order kinetics for a least one-half life under all conditions studied (see also ref. 4). The effect of temperatures from 5 to 30 °C on this reaction in the absence of added ions and in the presecne of $MgCl_2$ (0.04 M) and TEA (0.5 mM) is shown in Fig. 1. The following equation from transition rate theory relates the rate constants for decarbamylation to temperature[12].

$$\log k/T = \frac{-\Delta H^{\ddagger}}{2.3\,RT} + \frac{\Delta S^{\ddagger}}{2.3\,R} + \frac{\log R}{nh}$$

where k = apparent first order decarbamylation rate constant, and h = Planck constant.

This equation predicts a linear relationship between $\log k/T$ and $1/T$, the slope allowing calculation of the enthalpy of activation ($\Delta H^{\ddagger}$) and the intercept the

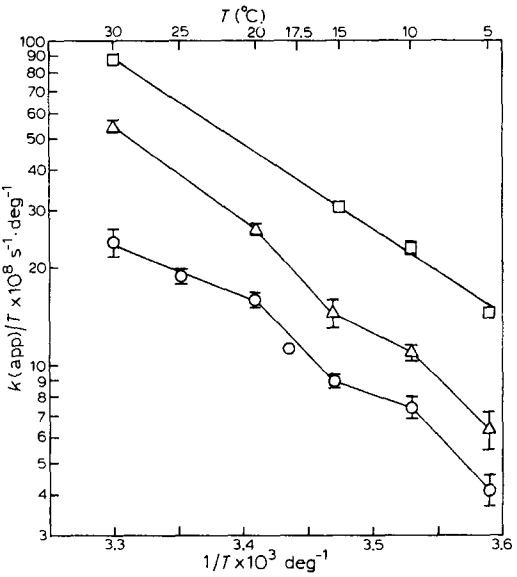

Fig. 1. Temperature dependence of decarbamylation of dimethylcarbamylated acetylcholinesterase. The decarbamylation rate was measured as described in Experimental. Where bars are shown the data represents the mean ± S.E. of from two to five determinations, usually three. ○—○, control (no added ions); △—△, 0.5 mM TEA; □—□, 0.04 M $MgCl_2$.

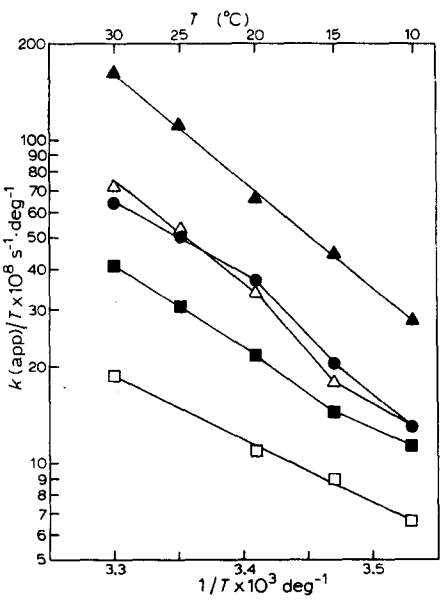

Fig. 2. Temperature dependence of decarbamylation of dimethylcarbamylated acetylcholinesterase. Conditions as described in Fig. 1. □—□, 0.5 mM TMA; ■—■, 0.2 mM $CaCl_2$; △—△, 50 mM NaCl; ●—●, 50 mM KCl; ▲—▲, 10 μM gallamine triethiodide.

