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KINETIC ANALYSIS OF CALCIUM ACTIVATION OF BRAIN ACETYLCHOLINESTERASE FORMS

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Summary

Calcium activation of acetylcholine hydrolysis by bovine brain acetylcholinesterase (Acetylcholine hydrolase, EC 3.1.1.7) forms has been analyzed in terms of changes in kinetic constants and thermodynamic activation parameters. De-acetylation was determined to be the major rate-influencing step in acetylcholine hydrolysis by both 60 000- and 240 000-dalton forms of the brain enzyme and 10 mM Ca^{2+} increased the rate constant for this step (k_{+3}) by approximately 30% for both forms. For the smaller acetylcholinesterase form the effects of Ca^{2+} on de-acetylation was equivalent to its effect on the overall rate constant (k) and occurred without an effect on pK. In the case of the 240 000-dalton species, the overall rate constant was increased by Ca^{2+} by 33% at pH 8.0 and 81% at pH 7.25 and involved a pK shift of —0.2 pH units. For both enzyme forms the rate constants for acetylation (k_{+2}) were increased by Ca^{2+}. Thermodynamic analysis suggested that Ca^{2+} activation of the acetylation step was entropically driven. Differences between the two enzymes forms in terms of Ca^{2+} appear to result from association of low molecular weight species.

Introduction

Acetylcholine hydrolysis by bovine erythrocyte, electric eel, and bovine brain caudate nucleus acetylcholinesterase (Acetylcholine hydrolase, EC 3.1.1.7) is accelerated in the presence of Ca^{2+} ions [1—3]. A number of investigators have proposed that this acceleration is effected by an interaction between Ca^{2+} and a peripheral anionic binding site for cholinergic ligands

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[4—6]. In the present study, analyses of thermodynamic activation parameters and other kinetic constants of brain acetylcholinesterase catalyzed acetylcholine hydrolysis have been employed to examine possible mechanisms of Ca^{2+} activation of the enzyme. Two molecular weight forms of brain acetylcholinesterase, isolated from bovine caudate nucleus tissue have been studied. Brain enzyme from this source has been characterized previously in terms of molecular weight, inhibitor sensitivity, glycoprotein nature [7—9], and more recently, active site numbers [10]. These studies provide a basis for the present thermodynamic investigation.

Calcium ions have been implicated in various aspects of cholinergic neurotransmission. In particular, Ca^{2+} activity has been suggested to play a central role in both presynaptic neuronal membrane excitation-transmitter release coupling as well as neurotransmitter-receptor mediated membrane conductance changes [11—12]. Since significant changes in the time course of cholinergic responses depending upon the level of acetylcholinesterase activity have been noted [13,14], Ca^{2+}-mediated allosteric control of acetylcholinesterase activity could be of functional importance.

Evaluation of thermodynamic activation parameters and kinetic constants has proved useful in analysis of ionic effects on several enzyme systems, including the activating effect of K^+ and Mn^{2+} on pyruvate kinase [15]. Similarly, inhibition of deacetylation, accounting for the inhibitory effect of F^- on acetylcholinesterase catalytic rate, was deduced in part from analysis of activation energy [16]. The two-step, acylation-deacylation catalytic process of acetylcholinesterase is also shared by trypsin and chymotrypsin [17—20]. Studies in these systems have utilized activation parameter analysis to determine the rate determining step as a function of substrate used [21]. The basic approach appears applicable to the characterization of the reaction steps that may be influenced by ionic rate modifiers. Furthermore, some indication of the nature of active site groups may be revealed by studies of the effect of pH on catalytic rate constants and on the K_m. The nature of the temperature dependence of the rate constants, measured at different pH values, may reveal processes such as the temperature effect on the equilibrium between two forms of the enzyme, as is seen for trypsin or provide information as to enthalpy and entropy of activation changes accompanying changes in ionization of reaction intermediates [22].

Methods

Materials

General chemicals used were analytical-reagent grade. Acetylthiocholine iodide, acetylcholine chloride, acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7, type III, electric eel) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.. Gelatine (U.S.P.) was obtained from Braun-Knecht-Herman Co., San Francisco, CA, U.S.A.. $CaCl_2$, NaCl, and NaOH were procured from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A..

Sephadex G-200 materials were obtained from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. and the collodion bags (8 ml capacity) were from Scheicher and Schuell, Keene, NH, U.S.A..

Preparation of bovine brain acetylcholinesterase forms

Acetylcholinesterase forms were partially purified from caudate-nucleus tissue of ox brains (obtained from a local slaughterhouse) by a previously reported method [7]. The procedure involved solubilization in 0.32 M sucrose containing 1 mM EDTA then fractionation with $(NH_4)_2SO_4$. Acetylcholinesterase forms of differing molecular weights were then separated by Sephadex G-200 chromatography. The Sephadex G-200 columns were equilibrated with and the samples were run in 30 mM Tris · HCl buffer pH 7. To obtain sufficiently high enzyme activities for the kinetic experiments, the G-200 fractions associated with the different molecular weight forms were concentrated using collodion bag filtration at 4° C. This procedure involves placing the sample into 8-ml capacity collodion bags (exclusion limit, 40 000 daltons). The bags were slipped onto a glass tube open to the atmosphere and then inserted into a larger glass chamber attached to the laboratory vacuum line. The degree of concentration required was determined by the initial enzyme activity associated with the pooled fractions for each form but generally was approximately four-fold. Calibrated G-200 chromatography and gradient gel electrophoresis were used in molecular weight determinations of the two acetylcholinesterase forms examined in the kinetic study [10]. The larger molecular form had a molecular weight of approximately 240 000, whereas the molecular weight of the smaller species was estimated by gradient gel electrophoresis as 60 000 and by G-200 chromatography as 75 000. The determination of precise concentration of each enzyme form used in kinetic experiments involved comparison of observed activity with the known activity of the purified form of the enzyme [7,23]. This method was accurate to within 3% when the enzyme activity of purified brain acetylcholinesterase forms was correlated with protein concentration determine by the fluorescamine procedure [10,24]. The catalytic site concentration associated with each form was based on a previous study in which the 240 000-dalton species was determined to contain four active sites and the 60 000-dalton form one active site [10]. In the instances in which electric eel acetylcholinesterase was studied, the commercial enzyme preparation from Sigma (type III) was used without further purification.

