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Role of Phospholipid (Cardiolipin) in the Modulation of Substrate and Inhibitor Interactions with Erythrocyte Acetylcholinesterase

BASIL D. ROUFOGALIS AND GUY BEAUREGARD

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

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SUMMARY

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The hydrolysis of acetylcholine by bovine erythrocyte acetylcholinesterase shows a break in the Arrhenius plot around 20°. Evidence is presented that the cardiolipin-associated enzyme is modulated by a temperature dependent conformational rearrangement of the active site induced by the binding of substrate and inhibitor to the enzyme. Neither carbamylation by *o*-nitrophenoldimethylcarbamate, decarbamylation nor the maximum velocity of acetylcholine hydrolysis (rate limited by deacetylation) showed a non-linear Arrhenius plot. The break in the Arrhenius plot of acetylcholine hydrolysis appeared to parallel the nonlinear temperature dependence of $V_{max}/K_{m(app)}$ which reflects the conformational rearrangement of the enzyme-substrate complex, the rate limiting step at subsaturating substrate concentrations. Treatment of the enzyme with high salt and phosphate, which abolishes the non-linear Arrhenius plot and permits the extraction of cardiolipin from the enzyme by chloroform/methanol, gave linear plots for the temperature dependence of $V_{max}/K_{m(app)}$, indicating that in these conditions the rate-limiting step was altered or no longer modulated by temperature.

Above the transition temperature fluoride inhibited the cardiolipin-associated enzyme with a Hill slope greater than 1.0 ($-n = 1.4$), whereas below the transition temperature or in high salt and phosphate treated cardiolipin-dissociated enzyme the corresponding negative Hill slope was 1.0. The rate of irreversible inhibition at the active site by N,N'-dicyclohexylcarbodiimide also exhibited a break at around 20°, the large increase in the entropy of the reaction above this temperature being consistent with a conformational rearrangement of the active site.

INTRODUCTION

Recently it has been shown that a number of membrane-bound proteins are regulated by their phospholipid environment. In a previous report we showed that cardiolipin was associated with bovine erythrocyte acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) and was responsible for an anomalous break in the Arrhenius plot of the enzyme activity (1). The cardiolipin appeared to be bound to the enzyme by ionic as well as hydrophobic interaction, as it could not be removed by nonionic detergent (Lubrol WX) and was removed by chloroform/methanol extraction only in the presence of high salt (1). The break in the Arrhenius plot was abolished by treatment of the enzyme complex with high (1.8 M) salt in Ca^{2+} chelating conditions, presum-

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ably as a result of disruption of the cardiolipin-enzyme interaction. The disruption was slowly reversible over a few days (unpublished observation) and the break was recovered when $CaCl_2$ was added back to the enzyme (1). The association of acetylcholinesterase with phospholipid has also been observed in human erythrocytes (2, 3).

Anomalous Arrhenius plots have been observed with a variety of enzymes (4–7), particularly those that are membrane bound (8–10). Generally, the precise cause of the Arrhenius plot anomalies has not been elucidated, and may result from a variety of temperature-dependent changes in the reaction sequences of active protein complexes (7). The anomalous Arrhenius plots have been approximated by smooth curves, discontinuous curves or sharp breaks (4–7, 11). For membrane bound enzymes the Arrhenius plots have usually been fitted by intercepting straight lines, as the breaks often correspond to phase changes of the lipids surrounding the protein (8, 9, 12).

In the present study we have investigated the nature of the break in the temperature dependence of bovine erythrocyte acetylcholinesterase by examining the influence of the cardiolipin-acetylcholinesterase interaction on the individual steps in the reaction sequence of acetylcholine hydrolysis and the inhibition of the enzyme by selected inhibitors. The results suggest that cardiolipin modulates a temperature-dependent conformational change of the active site induced by the binding of substrate or inhibitor to the enzyme.