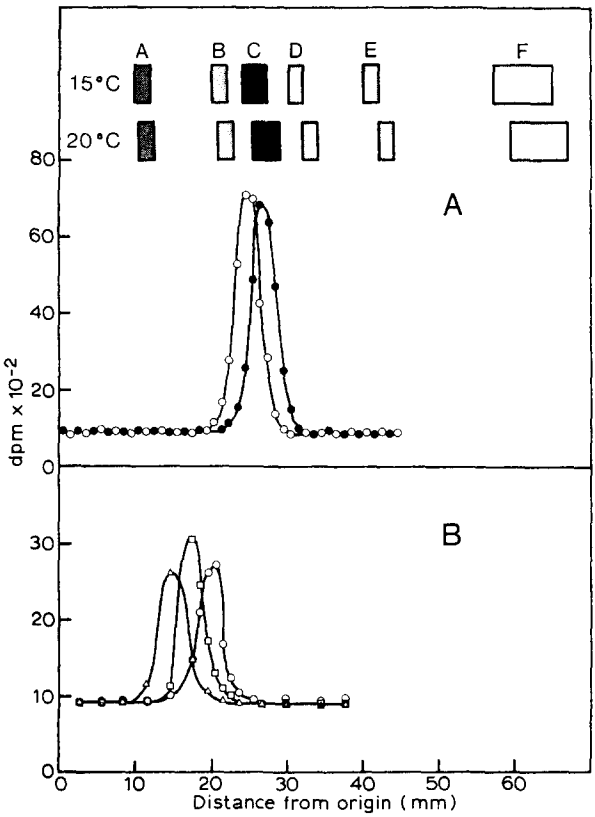

Fig. 3. Acrylamide gel electrophoresis of acetylcholinesterase. Electrophoresis was performed on 5 % acrylamide gel as described in Experimental. Protein staining by Napthol Blue Black reagent gave protein bands A and C when 20 μg protein were applied; A, B and C with 80 μg protein; and A to F with 800 μg protein. Acetylcholinesterase activity in the gel slices was determined by a modification of a radiometric assay described by Berg and Maickel[10], as follows. After incubation of the gel slice in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) at 0–4 °C for 12 h, a 50-μl aliquot of the medium was added to 0.15 ml of 0.1 M phosphate buffer (pH 7.4). The reaction was started by addition of 20 μl of a mixture of acetyl-1-[^{14}C]choline iodide and acetylcholine perchlorate to a final total concentration of 1 mM acetylcholine containing 19.9 nCi per sample. After incubation for 30 min at 25 °C the reaction was stopped by addition of 0.2 ml abs. ethanol. To each sample was added 1 ml of a slurry of cation exchange resin, Na^+ form, containing approximately 0.35 g of resin. Samples were mixed thoroughly by inversion and centrifuged briefly in an International Model HN laboratory centrifuge. An aliquot (0.5 ml) was added to 10 ml of Bray's[22] solution and counted in a refrigerated Picker Liquimat scintillation counter at an average efficiency of 88%, using the Picker external standardization system. A count of 850 dpm was obtained in the absence of enzymic hydrolysis. (A) ○—○, 15 °C; ●—●, 20 °C. (B) ○—○, control; □—□, phospholipase A-treated; △—△, phospholipase C-treated, at 15 °C.

entropy of activation (ΔS^*). In the absence of added ions breaks in the relationship of temperature to the reaction rate were obtained in the control plot (Fig. 1). Decarbamylation rates which were lower than predicted occurred around 15 °C and above 20 °C. In the presence of TEA (0.5 mM), which accelerated the decarbamylation rate about 2-fold, the discontinuity at 15 °C was still present (Fig. 1). In contrast, $MgCl_2$ (0.04 M), which accelerated the decarbamylation reaction about 4-fold, abolished the discontinuities. In this case the Arrhenius-type plot was linear over the temperature range 5 to 30 °C.

The temperature was also varied from 10 to 30 °C in the presence of $CaCl_2$ (0.2 mM), NaCl (50 mM), KCl (50 mM), gallamine triethiodide (10 μM) and TMA (0.5 mM). The results are shown in Fig. 2. All ligands except TMA accelerated the decarbamylation reaction from 1.5- to 4-fold. TMA slightly inhibited the decarbamylation reaction, particularly at the higher temperatures. Again the plots in the presence of $CaCl_2$, NaCl and KCl suggest discontinuities around 15 °C, similar to the control plot in Fig. 1. Discontinuities at 15 °C are not evident in the presence of gallamine or TMA, the Arrhenius-type plots being linear in their presence.

Acrylamide gel electrophoresis and phospholipase digestion

At both 15 and 20 °C electrophoresis of acetylcholinesterase gave a single sharp band of enzyme activity on 5% acrylamide gel, as shown in Fig. 3A. Similar results were also obtained on 7.5% acrylamide gels. Whereas Grafius *et al.*[13] found evidence of a proteolipid matrix in a fast component (70 S) of eel acetylcholinesterase, the purified preparation of acetylcholinesterase used in this study was found to contain only a small quantity of total phosphate (equivalent to less than 4.3 μg of phospholipid per mg of protein), and this was not decreased further by digestion with phospholipase C for 2 h. Neither the enzymic activity toward acetylcholine nor the electrophoretic pattern after phospholipase A or C digestion were affected in the manner expected from breakdown of a proteolipid complex (Fig. 3B). The slight retardation of mobility after phospholipase treatment is possibly due to loss of negative charge produced by neuraminidase and/or proteolytic activity present in the phospholipase preparations.

Kinetics of acetylcholine hydrolysis

The effect of the various ligands on the kinetics of acetylcholine hydrolysis at 10 °C were investigated at the same concentrations used in the decarbamylation studies. In Table I are shown the effects of the ligands on both the maximum velocity

TABLE I

EFFECT OF LIGANDS ON ACETYLCHOLINE HYDROLYSIS AT 10 °C

Acetylcholine hydrolysis was determined titrimetrically at 10 °C as described previously[6]. The assays were performed at acetylcholine perchlorate concentrations varied from 0.05 mM to 0.4 mM at pH 7.4, in the absence of added buffers or salts other than those ligands being investigated. V and apparent K_m for acetylcholine hydrolysis were determined by least squares analysis of data in the form of v *vs* $v/[s]$. The acetylcholinesterase had half the specific activity of that used in the previous study[6], but the K_m for acetylcholine was similar.

