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Catalysis by Acetylcholinesterase. The Rate-Limiting Steps Involved in the Acylation of Acetylcholinesterase by Acetic Acid Esters and Phosphorylating Agents

Terrone L. Rosenberry

Departments of Biochemistry and Neurology, College of Physicians and Surgeons, Columbia University, New York, N. Y., USA

Inferences about the catalytic mechanism of acetylcholinesterase are frequently made on the basis of a presumed analogy with chymotrypsin. Although both enzymes are serine hydrolases, several differences in the steady-state kinetic properties of the two have been observed. In this report particular attention is focused on the second-order reaction constant, k_{cat}/K_{app}. While the reported pH dependence and deuterium oxide isotope effect associated with this parameter for chymotrypsin are generally consistent with simple models involving rate-limiting general acid-base catalysis, this study finds a more complicated situation with acetylcholinesterase. The apparent pK_a of k_{cat}/K_{app} for acetylcholinesterase varies between 5.5 and 6.3 for *neutral* substrates and involves non-linear inhibition by H^+. Deuterium oxide isotope effects of k_{cat}/K_{app} range from 1.1 for acetylcholine to 1.9 for *p*-nitrophenyl acetate. The bimolecular reaction rate appears rate-limiting for acetylcholine at low concentrations, while a *rate-limiting induced-fit* step is proposed to account for apparent pK_a values and low deuterium oxide isotope effects associated with low concentrations of phenyl acetate and isoamyl acetate. Other neutral acetic acid esters give apparent pK_a values and deuterium oxide isotope effects consistent with rate-limiting general-base catalysis at all substrate concentrations. The pH dependence of second-order acylation by two phosphorylating agents was also examined; one of these agents, diisopropylfluorophosphate, gave the very unusual observation of an increase in acylation rate as the pH decreased from 6 to 5.

INTRODUCTION

Acetylcholinesterase (E. C. 3.1.1.7) is classified as a serine hydrolase along with other esterases and peptidases which show essentially irreversible active-site phosphorylation[1]. Equivalent-weight determinations and peptide analysis indicate that phosphorylation occurs only at a single serine residue, and the amino acid sequence about this residue shows significant homology among the enzymes in this class[1,2]. Three-dimensional structures of several serine hydrolases have been determined by X-ray crystallography, and further striking structural similarities have thus been revealed[3]. The native polypeptide conformations of chymotrypsin, trypsin, and elastase are nearly superimposable. Blow *et al.*[4] have reported that a dominant feature of these structures is a »charge-relay« system of hydrogen bonds formed by the active

site serine hydroxyl, a histidine imidazole side chain and a carboxylate side chain in linear array. The evolutionary importance of this charge relay is demonstrated by its identification in subtilisin and other serine hydrolases structurally unrelated to chymotrypsin[3].

The discovery of the charge-relay structure concides with previous inferences about the catalytic mechanism of serine hydrolases from kinetic studies. A minimal catalytic mechanism involves the enzyme species in Scheme 1.

$$\begin{array}{ccccccc} E & + RX & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E \cdot RX & \xrightarrow{-X,\ k_2} & ER & \xrightarrow{+H_2O,\ k_3} E + ROH \\ + & & & + & & + & \\ H & & & H & & H & \\ K_{a1} \Updownarrow & & & K'_{a1} \Updownarrow & & K''_{a1} \Updownarrow & \\ EH & + RX & \rightleftharpoons & EH \cdot RX & & ERH & \end{array}$$

The proposed intermediates include the initial Michaelis complex E · RX and the acyl enzyme ER, for which evidence has long been obtained[1,5,6]. The pH dependence of substrate hydrolysis for chymotrypsin and other serine hydrolases suggests general acid-base catalysis by a group in the free enzyme with a pK_{a1} of 6 to 7. The deacylation rates k_3 of series of acyl chymotrypsins follow a Hammett relationship with a positive ρ value similar to that observed for general base catalysis of the non-enzymic hydrolysis of corresponding model esters[7], and the k_3 is typically reduced in deuterium oxide by a factor of two to three[8]. The second-order chymotrypsin acylation rates for a series of substituted phenyl acetates also follow a positive ρ (ref. 9). These observations suggest that general base catalysis occurs during the rate-limiting step for both acylation and deacylation of chymotrypsin. For the substituted phenyl acetates there appears to be concomitant loss of the leaving group X during acylation, but more extensive investigation indicates that partial protonation generally accompanies loss of the leaving group[10]. Crystallographic analysis of acyl chymotrypsins has suggested that HIS-57 acts successively during acylation, first as a general base for the attack of SER-195 on the carbonyl carbon and then as a general acid to assist loss of the leaving group[11]. Because of the symmetry of the proposed action of HIS-57, deacylation is presumed to occur by a similar process.