MATERIALS AND METHODS

Acetylcholinesterase assay. Acetylcholinesterase partially purified from bovine erythrocytes was from Sigma. The enzyme preparation contained 20 mg acetylcholinesterase, 100 mg gelatin and 30.7 mg NaCl in 5 ml of sodium phosphate buffer, pH 7.6. Arrhenius plots of the untreated enzyme were obtained as described previously (1). The enzyme was treated with high salt in phosphate by dissolving 0.25 mg of the Sigma enzyme preparation in 1 ml of 1.8 M sodium chloride and 2 mM sodium phosphate pH 7.4 and then dialyzing in distilled water overnight (1) or by dissolving the enzyme in a concentration ratio of 100 mg/ml of water. The latter method produced the same effect as adding salt, since the high concentration of the enzyme preparation (100 mg/ml) resulted in a high salt and phosphate concentration, due to the presence of these in the Sigma preparation. Acetylcholinesterase was measured by a radiometric assay as previously described (13).

Fluoride inhibition. The degree of fluoride inhibition at various temperatures was determined as described by Morero *et al.* (14). Essentially, aliquots (10 μl) of acetylcholinesterase (0.25 mg/ml) were preincubated at the required temperature and incubated for 10 min in a final volume of 0.2 ml of 0.1 M sodium phosphate pH 8, containing 0.87 mM $MgCl_2$, acetyl-1-[^{14}C]choline (10.4 nCi) and acetylcholine perchlorate, to a final acetylcholine concentration of 0.55 mM, and various concentrations of sodium fluoride.

Carbamylation and decarbamylation rates. Carbamylation (15) and decarbamylation (13) rates were determined with *o*-nitrophenoldimethylcarbamate as previously described, with the following modifications. In the decarbamylation experiment the concentration of Sigma enzyme preparation in the initial carbamylation step was reduced to 33 mg/ml and then diluted 1000-fold in 0.1 M sodium phosphate pH 7.4 to begin the decarbamylation at various temperatures. The recovered enzyme activity in 0.2 ml aliquots was assayed with 1 mM acetylcholine containing 76 nCi/tube of acetyl-1-[^{14}C] choline iodide for 5 min.

Determination of V_{max} and $K_{m(app)}$. $K_{m(app)}$ and V_{max} were determined at various temperatures at three substrate concentrations (0.25 mM, 0.5 mM and 1.0 mM) assayed in quadruplicate. $K_{m(app)}$ and V_{max}, and their standard errors, were determined from the mean velocities by the computerized weighted statistical analysis of Wilkinson (16).

Source of chemicals. Inorganic salts were of analytical reagent grade. Acetyl-1-[^{14}C] choline iodide (New England Nuclear), acetylcholine perchlorate (British Drug

Houses), sodium fluoride (Mallinckrodt) and DCC[1] (Nutritional Biochemicals) were used as received. *o*-Nitrophenoldimethylcarbamate was prepared as previously described (13).

RESULTS

Hydrolysis of acetylcholine (1 mM) by Sigma bovine erythrocyte acetylcholinesterase gave a non-linear Arrhenius plot, which was fitted by two straight lines with a break at 20° (see ref. 1 and Fig. 3). Since acetylcholine hydrolysis proceeds through a number of intermediate steps, we have examined the temperature dependence of the various rate constants. At Sigma enzyme concentrations where the Arrhenius plots of acetylcholine hydrolysis were non-linear (0.25–33.3 mg/ml) the corresponding rate of carbamylation of the enzyme by *o*-nitrophenoldimethylcarbamate and the decarbamylation rate gave linear Arrhenius plots (data not shown).

Maximum velocity and Michaelis complex. The temperature dependence of $K_{m(app)}$ and V_{max} were examined to determine if the non-linear Arrhenius plot depended on the degree of substrate saturation of the enzyme. A gap or discontinuity in the van't Hoff plot of the temperature dependence of $K_{m(app)}$ occurred at around 20°, while the corresponding plot for the acetylcholinesterase treated with high salt in phosphate buffer was linear over the same temperature range (data not shown). The variations in $K_{m(app)}$ with temperature were small but quadruplicate determinations were of sufficient precision to demonstrate significant changes with temperature. No temperature dependence was apparent in previous studies (11, 17). A second experiment (in quadruplicate) confirmed the pattern of temperature dependence of $K_{m(app)}$ of the untreated enzyme. The significance of the temperature dependence of K_m has been discussed by Dixon and Webb (7) and Silvius *et al.* (10). A similar reversal in sign of the van't Hoff plot slopes as found in the present study was found for the K_m of phosphorylase in the presence of an effector (18).