Kinetic measurements

Kinetic measurements, based upon titration of protons released with acetylcholine hydrolysis, were made with a Radiometer automatic titrator (TTT1C) and Titragraph (SBR2C) using the pH-stat mode. A Radiometer G-202C sensing electrode and a Beckman 40249 Ag/AgCl reference electrode were employed for pH measurement. In addition, a Radiometer T-301 continuous temperature compensation electrode was used throughout. Dual calibrated glass syringes allowed simultaneous additions of the 5 mM NaOH titrant (CO_2-free) as well as 5 mM acetylcholine to replace hydrolyzed substrate. The 100 ml reaction volume was contained in a 125 ml capacity water-jacketed reaction vessel. A slow stream of N_2 covering the surface of the mixture, eliminated small blanks arising from absorption of atmospheric CO_2. A magnetic stirring bar provided adequate mixing of the reaction solution. Voltage output proportional to the pH was taken from the automatic titrator and fed into a Fairchild Digital Multimeter model 7000A. Output from the multimeter was in turn fed into a Ameri-

can Instrument Co. BCD printer model 4500. Sampling every 5 s and printing voltages proportional to the pH permitted monitoring of the pH-stat system and assured accuracy of pH measurement to ±0.01 pH units. Reaction mixture temperature was set and maintained by a Forma Scientific water bath model 2096 accurate to ±0.1°C. The reaction mixture temperature during the reaction was monitored using a Yellow Springs Instrument tele-thermometer system with a Hitachi-Perkin-Elmer model 159 recorder providing continuous graphical output.

The reaction mixture, unless otherwise indicated, initially contained 1.2 mM acetylcholine, which was shown to saturate the 240 000-dalton form of the enzyme or 0.8 mM which was saturating for the 60 000-dalton species, and 0.01% gelatin. Since it was determined that substrate inhibition is not exhibited except at much higher substrate concentrations than those used, the maximal enzyme velocities obtained probably reflect substrate saturating conditions. In these cases, representing the low ionic strength control conditions, the reaction was started by the addition of 50 μl of enzyme (see Table I for concentration). Control assays were performed in the absence of added inorganic salts, since salts usually employed have specific effects at the peripheral site on the enzyme and could interfere with the characterization of the Ca^{2+} interaction effect [25]. In studies examining the influence of pH and temperature on reaction rate, initial pH values were altered by adding dilute NaOH or HCl and the rate of the new pH was then measured. After determining the reaction rate pH dependence under the above conditions, the pH dependence was re-determined after the addition of 10 mM $CaCl_2$. After a series of pH vs. rate determinations with the same enzyme sample, the rate at the first pH value of the series was redetermined. Since, within stated experimental error, the initial and final rates were equal, neither enzyme denaturation or choline inhibition appear to occur under these conditions. After the effect of pH had been measured at the initial temperature in the presence and absence of $CaCl_2$ a new reaction mixture was prepared and after a 30 min equilibration time, the procedure was repeated at a different temperature. The pK of the reaction was determined from the pH vs. rate relationship for each set of conditions. The pH vs. velocity curve was in every case based upon 9—14 data points over the pH range 7.0—8.4. In the pK determinations an ideal ionization curve was fitted to the data points [26]. Volume changes, accompanying the additions of NaOH, acetylcholine, $CaCl_2$, and HCl were limited to less than 5% which was within the experimental error of determinations of the rate constants (5%). In preliminary experiments with eel enzyme 100 mM NaCl was present throughout.

The K_m was estimated in duplicate experiments using a computer generated hyperbolic best fit program [27]. In these experiments, the enzyme was added to the reaction mixture containing 0.01% gelatin and then substrate added incrementally until saturation was reached. A new reaction mixture containing 10 mM $CaCl_2$ was prepared and the procedure repeated. The K_m values (25°C) were obtained from measurements at substrate concentrations including at least two concentrations below K_m and are given as the mean of the two determinations. The duplicate K_m values agreed within ±4% and the computer-generated error estimate for the K_m in each determination was in no case greater than 10% and usually no greater than 5%.

Calculations were based upon the following relationships:

$$v = \frac{V[S]}{K_m + [S]} \tag{1}$$

$$V = k[E] \tag{2}$$

where v is the observed reaction velocity, S is the substrate concentration, k is the reaction rate constant, and E is the number of enzyme catalytic sites present.