Ligand (mM)	*Kinetics of acetylcholine hydrolysis*	
	V (μmoles/mg per h)	*Apparent K_m (μM)*
Control	41	20.0
TMA (0.5)	40	35.7
TEA (0.5)	57	108.2
$CaCl_2$ (0.2)	68	21.1
NaCl (50)	83	78.1
KCl (50)	90	133.9
$MgCl_2$ (40)	120	318.2
Gallamine (0.01)	47	483.6

and apparent K_m of acetylcholine hydrolysis. These results were similar to data obtained previously at 25 °C (ref. 6).

DISCUSSION

Analysis of discontinuities

Enthalpies and entropies of decarbamylation of dimethylcarbamylacetylcholinesterase were calculated from the linear segments of the discontinuous Arrhenius-type plots of Fig. 1 and Fig. 2. The results are shown in Table II. Also included are thermodynamic parameters calculated over the whole temperature range (5 to 30 °C or 10 to 30 °C, as applicable). These average values were obtained by ignoring discontinuities when these occurred and applying a least squares fit to the data. This was done to allow easier comparison of overall effects of ligands on the thermodynamic parameters for the reaction under investigation.

The discontinuity in the control plot at 15 °C is characterized by a decrease in activation enthalpy of the reaction from 18 kcal/mole in the 5 to 10 °C range to 6.2 kcal/mole near the transition temperature (10 to 15 °C), and an unfavorable decrease in activation entropy from −16 e.u. to −58 e.u. A low enthalpy (7.2 kcal/mole) and a large negative entropy (−54 e.u.) also characterizes the control reactions at temperatures above 20 °C. The considerable changes in activation enthalpy and entropy characterizing the discontinuities in this study are consistent with a temperature dependent configurational transition between two enzyme states[14]. Similar discontinuities have been shown in a surprisingly large number of enzymes (see ref. 14), but generally such discontinuities are characterized by a single sharp break between two configurational states having a large difference in activation enthalpies (70–130 kcal/mole). Our data on acetylcholinesterase is more consistent with a mechanism whereby the transition between two states of the enzyme occurs *via* a third, less catalytically active state of the enzyme at around 15 °C (Fig. 1). Making

TABLE II

THERMODYNAMIC PARAMETERS FOR THE DECARBAMYLATION OF ACETYLCHOLINESTERASE

The activation enthalpies ($\Delta H^{\ddagger}$) and entropies ($\Delta S^{\ddagger}$) were calculated from linear segments of the Arrhenius-type plots shown in Figs 1 and 2. The values in the 5–30 °C column were obtained by least squares fit of data in Fig. 1 by ignoring discontinuities when these occurred, and the values in the 10–30 °C column were similarly obtained from Fig. 2. $\Delta H^{\ddagger}$ is expressed in kcal/mole and $\Delta S^{\ddagger}$ in entropy units (e.u.).

Ligand (mM)	*Temperature range (°C)*											
	5–10		*10–15*		*15–20*		*20–30*		*5–30*		*10–30*	
	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
Control	18.0	−16	6.2	−58	19.1	−13	7.2	−54	13.2*	−34		
TMA (0.5)											8.7	−49
$MgCl_2$ (40)									11.8	−36		
Gallamine (0.01)											13.9	−28
TEA (0.5)	14.9	−27	9.3	−47	19.9	−9	12.9	−34	14.4	−29		
$CaCl_2$ (0.2)			7.9	−51	13.6	−31	11.3	−39			11.4	−39
NaCl (50)			10.2	−43	21.3	−4	13.6	−31			15.4	−24
KCl (50)			13.4	−32	20.0	−8	9.6	−44			13.7	−30

* 5–20 °C.

this assumption allows calculation of the activation enthalpies and entropies of the states of the enzyme above and below the transition temperature (Table II) namely, 13 to 18 kcal/mole and −34 to −16 e.u. for the low temperature form (5 to 20 °C) and 7.2 kcal/mole and −54 e.u. for the high temperature form (20 to 30 °C). The existence of two reversible configurational forms of acetylcholinesterase (P and D) was first suggested by Changeux[1]. A number of authors have used a two-state model to analyze kinetic data for the binding of neuromuscluar blocking agents[15] and atropine[16] to the enzyme. It is also of interest that a recent theoretical analysis of ligand effects on the nicotinic cholinergic receptor is consistent with models suggesting the presence of two ligand-dependent configurational states of the cholinergic receptor[17].