[1] The abbreviation used is: DCC, dicyclohexylcarbodiimide.

Since $K_{m(app)}$ is a complex of a number of rate constants $(k_{-1} + k_2/k_1)(k_{cat}/k_2)$ (19), a further analysis of the individual constants was made. Plots of log $V_{max}/K_{m(app)}$ versus 1/T and log V_{max} versus 1/T are shown in Fig. 1A for untreated acetylcholinesterase and Fig. 1B for high salt and phosphate-treated acetylcholinesterase. With both enzyme forms the Arrhenius plot of the variation of V_{max} with temperature was linear. By contrast, the Arrhenius plot of log $V_{max}/K_{m(app)}$ versus 1/T for untreated acetylcholinesterase showed a sharp transition or gap around 20°, with different slopes above and below the transition temperature (Fig. 1A). The corresponding plot for the high salt and phosphate-treated acetylcholinesterase was linear (Fig. 1B). The energies of activation associated with the partial reactions of acetylcholinesterase in the cardiolipin-associated and cardiolipin-dissociated enzyme states are summarized in Table 1.

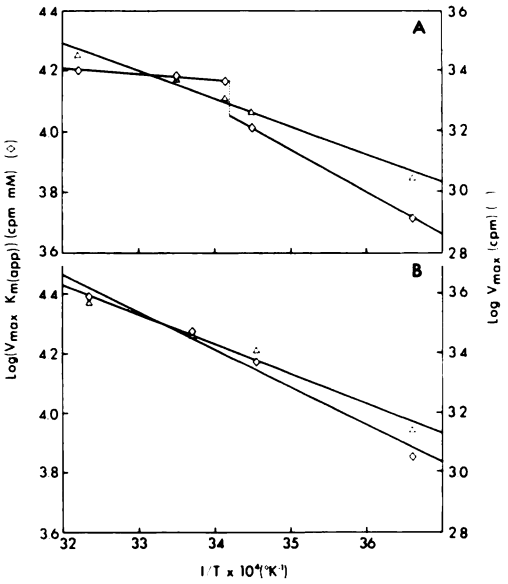

FIG. 1. *Variation of V_{max} and $V_{max}/K_{m(app)}$ with temperature*

A: Untreated acetylcholinesterase preparation. (△), variation of V_{max}; (◇), variation of $V_{max}/K_{m(app)}$. B: Acetylcholinesterase concentrated to 100 mg/ml and diluted 0.25 mg/ml before assay. (△), variation of V_{max}; (◇) variation of $V_{max}/K_{m(app)}$. Each point represents the average of four determinations. $K_{m(app)}$ was obtained from four determinations at acetylcholine concentrations of 0.25, 0.5 and 1.0 mM at each temperature.

Hill slope of fluoride inhibition. Fluoride ion inhibition of acetylcholinesterase was used to examine changes in allosteric behavior of acetylcholinesterase at different temperatures in the cardiolipin-associated enzyme (14). A sharp change in the Hill slope (20) of fluoride inhibition occurred around 20° (Fig. 2A). The negative Hill slope changed from 1.0 (0.963 and 0.993) at temperatures below 20° (0° and 16.5°) (Fig. 2A) to 1.4 (1.48 and 1.33) at temperatures above 20° (25.7° and 38°) (Fig. 2B). A plot of the negative Hill slope against temperature (Fig. 2D) showed a sharp change at 20°. Treatment of the acetylcholinesterase with 1.8 M NaCl in 2 mM sodium phosphate pH 7.4, resulted in a negative Hill slope of fluoride inhibition of 1.0 at temperatures both lower and higher than the transition temperature (0.96 at 0° and 0.99 at 26°) (Fig. 2C).