Determination of acetylation rate constants (k_{+2}), deacetylation rate constants (k_{+3}), and thermodynamic activation parameters

Fig. 1 illustrates an example of the Arrhenius relationship for brain acetylcholinesterase catalyzed acetylcholine hydrolysis in the presence of 10 mM $CaCl_2$. The pronounced curvature (log k) has been previously reported [28] for eel acetylcholinesterase and is in agreement with the two step catalytic mechanism. The mechanism has been described as follows:

$$\text{Enzyme-substrate complex} \xrightarrow{k_{+2}} \text{Choline + acetyl enzyme} \tag{3}$$

$$\text{Acetyl enzyme + } H_2O \xrightarrow{k_{+3}} \text{Acetic acid + enzyme} \tag{4}$$

Providing that k_{+2} is rate determining over the entire experimental temperature range, plots of log k_{+2} vs. T^{-1} should yield straight lines. The same argument would follow for plots of log k_{+3} vs. T^{-1}. Generally, for a two-step process, each with a different temperature dependence, one straight line would be seen over one temperature range in which one step was rate limiting and a second, differ-

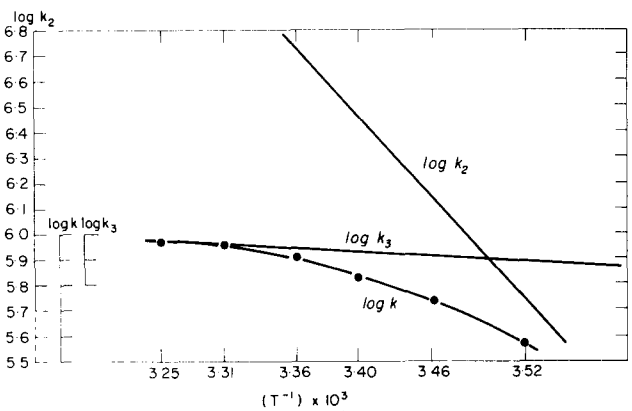

Fig. 1. Representation of derived rate constants for acetylaton (k_{+2}) and deacetylation (k_{+3}) from the Arrhenius plots of brain acetylcholinesterase (240 000-dalton form) catalyzed acetylcholine hydrolysis in 10 mM $CaCl_2$. Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, 1.2 mM acetylcholine, $1.62 \cdot 10^{-12}$ mol acetylcholinesterase, 10 mM $CaCl_2$ pH 7.75 in a total volume of 100 ml. Values of log k (k expressed min^{-1}) were computed from the relationship $V = k[E]$. k_{+3} values were derived from the asymptote of the log k vs. reciprocal absolute temperature plot and k_{+2} values were computed from the relationship $k = (k_{+2}^{-1} + k_{+3}^{-1})^{-1}$.

ent straight line would be seen corresponding to the temperature range in which the second step is rate-limiting. These two straight lines would be joined by a curved portion in the temperature range in which both velocity constants have similar values. It follows that, as the temperature is increased, the rate approaches that having the lower activation energy and as a consequence the two-step process should yield a curve flattening towards the higher temperatures [28]. Curved Arrhenius plots for the brain enzyme were consistently observed for the 24 Arrhenius plots utilizing the 240 000-dalton brain form and were also observed for the 24 Arrhenius plots using the 60 000-dalton brain acetylcholinesterase form. Arrhenius plot curvatures similar to those observed by Wilson and Cabib [28] were also observed with eel enzyme.

The preceding kinetic mechanism description, assuming steady-state conditions, yields the following relationships [28]:

$$v = \frac{V[S]}{[S] + K_m} = \frac{k[E][S]}{[S] + K_m} \tag{5}$$

where

$$k = (k_{+2}^{-1} + k_{+3}^{-1})^{-1} \tag{6}$$

and

$$K_m = \left(\frac{k_{-1} + k_{+2}}{k_{+1}} \cdot \frac{1}{1 + \frac{k_{+2}}{k_{+3}}}\right) \tag{7}$$

As shown in Fig. 1, an asymptote may be drawn to the curve. This line approximates the temperature dependence of the de-acetylation rate constant, k_{+3} [28]. Using the experimental k and the estimated k_{+3}, k_{+2} (acetylation) may be calculated from the relationship given in Eqn. 6 and, as a consequence, its temperature dependence determined. The asymptote method is one of the most important methods for the separation of acetylation and deacetylation [29]. The curved Arrhenius plots derived from brain enzyme were best-fit to hyperbolas with resulting correlation coefficients of 0.97 to greater than 0.99 which were in all cases higher than the correlation coefficient determined for best-fit to a straight line. Subsequently, the asymptotes were determined and values of k_{+3} and k_{+2} calculated in the manner described above. Error associated with k_{+3} values determined in this way was considered to be between 10 to 20%. This error estimate followed from the determination of the confidence interval of the slope of the asymptote.

It is possible to obtain an estimate of the value K_m' by multiplying Eqn. 7 by $(1 + (k_{+2}/k_{+3}))$, yielding:

$$\frac{(k_{-1} + k_{+2})}{k_{+1}} \tag{8}$$

In certain experiments the effect of pH on K_m' values of k_{+2} and k_{+3} were derived by the asymptote method [29].

Activation enthalpies and entropies were estimated from plots of log k_{+2} or log k_{+3} vs. reciprocal absolute temperature with calculations based upon the

following Arrhenius and transition-state-theory relationships [29].

$$\log k_n = \frac{-E_{act}}{2.303R} + \log A \tag{9}$$

$$\Delta H^{\ddagger} = E_{act} - RT \tag{10}$$

$$\Delta G^{\ddagger} = -RT \ln \frac{kh}{k_b T} \tag{11}$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \tag{12}$$

As can be seen from Eqn. 9, plots of log k_n vs. T^{-1} result in slopes equal to $-E_{act}/2.303\,R$, where E_{act} is the energy of activation, R is the gas constant, and A is a statistical factor. $\Delta H^{\ddagger}$ is calculated from the relationship given in Eqn. 10. Free energies of activation, $\Delta G^{\ddagger}$ can be calculated from Eqn. 11 in which k_b is the Boltzman constant and T is the absolute temperature and $\Delta S^{\ddagger}$ is derived from Eqn. 12.