Effect of ligands

The ligands investigated in this study, with the exception of TMA, accelerated the decarbamylation reaction from 2- to 5-fold (Fig. 1 and Fig. 2). There is no simple quantitative correlation between the degree of activation and the effect of the ligands on the overall enthalpy or entropy of the reaction (Table II).

The Arrhenius-type plots are linear in the presence of $MgCl_2$ (0.04 M), gallamine (10 μM) and TMA (0.5 mM) (Figs 1 and 2). No discontinuities in the temperature dependence of the hydrolysis of acetylcholine were found in a study at high ionic strength by Wilson and Cabib[18], and the same result was obtained by us in a similar study at low ionic strength. We interpret this to mean that substrate would also protect against discontinuities in the Arrhenius plots. If it is assumed that the discontinuities are due to the existence of at least two states of the carbamylated enzyme, as discussed above, it follows that $MgCl_2$, gallamine, TMA, and possibly substrate preferentially bind to one form of the enzyme, which in this way is stabilized over a wide range of temperature. The activation enthalpies and entropies of the decarbamylation reaction in the presence of 0.5 mM TMA are 8.7 kcal/mole and −49 e.u., respectively. TMA is a depolarizing agent[1] and does not accelerate either the decarbamylation reaction (Fig. 2) or acetylcholine hydrolysis at 10 °C (Table I). Hence we propose that TMA (and possibly substrate) stabilize an entropically unfavorable high-temperature form of the enzyme (D in the terminology of Changeux[1]), which is also characterized by similar thermodynamic parameters (7.2 kcal/mole and −54 e.u.). On the other hand activation enthalpies and entropies of the decarbamylation reaction in the presence of gallamine (10 μM) and $MgCl_2$ (0.04 M) are 14 kcal/mole and −28 e.u. and 12 kcal/mole and −36 e.u., respectively. $MgCl_2$ and gallamine are potent accelerators of the decarbamylation reaction (Figs 1 and 2), and in contrast to TMA, gallamine is a nondepolarizing neuromuscular blocking agent. Hence we propose that $MgCl_2$ and gallamine stabilize a less ordered form of the enzyme, possibly the low-temperature form (P in the terminology of Changeux[1]), which is also characterized by similar thermodynamic parameters (13 to 18 kcal/mole and −34 to −16 e.u.). A similar temperature stabilization of D-amino acid oxidase by D-methionine and fumarase by L-malate was found by Massey *et al.*[14].

In contrast to the ligands discussed above, TEA (0.5 mM), $CaCl_2$ (0.2 mM), NaCl (50 mM) and KCl (50 mM) did not completely abolish the discontinuous temperature dependence at 15 °C. At the concentrations tested these ligands are moderately potent in accelerating the decarbamylation reaction (Figs 1 and 2), and have been shown previously to act predominantly at an allosteric activator site on the en-

zyme[6,7]. The predominantly small effects of these ligands on the apparent K_m of acetylcholine hydrolysis at 10 °C found in the present study (Table I) is also consistent with this interpretation. It is suggested that these ligands can bind to high- and low-temperature (and possibly other[8]) forms of the enzyme and that at the concentrations used they have only small effects on the equilibrium position and/or activation energies of the various forms. This is similar to the situation described by Massey *et al.*[14] for the binding of D-alanine to D-amino acid oxidase.

Structural changes

Acrylamide gel electrophoresis of acetylcholinesterase under conditions of low ionic strength failed to reveal either the presence of isoenzymes or gross structural changes in aggregation of the enzyme around the transition temperatures (Fig. 3A). Hence the suggested changes in state of the enzyme at different temperatures must involve small conformational or configurational changes in structure not detectable by this technique.

In a number of cases, in particular (Na^+–K^+)-ATPase[19], temperature-dependent discontinuities in activity have been related to changes in state of essential phospholipids. Treatment of the acetylcholinesterase preparation with phospholipase C failed to decrease the already low phosphate levels, and treatment with phospholipase A or C failed to alter either enzyme activity or electrophoretic mobility (Fig. 3B) in the direction of increased enzyme mobility expected for destruction of a large phospholipid–protein aggregate, such as the fast (70 S) lipoprotein complex from electric eel described by Grafius *et al.*[13]. The molecular weight of acetylcholinesterase in our erythrocyte preparation was found to be approximately 250 000 by sodium dodecyl sulphate acrylamide gel electrophoresis[20] (Roufogalis, B. D. and Wickson, V. M., unpublished). A similar molecular weight (260 000) has been found for crystalline acetylcholinesterase from electric eel[21]. These results argue against the possibility that phospholipids play a major role in the temperature-dependent transitions in this enzyme preparation, but rather suggest a change in protein conformation. Such conformational changes may have important physiological consequences for the control of the biological functions of this enzyme.

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