N,N'-Dicyclohexylcarbodiimide inhibition. To determine if the anomalous break in the Arrhenius plot resulted from the initial binding or activation step in the reaction sequence, we examined the temperature dependence of the irreversible inhibition of acetylcholinesterase by DCC, a hydrophobic antagonist which probably binds to the active site (21). The Arrhenius plot of the rate of DCC inhibition of the untreated enzyme gave a break at around

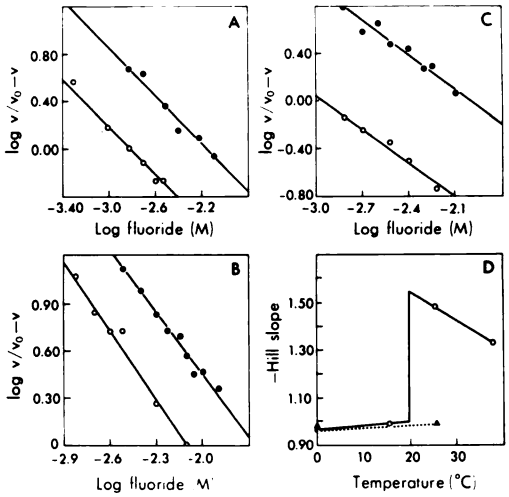

FIG. 2. *Hill slope of fluoride inhibition of acetylcholinesterase*

A: Hill plot of untreated acetylcholinesterase at 0° (●) and 16.5° (○). The Hill slope was was −0.963 at 0° and −0.993 at 16.5°. B: Hill plot of untreated acetylcholinesterase at 25.7° (○) and 38° (●). The Hill slope was −1.48 at 25.7° and −1.33 at 38°. C: Hill plot of acetylcholinesterase pretreated with 1.7 M NaCl and 2 mM sodium phosphate pH 7.4, at 0° (●) and 26° (○). The Hill slope was −0.96 at 0° and −0.99 at 26°. D: Variation of Hill slope with temperature for untreated acetylcholinesterase (○) and acetylcholinesterase treated with 1.7 M NaCl and 2 mM sodium phosphate pH 7.4 (▲). v is the velocity in the presence of fluoride and v_0 is the control velocity in the absence of fluoride (see MATERIALS AND METHODS).

TABLE 1

Energies of activation for various reactions of acetylcholinesterase (in kcal/mole)

Reaction	Cardiolipin-associated	Cardiolipin-dissociated
DCC inhibition	23.8[a], 9.2[a]	20.4
Carbamylation	13.5	—
Decarbamylation	13.8	15.2
$V_{max}/K_{m(app)}$[b]	0.92, 6.8	5.8
v[c]	1.3, 6.7	4.5
V_{max}[d]	3.8	4.6

[a] When two numbers appear on the same line, they refer to above and below the break, respectively.

[b] Average of two experiments (each with quadruplicate determinations).

[c] Average of three experiments.

[d] Average of two experiments for cardiolipin-associated acetylcholinesterase and a single experiment (in quadruplicate) for cardiolipin-dissociated acetylcholinesterase.

20° (Fig. 3A). In contrast to the temperature dependence of acetylcholine hydrolysis, the energy of activation at temperatures above the transition temperature was higher than at temperatures below the transition temperature. The corresponding inhibition by DCC of the cardiolipin-dissociated acetylcholinesterase, as for acetylcholine hydrolysis, gave a linear Arrhenius plot (Fig. 3B). The energies of activation of the rate of DCC inhibition are summarized in Table 1.