Results

Effect of pH on kinetic constants

As shown in Table I, the rate constants for acetylation (k_{+2}), catalyzed by the 240 000-dalton brain acetylcholinesterase form, were more pH dependent, both in the presence and absence of Ca^{2+}, than those for deacetylation (k_{+3}). As the pH was increased from 7.25 to the pH optimum of 8.0, acetylation was enhanced approximately 4-fold in the absence and about two-fold in the presence of Ca^{2+}. In contrast, deacetylation, either in the presence or absence of Ca^{2+}, increased by only 35% as the pH optimum was approached. At all pH values, Ca^{2+} addition resulted in a marked acceleration (2—4-fold) of acetylation, whereas Ca^{2+} accelerated deacetylation by only 30%. The ratios of acetylation/deacetylation show that deacetylation is the major rate influencing step for acetylcholine hydrolysis catalyzed by this brain enzyme form at 20°C. In addition, the ratios indicate that the influence of k_{+2} on the observed k (see Eqn. 6) varies from 33% (pH 7.25) to 12% (pH 8) in the absence of Ca^{2+} and from 17% (pH 7.25) to 10% (pH 8.0) in the presence of Ca^{2+}. As indicated in Table II, the ratio of acetylation/deactylation shows that deacetylation is also the major rate influencing step for acetylcholine hydrolysis catalyzed by the 60 000-dalton form and that the influence of acetylation on the overall rate varies in a manner similar to that observed for the 240 000-dalton species. Enhancement of acetylation by Ca^{2+} is also noted in the 60 000-dalton form, although the degree of rate acceleration is less than that observed in the larger species. In the smaller form, the ratio of the overall velocity constant, k, (with Ca^{2+}/without Ca^{2+}) varies with pH in a manner that parallels the pH dependence of the ratio of the major rate influencing rate constant k_{+3} (with/without Ca^{2+}). In contrast, in the reaction catalyzed by the 240 000-dalton form, the ratio of the overall velocity constant, k, (with Ca^{2+}/without Ca^{2+}), increased markedly (from 33 to 81%) with decreasing pH, whereas the ratio of the deacetylation rate constant, k_{+3} (with/without Ca^{2+}) remained relatively constant (average value of 1.31). This observation points out the importance of the k_{+2}/k_{+3} ratio

TABLE I

pH DEPENDENCE OF THE OVERALL RATE CONSTANT (k), ACETYLATION RATE CONSTANT (k_{+2}) AND DEACETYLATION RATE CONSTANT (k_{+3}) OF THE 240 000-DALTON BRAIN ACETYLCHOLINESTERASE FORM

Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, 1.2 mM acetylcholine, $1.62 \cdot 10^{-12}$ mol acetylcholinesterase, at 20°C, in a total volume of 100 ml. The $CaCl_2$ concentration was 10 mM. The data was obtained from two sets of experiments with errors associated with the experimentally determined k and the derived k_{+3} values as reported in Methods. $+Ca^{2+}$ refers to with Ca^{2+}. $-Ca^{2+}$ refers to without Ca^{2+}.

	pH			
	7.25	7.5	7.75	8.0
$10^{-3}k$ (s^{-1}) ($-Ca^{2+}$)	5.2	6.0	7.2	9.5
$10^{-3}k$ (s^{-1}) ($+Ca^{2+}$)	9.3	9.5	10.6	12.6
$\frac{k + Ca^{2+}}{k - Ca^{2+}}$	1.81	1.58	1.49	1.33
$10^{-3}k_{+2}$ (s^{-1}) ($-Ca^{2+}$)	14.8	16.3	30.1	60.1
$10^{-3}k_{+2}$ (s^{-1}) ($+Ca^{2+}$)	58.1	58.3	112.3	123.4
$\frac{k_{+2} + Ca^{2+}}{k_{+2} - Ca^{2+}}$	3.91	3.57	3.64	2.02
$10^{-3}k_{+3}$ (s^{-1}) ($-Ca^{2+}$)	7.8	9.3	9.3	11.3
$10^{-3}k_{+3}$ (s^{-1}) ($+Ca^{2+}$)	11.2	11.3	12.7	14.0
$\frac{k_{+3} + Ca^{2+}}{k_{+3} - Ca^{2+}}$	1.43	1.21	1.36	1.24
$\frac{k_{+2} - Ca^{2+}}{k_{+3} - Ca^{2+}}$	1.9	1.8	3.3	5.4
$\frac{k_{+2} + Ca^{2+}}{k_{+3} + Ca^{2+}}$	5.2	5.1	8.9	8.8

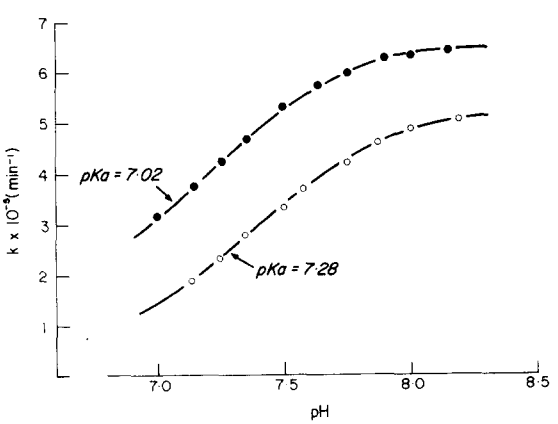

Fig. 2. Reaction rate vs. pH profiles of the 240 000-dalton brain acetylcholinesterase-catalyzed acetylcholine hydrolysis. Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, 1.2 mM acetylcholine, $1.62 \cdot 10^{-12}$ mol acetylcholinesterase, at 20°C in a total volume of 100 ml. ○, in the absence of added ions; ● in the presence of 10 mM $CaCl_2$. Each data point was the mean of two determinations with error associated with each value as reported in text.

TABLE II

pH DEPENDENCE OF THE RATIOS OF THE OVERALL RATE CONSTANTS (k), ACETYLATION RATE CONSTANTS (k_{+2}), AND DEACETYLATION RATE CONSTANTS (k_{+3}) WITH AND WITHOUT 10 mM $CaCl_2$ FOR THE 60 000-DALTON BRAIN ACETYLCHOLINESTERASE FORM

Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, 0.8 mM acetylcholine, $1.52 \cdot 10^{-12}$ mol acetylcholinesterase, at 20°C, in a total volume of 100 ml. The data was obtained from two sets of experiments with errors associated with the experimentally determined k and the derived k_{+3} values as reported in Methods.

	pH			
	7.00	7.25	7.50	7.75
$\frac{k + Ca^{2+}}{k - Ca^{2+}}$	1.13	1.23	1.47	1.41
$\frac{k_{+2} + Ca^{2+}}{k_{+2} - Ca^{2+}}$	1.34	1.47	1.91	2.74
$\frac{k_{+3} + Ca^{2+}}{k_{+3} - Ca^{2+}}$	1.20	1.23	1.48	1.38
$\frac{k_{+2} + Ca^{2+}}{k_{+3} + Ca^{2+}}$	3.51	5.02	10.43	12.41
$\frac{k_{+2} - Ca^{2+}}{k_{+3} - Ca^{2+}}$	3.12	4.23	7.80	5.91

in determining to what extent the more pH-dependent k_{+2} step will influence the observed k, in accordance with the reciprocal rate constant relationships (Eqn. 6).

Analysis of pH vs. rate profiles for acetylcholine hydrolysis catalyzed in the

TABLE III

THERMODYNAMIC ACTIVATION PARAMETERS FOR ACETYLATION AND DEACETYLATION IN THE PRESENCE AND ABSENCE OF Ca^{2+} FOR THE 250 000- AND 60 000-DALTON BRAIN ACETYLCHOLINESTERASE FORMS

Enzyme activity was measured as resported in the legends of Tables I and II. The pH at which the determinations were made was 8.0 in the case of the 240 000-dalton form and 7.75 for the 60 000-dalton species. Thermodynamic activation parameters were calculated as indicated in Methods. Ca^{2+} effects at four pH values for each species were considered (data not shown) and differences were analyzed utilizing the paired observation test. Ca^{2+}-mediated differences in $\Delta G^{\ddagger}$ were significant ($P < 0.025$); differences in $\Delta H^{\ddagger}$ were not significant ($P < 0.8$); differences in $\Delta S^{\ddagger}$ were significant ($P < 0.05$).

Brain form (mol. wt.)	Reaction step	$\Delta G^{\ddagger}$ (kJ · mol^{-1})		$\Delta H^{\ddagger}$ (kJ · mol^{-1})		$\Delta S^{\ddagger}$ (J · mol^{-1} · K^{-1})	
		With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}	With Ca^{2+}	Without Ca^{2+}
240 000	Acetylation	44.3	46.0	75.2	74.8	+105.5	+98.2
240 000	De-acetylation	48.6	49.1	1.7	1.7	−160.2	−162.0
60 000	Acetylation	43.6	45.2	76.6	77.3	+112.7	+109.8
60 000	De-acetylation	48.1	48.8	1.3	1.7	−159.9	−160.9

presence and absence of Ca^{2+} by the 240 000-dalton brain form revealed a pK shift of approximately 0.22 ± 0.05 pH units. An example of their effect is seen in Fig. 2. This shift was not temperature-dependent and was consistently observed. Ca^{2+} addition does not influence the pH vs. rate relationships for the 60 000-dalton form.

Calcium effects on thermodynamic activation parameters

As seen in Table III, the Ca^{2+}-mediated increase in the acetylation and deacetylation rate constants at pH 8 for the 240 000-dalton form and at pH 7.75 for the 60 000-dalton form are reflected in the more favorable free energies of activation in the presence of Ca^{2+}. The enthalpies of activation remained relatively constant in the presence or absence of Ca^{2+} and as a result, the rate accelerations are probably due to more favorable entropies of activation in the presence of Ca^{2+} compared with the control case. A statistical analysis of the pH dependence (pH 7.0—8.0) of the activation parameters for acetylation and deacetylation for the brain enzyme forms suggests that the Ca^{2+} mediated more favorable acetylation. $\Delta G^{\ddagger}$ values for both brain forms is due to a more favorable $\Delta S^{\ddagger}$ value with little likelihood of a change in $\Delta H^{\ddagger}$. This conclusion follows from results of the paired observation test comparing $\Delta S^{\ddagger}$ ± Ca^{2+} ($P < 0.0001$) and comparing $\Delta H^{\ddagger}$ ± Ca^{2+} ($P < 0.9$). In the case of deacetylation, no clear cut distinction in the relative contribution of $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ in the Ca^{2+} acceleration process could be resolved.