DISCUSSION

The non-linear Arrhenius plot of the hydrolysis of acetylcholine (1 mM) by purified bovine erythrocyte acetylcholinesterase is abolished by treatment of the enzyme with high salt in Ca^{2+} chelating conditions (see Fig. 3 and reference 1). It is thought that

these conditions disrupt the tight association between Ca^{2+}, cardiolipin and acetylcholinesterase (1) (in these conditions the enzyme is referred to as the cardiolipin-dissociated state). We have now attempted to identify the step(s) in the reaction sequence which are modulated by changes in temperature and are altered by the high salt and Ca^{2+} chelating conditions. A detailed scheme describing the partial reactions of acetylcholine hydrolysis was recently proposed by Rosenberry (19):

$$E + RX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E.RX \underset{k'_{-1}}{\overset{k_1'}{\rightleftharpoons}} (E.RX)' \xrightarrow{k_2}$$

$$ER \xrightarrow[H_2O]{k_3} E + ROH$$

where $E.RX$ is the initial enzyme-substrate Michaelis complex, $(E.RX)'$ is the activated enzyme-substrate complex induced by the substrate, and ER is the acetylated enzyme intermediate. At saturating substrate concentrations the deacetylation step, k_3, is rate limiting in acetylcholine hydrolysis, so that $V_{max} \simeq k_3 [E_o]$ (22). Since the Arrhenius plot of V_{max} was linear, the deacetylation step, k_3, cannot be the temperature-modulated step in the reaction sequence. This is consistent with the linear Arrhenius plot obtained for the decarbamylation reaction, which is a model for the deacetylation step in acetylcholine hydrolysis (23). Thus it appears that the temperature-modulated steps occur before the deacetylation step, or alternatively, that the non-linear Arrhenius plot results from different temperature coefficients of the acetylation and deacetylation steps which determines which step becomes rate-limiting at different temperatures (7, 11). Studies showing non-linear temperature dependence of inhibitor binding (see below) make the second possibility less likely.

The temperature dependence of $K_{m(app)}$ showed a break at a similar temperature to that of the break in the Arrhenius plot of the velocity of acetylcholine hydrolysis. $K_{m(app)}$ is a complex of all the rate constants, whereas $k_{(cat)}/K_{m(app)}$ $(= k_1'k_2/K_s(k'_{-1} + k_2))$ reflects the acetylation steps prior to deacetylation (19). Since in this study $V_{max}/K_{m(app)}$ $(= k_{(cat)} [E_o]/K_{m(app)})$ gave a break in the Arrhenius plot at a similar temperature to that of acetylcholine hydrolysis, the break appears to reflect a step in the acetylation reaction, which is rate limiting at sub-saturating substrate concentrations (19). Carbamylation of cardiolipin-associated acetylcholinesterase, which is a model of the acetylation reaction in acetylcholine hydrolysis (24), gave a linear Arrhenius plot. Under the conditions of the experiment the linear temperature dependence of this step may reflect the fact that k_2 is rate limiting ($k_2 \ll k_1'$) (25, 26). The non-linear temperature dependence of $V_{max}/K_{m(app)}$ for acetylcholine hydrolysis thus may reflect a rate-limiting step prior to k_2 (i.e., k_1' or k_1). (It is also possible that the formation of the transition state complex ($E.RX$) by *o*-nitro-

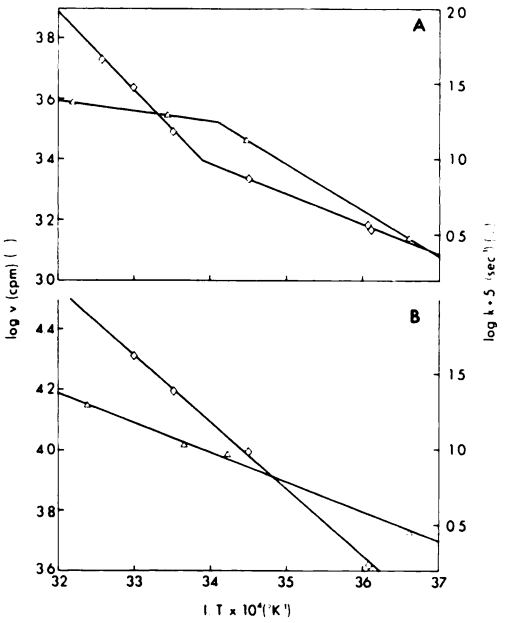

FIG. 3. *Temperature dependence of the inhibition of acetylcholinesterase by DCC*

A: (◇), Arrhenius plot of the DCC inhibition rate (k) for untreated acetylcholinesterase; (△), Arrhenius plot for acetylcholine hydrolysis by untreated acetylcholinesterase. B: (◇), Arrhenius plot of DCC inhibition rate (k) for acetylcholinesterase concentrated to 100 mg/ml and diluted to 0.25 mg/ml before reaction with DCC (cardiolipin-dissociated acetylcholinesterase), (△), Arrhenius plot of acetylcholine hydrolysis by cardiolipin-dissociated acetylcholinesterase. Results represent typical experiments.

phenoldimethylcarbamate is different from that of other acylating agents (19)).