Calcium effects on K_m

Table IV illustrates that, in the case of the larger enzyme form, the K_m is higher in the presence of Ca^{2+} at all pH values except 7.25. Similarly, K_m', Table V, is also higher in the presence of Ca^{2+}. K_m' is equal to $(k_{-1} + k_{+2})/k_{+1}$ (Eqn. 8) and the effect of Ca^{2+} on K_m' can be further analyzed by examining the influence of Ca^{2+} on the acetylation rate constant (k_{+2}). If the value of k_{+2} contributed significantly to the value of K_m', the pH-dependent changes in the value

TABLE IV

INFLUENCE OF pH AND Ca^{2+} ON THE K_m FOR ACETYLCHOLINE HYDROLYSIS CATALYZED BY THE 240 000-DALTON BRAIN ENZYME FORM

Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, acetylcholine concentration from 0.04 to 3 mM, $1.63 \cdot 10^{-12}$ mol acetylcholinesterase, 10 mM $CaCl_2$, at 25°C. Computer-generated best-hyperbolic fit analysis was used for the K_m determination with computer-generated error estimate of no greater than 10%. As estimated,

$$K_m = \frac{(k_{-1} + k_{-2})}{k_{+1}} \cdot \frac{1}{1 + (k_{+2}/k_{+3}))}$$

pH	$10^4 K_m$ (M)		$\frac{K_m + Ca^{2+}}{K_m - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
8.0	0.7	1.8	2.5
7.75	1.3	2.3	1.7
7.50	1.2	1.5	1.3
7.25	1.5	1.5	1.0

TABLE V

INFLUENCE OF pH AND Ca^{2+} ON K_m', AND ON k_{+2} (25°C) FOR ACETYLCHOLINE HYDROLYSIS CATALYZED BY THE 240 000-DALTON BRAIN ENZYME FORM

To obtain K_m', $(k_{-1} + k_{+2})/k_{+1}$, the values of K_m (Table IV) are multiplied by $(1 + (k_{+2}/k_{+3}))$ (see Eqn. 6) in which values of the acetylation and deacetylation rate constants at 25°C are estimated by the procedure described in Methods.

pH	$10^4 K_m'$ (M)		$\frac{K_m' + Ca^{2+}}{K_m' - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
8.00	4.2	16	3.9
7.75	8.7	15	1.7
7.50	4.5	12	2.7
7.25	4.7	11	2.3

pH	$10^{-3} k_{+2}$ (s^{-1})		$\frac{k_{+2} + Ca^{2+}}{k_{+2} - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
8.00	58.3	146.6	2.5
7.75	56.6	121.6	2.2
7.50	20.0	80.0	4.0
7.25	18.3	66.6	3.6

of the acetylation rate constant should be parallelled by pH-dependent changes in the value of K_m'. As seen in Table V, in the absence of Ca^{2+}, there is a more than 3-fold pH-dependent (pH 8—pH 7.25) decrease of k_{+2}, whereas the K_m' value does not change in a parallel manner. In the presence of Ca^{2+}, there is a 1.4-fold decrease in K_m' with decreasing pH, concomitant with a 2.2-fold decrease in value of k_{+2}. These results suggest in determining the value of K_m' that, in the absence of Ca^{2+}, the acetylation rate constant may not contribute significantly and therefore that K_m' may approximate K_s. Since in the presence of Ca^{2+} the extent of the contribution of k_{+2} to the value of K_m' is less apparent, it is not clear to what extent that change in K_m' due to Ca^{2+} may be due to changes in k_{-1} and/or k_{+1}. In addition, Table VI indicates that for the smaller, 60 000-dalton brain enzyme form, the K_m is higher in the presence of Ca^{2+} at all pH

TABLE VI

INFLUENCE OF pH AND Ca^{2+} ON THE K_m FOR ACETYLCHOLINE HYDROLYSIS BY THE 60 000-DALTON BRAIN ACETYLCHOLINESTERASE FORM

pH	$10^4 K_m$ (M)		$\frac{K_m + Ca^{2+}}{K_m - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
8.00	2.3	12.0	5.2
7.75	3.1	13.0	4.2
7.50	2.6	11.0	4.2
7.25	2.0	7.8	3.9

TABLE VII

INFLUENCE OF pH AND Ca^{2+} ON THE K_m AND ON k_{+2} (25°C) FOR ACETYLCHOLINE HYDROLYSIS BY THE 60 000-DALTON BRAIN ACETYLCHOLINESTERASE FORM

pH	$10^4 K_m$ (M)		$\frac{K_m + Ca^{2+}}{K_m - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
7.75	2.9	29.0	10.2
7.50	3.9	17.0	4.2
7.25	1.8	7.8	4.3

pH	$10^{-4} k_{+2}$ (s^{-1})		$\frac{k_{+2} + Ca^{2+}}{k_{+2} - Ca^{2+}}$
	Without Ca^{2+}	With Ca^{2+}	
7.75	10.8	36.1	3.4
7.50	15.3	22.1	1.4
7.25	9.5	14.6	1.5

values. The pH dependence of K_m' and the effect of Ca^{2+} on K_m' can be further analyzed by examining the influence of Ca^{2+} on k_{+2}. As seen in Table VII, in the absence of Ca^{2+}, there appears a pH dependent biphasic change in K_m'. This pH-dependent change is paralleled by changes in k_{+2}. In the presence of Ca^{2+} an approximate 3-fold decrease in K_m' is also observed in the estimated k_{+2} value. These results suggest that k_{+2} may contribute significantly to the value of K_m' and note a difference in behavior between the 240 000- and 60 000-dalton forms of the enzyme.