Rosenberry (19) showed that for acetylcholine hydrolysis the assumption of virtual equilibrium does not apply, since $k_2 > k'_{-1}$. In these conditions $k_{(cat)}/K_{m(app)} \simeq k_1'/K_s$. These constants could not be readily separated by the steady-state kinetic procedures used. However, as the process of binding (K_s) is less likely to exhibit an anomalous temperature dependence (27), and as k_1' is considered to be the rate limiting step (19), it seems possible that the break in the Arrhenius plot of acetylcholine hydrolysis reflects the conformational change in the enzyme induced by substrate binding (k_1'). Biphasic Arrhenius plots in enzymes with flexible active sites were predicted by Koshland (6). The close correlation between the energy of activation of $V_{max}/K_{m(app)}$ and the velocity of acetylcholine hydrolysis at subsaturating substrate concentration (Table 1) supports the suggestion that k_1' is rate limiting in acetylcholine hydrolysis. Studies with inhibitors seem to be consistent with this interpretation (see below), but definitive evidence will require rapid, non-equilibrium kinetic studies. In cardiolipin-dissociated acetylcholinesterase the rate-determining step is either altered or becomes insensitive to modulation by temperature, possibly because the active site is in an unrestrained or "open" conformation (see ref. 6). The energy of activation of the velocity of acetylcholine hydrolysis by the cardiolipin-dissociated state is similar to the activation energy of V_{max} (Table 1).

The temperature dependence of the Hill slope of fluoride inhibition was examined to determine whether acetylcholinesterase exhibited distinct properties above and below the transition temperature. The Hill slope of fluoride inhibition of acetylcholinesterase has been shown to be sensitive to the degree of unsaturation of membrane lipids (14), but it is unknown whether Hill slopes greater than one represent true cooperativity of fluoride binding or the random binding of two molecules of fluoride without true cooperativity (28). Fluoride inhibition of the cardiolipin-associated enzyme had a Hill slope greater than 1.0 above the transition temperature. At all temperatures in high salt and phosphate-treated enzyme, or below the transition temperature in the untreated enzyme, fluoride inhibited with a Hill slope of 1.0. Under the conditions of the present experiment fluoride inhibition is uncompetitive (20, 29, 30), suggesting that fluoride binds preferentially to the ERX complex, possibly blocking the conformational change of ERX to (ERX)' (19). The change in the kinetics of fluoride inhibition with temperature is consistent with the proposal that temperature modulates the substrate-induced conformational change of the active site in the presence of cardiolipin. The nature of the temperature-dependent changes responsible for the modulation in the cardiolipin-associated enzyme is unknown at present and is being investigated further.

Finally, the suggestion that the non-linear Arrhenius plot reflects a conformational change resulting from the binding of acetylcholine was consistent with the break observed in the Arrhenius plot of the rate of irreversible inhibition by DCC, a ligand considered to react with a carboxyl group in a hydrophobic area of the active site (21). The transition in the rate of reaction around 20° in this experiment is independent of the influence of changes in the extent of substrate saturation of the enzyme with temperature or the influence of temperature on successive reaction steps which could have influenced the interpretation of the anomalous Arrhenius plot in acetylcholine hydrolysis (10, 7). The unusual increase in the rate of DCC inhibition above 20° has also been observed with reversible inhibitors of fumarase (31). The large increase in entropy of the reaction from −48 to +10 e.u. as the temperature was raised above 20° supports the suggestion of a conformational rearrangement in the active site, which may be constrained by cardiolipin in the "low temperature" state.

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