Discussion

The bell-shaped pH-activity profile for acetylcholinesterase has been interpreted as representing two essential ionizable groups at the active site. One titratable group with a pK of about 6.7 has been suggested to be an imidazole group, associated with an essential active site histidine [17,30]. The involvement of the serine hydroxyl moiety has been suggested both by a second pK value of approximately 9.5 and by the recovery of serine phosphate from hydrolysates of di-isopropylphosphonofluoridate inhibited enzyme [31,32]. In the present study we have examined the Ca^{2+} mediated acceleration of acetylcholine hydrolysis catalyzed by the 60 000- and the 240 000-dalton brain acetylcholinesterase forms. Examiniation of Ca^{2+} acceleration has included analysis of Ca^{2+} influences on acetylation and deacetylation as well as effects on the apparent reaction pK, K_m, and K_m'.

Deacetylation was found to be the major rate-influencing step in acetylcholine hydrolysis catalyzed either by the 60 000- or the 240 000-dalton brain enzyme form. This finding was in accord with that proposed for eel acetylcholinesterase-catalyzed acetylcholine hydrolysis [28]. In the case of both brain enzyme forms, Ca^{2+} enhances deacetylation by about 30% over the pH range 7.25—8.0. With the 60 000-dalton form the acceleration effect on deacetylation (k_{+3}) parallels the Ca^{2+} effect on the ratio of the overall rate constant, k,

with/without Ca^{2+} and occurs without an effect on the pK. By contrast, the ratio of the overall rate constant, k, with/without Ca^{2+} for the 240 000-dalton brain enzyme form increases from 1.33 to 1.81 as the pH decrease from 8.0 to 7.25 despite a constant 30% acceleration of deacetylation over the same pH range. A pK shift of approximately 0.2 unit is noted for the 240 000-dalton form. It is suggested that a combination of the 30% enhancement of k_{+3} as well as the pK shift appears to account for the pH dependent degree of Ca^{2+} acceleration in the 240 000-dalton form. As shown in Fig. 3, a control curve a represents the pH vs. rate profile in the absence of Ca^{2+} (ideal ionization curve fit to data points). Curve b is the ideal curve form curve a, shifted 0.2 pH units, representing the 0.2-unit pK shift observed with Ca^{2+} in the larger brain form. Finally, this curve is shifted upwards by a factor of 1.3, accounting for the Ca^{2+}-mediated 30% increase in deacetylation (curve c). The observed experimental points in the presence of Ca^{2+} (solid circles) show a coincidence with the theoretical curve which argues in favor of Ca^{2+} effect on both pK and deacetylation as possible mechanisms for the pH-dependent degree of Ca^{2+} acceleration in the 240 000-dalton form. Since the Ca^{2+} effects on acetylation and deacetylation are similar in both the 60 000- and 240 000-dalton forms, the major distinguishing feature in terms of Ca^{2+} acceleration appears to be the apparent pK shift in the higher mol. wt. species. The pK shift may reflect the different pH dependence of k_{+2} ($\pm Ca^{2+}$).

Although employing the pH dependence curve of enzyme activity to estimate ionization constant of groups involved in the catalytic process is common, it must be recognized that the estimated values are derived from the overall velocity constant. The rate constant of any equilibrium step prior to the rate determining step will become part of the kinetically determined pK value. As a result, the change in the apparent pK (0.2 pH unit) with Ca^{2+} may not repre-

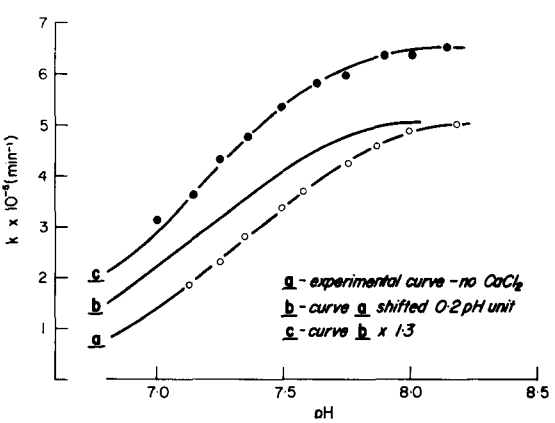

Fig. 3. Reaction rate versus pH profiles of the 240 000-dalton brain acetylcholinesterase-catalyzed acetylcholine hydrolysis. Enzyme activity was measured by the pH-stat method with standard assay conditions of 0.01% gelatin, 1.2 mM acetylcholine, $1.62 \cdot 10^{-12}$ mol acetylcholinesterase, at 20°C in a total volume of 100 ml. ○, in the absence of added ions; ● in the presence of 10 mM $CaCl_2$. The control curve of Ca^{2+} (ideal ionization curve fit to data points). Curve b is the ideal curve from curve a shifted 0.2 pH units, representing the 0.2-unit pK shift observed with Ca^{2+}. This theoretical curve is shifted upwards by a factor of 1.3 (curve c), accounting for the Ca^{2+} mediated 30% increase in deacetylation. The observed experimental points in the presence of Ca^{2+} (solid circles) show a coincidence with the theoretical curve.

sent a change in the ionization constant of a particular group at the active centers, but could reflect an alteration in the relative importance of the different steps in determining the overall rate constant [33,34].

In the present study both acetylation and the rate-determining deacetylation step are enhanced by Ca^{2+}. Thermodynamic analysis suggests that in considering acceleration, over the pH range 7.0—8.0 for both enzyme forms, the Ca^{2+}-mediated acceleration of acetylation is entropically driven. However, over the same pH range, it is not possible to resolve the entropic and enthalpic contribution to the Ca^{2+}-mediated acceleratory effect on deacetylation. In the case of the rate-limiting deacetylation step, the unfavorable entropy of activation, suggesting a very loose structure for the acetyl enzyme relative to the high degree of ordering of the protein and the reactant water molecule, is made somewhat less unfavorable in the presence of Ca^{2+}.

The multiplicity of brain acetylcholinesterase forms has been noted by others [35—37] and it has been suggested that the higher mol. wt. forms result from the association of low mol. wt. forms. As a consequence, the differences in kinetic behavior may occur as a result of molecular association of forms. Curavture of the Arrhenius plot for acetylcholinesterase-catalyzed acetylcholine hydrolysis has been noted for brain, eel [28], and erythrocyte enzyme [16]. However, a linear plot has been reported for insect acetylcholinesterase [38]. Differences in Arrhenius plot behavior may reflect fundimental mechanistic/kinetic differences between forms of acetylcholinesterase.

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References

1 Roufogalis, B.D. and Quist, E.E. (1972) Mol. Pharmacol. 8, 41—49
2 Marquis, J.K. and Webb, G.D. (1974) Biochem. Pharmacol. 23, 3459—3465
3 Gordon, M., Chan, S.L., Settle, W. and Trevor, A.J. (1976) Proc. Am. Soc. Biol. Chem. 35, 1733
4 Kitz, R.J., Braswell, L.M. and Ginsburg, S. (1970) Mol. Pharmacol. 6, 108—121
5 Roufogalis, B.D. and Wickson, V.M. (1975) Mol. Pharmacol. 11, 352—360
6 Taylor, P. and Lappi, S. (1975) Biochemistry 14, 1989—1997
7 Chan, S.L., Shirachi, D.Y., Bhargava, H.N., Gardner, E. and Trevor, A.J. (1972) J. Neurochem. 19, 2747—2758
8 Chan, S.L., Gardner, E. and Trevor, A.J. (1973) Proc. West. Pharmacol. Soc. 19, 181
9 Gardner, E., Chan, S.L., Thorne, D.R. and Trevor, A.J. (1975) Biophys. J. 15, 50a
10 Gordon, M., Chan, S.L. and Trevor, A.J. (1976) Biochem. J. 157, 69—76
11 Katz, B. and Miledi, R. (1965) Proc. R. Soc. 161, 496—503
12 Changeux, J.P., Podleski, T. and Meunier, J.C. (1969) J. Gen. Physiol. 54, 225s—244s
13 Katz, B. and Miledi, R. (1965) Proc. R. Soc. 161, 453—482
14 Katz, B. and Miledi, R. (1973) J. Physiol. 231, 549—574
15 Kayne, F.J. and Swelter, C.H. (1965) J. Am. Chem. Soc. 87, 897—900
16 Heilbronn, E. (1965) Acta Chem. Scand. 19, 1333—1346
17 Wilson, I.B. and Bergman, F. (1950) J. Biol. Chem. 185, 479—489
18 Wilson, I.B., Bergman, F. and Nachmansohn, D. (1950) J. Biol. Chem. 186, 781—790
19 Kaufman, S., Neurath, J. and Schwert, G.W. (1949) J. Biol. Chem. 177, 792—814
20 Butler, J.A.V. (1941) J. Am. Chem. Soc. 63, 2971—2974

21 Snoke, J.E. and Neurath, J. (1950) J. Biol. Chem. 182, 577—584
22 Gutfreund, J. (1972) Enzymes: Physical Principles, pp. 162—163, Wiley-Interscience, New York
23 Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88—95
24 Bohlen, P., Stein, S., Dairman, W. and Udenfriend, S. (1973) Arch. Biochem. Biophys. 155, 213—220
25 Roufogalis, B.D. and Thomas, J. (1968) Mol. Pharmacol. 4, 181—186
26 Dixon, M. (1953) Biochem. J. 55, 161—170
27 Cleland, W.W. (1967) Adv. Enzymol. 29, 1—32
28 Wilson, I.B. and Cabib, E. (1956) J. Am. Chem. Soc. 78, 202—207
29 Laidler, K.J. and Bunting, P.S. (1973) The Chemical Kinetics of Enzyme Action, 2nd edn., pp. 196—232
30 Spencer, T. and Sturtevant, J.M. (1959) J. Am. Chem. Soc. 81, 1874—1882
31 Schaffer, N.K., May, S.C. and Summerow, W.H. (1953) J. Biol. Chem. 202, 67—76
32 Schaffer, N.K., Michel, H.O. and Bridges, A.F. (1973) Biochemistry 12, 2946—2950
33 Bruice, T.C. and Schmir, G.L. (1959) J. Am. Chem. Soc. 81, 4552—4556
34 Jencks, W.P. (1969) Catalysis in Chemistry & Enzymology, pp. 471—477, 509—597, McGraw Hill, New York
35 Jackson, R.L. and Aprison, N.H. (1966) J. Neurochem. 13, 1351—1365
36 McIntosh, C.H.S. and Plummer, D.T. (1973) Biochem. J. 133, 655, 665
37 Adamson, E.D., Ayers, S., Deussen, Z.A. and Graham, C.F. (1975) Biochem. J. 147, 205—214
38 Hellenbrand, K. and Krupka, R.M. (1970) Biochemsitry 9, 4665—4672