TABLE I.

Apparent pK_a *Values Associated with Acetic Acid Ester Substrates*

Substrate	k_{cat}/K_{app}		k_{cat}	
	pK_1	pK_2	pK''_1	pK''_2
Acetylcholine 0.1 *M* NaCl	6.3	> 10.5	6.5	> 10.5
Phenyl acetate 0.1 *M* NaCl	5.5	10.5	6.56	> 10.5
1 *M* NaCl	5.5	9.8	6.1	10.1

Values were determined as outlined in the Methods.

In the absence of the three-dimensional structure for acetylcholinesterase, inferences about its mechanism have been based both on studies of its substrate catalysis and on analogies drawn from chymotrypsin[1,12,13]. Yet aside from the similarities which classify it as a serine hydrolase, acetylcholinesterase differs significantly from chymotrypsin both in size and in catalytic properties. Acetylcholinesterase 11 S is a tetramer of essentially identical catalytic subunits, each with a molecular weight[14] of about 75 000. The active site includes an anionic group which aids in the binding of cationic substrates[1,12,13]. It is also an *esterase* as opposed to a *peptidase;* acetyl-acetylcholinesterase is hydrolyzed some 10^6 times faster than acetylchymotrypsin[15] (Table II). Although chymotrypsin is rapidly acylated by specific ester substrates[16], its catalytic machinery appears to have evolved for the particular stabilization of the transition state for acylation with specific amides, especially those with amino acid amide leaving groups[17]. One might anticipate that the catalytic site structure of acetylcholinesterase would place greater emphasis on the presumably base-catalyzed initial attack of the enzyme nucleophile and relatively less on the presumably acid-catalyzed loss of the (protonated) alkoxide leaving group. As a consequence, steps in the catalytic pathway either prior to or subsequent to general base catalysis may become rate-limiting.

To decide whether the mechanism in Scheme 1 can adequately account for observations on acetylcholinesterase, some properties of the experimental parameters derived from Scheme 1 may be examined. Under steady-state conditions of substrate hydrolysis ([RX] $\gg E_{tot}$, where E_{tot} is the enzyme normality), the kinetic parameters k_{cat} and K_{app} are defined according to eq. 1.

$$v = \frac{k_{cat} E_{tot}}{1 + \frac{K_{app}}{[RX]}} \tag{1}$$

The pH dependence of k_{cat}/K_{app} (the second order rate) and k_{cat} (the first order rate) have long been formulated for Scheme 1 under the assumption that all reversibly-linked species are equilibrated[18]. If protonation of a single group inhibits enzyme acylation, as indicated in Scheme 1, the pH dependence of k_{cat}/K_{app} gives the pK_{a1} of this group in the free enzyme while the pK_a of k_{cat} is a weighted average of the pK'_{a1} and pK''_{a1} for this group in E · RX and ER[13]. The pH dependence of chymotrypsin is quite consistent with this formulation. A pK_{a1} of 6.8 for k_{cat}/K_{app} is observed for virtually all chymotrypsin substrates[8,19]; a similar pK'_{a1} is obtained for most E · RX species and a pK''_{a1} of 7.0 to 7.2 is seen with acetylchymotrypsin ER[8]. A much greater variation in apparent pK_a values has been reported for partially purified bovine erythrocyte acetylcholinesterase by Krupka[20,21]. The pH dependence of k_{cat}/K_{app} varied with the charge of the leaving group. For cationic substrates the apparent pK_{a1} for k_{cat}/K_{app} was about 6.3, while for neutral substrates pK_{a1} varied between 5.3 and 5.5. From all substrates for which acetylation is rate-determining, a pK_{a1} of 5.2 to 5.5 for E · RX was inferred, while deacetylation-limited substrates indicated a pK''_{a1} of 6.3 for the acetyl enzyme ER. Of particular interest in these studies is the suggestion that protonation of *two* groups in the free enzyme can affect activity. From

additional information discussed elsewhere[13,22], Krupka[21] proposed that these two groups represent different histidine residues which act successively as general acid-base groups during acylation and deacylation.

In this report a variability in the pK_{a_1} of k_{cat}/K_{app} for several substrates of the eel 11 S enzyme is confirmed[22]. An alternative explanation to the proposal of two catalytic groups will be examined. This explanation postulates that the rate-limiting step for acylation with several substrates occurs *prior to* general base catalysis. In particular, the pH dependence and the deuterium isotope effect associated with k_{cat}/K_{app} for several substrates of highly purified eel 11 S acetylcholinesterase will be reported and discussed.

MATERIALS AND METHODS

Acetylcholinesterase from electric organs of eel *Electrophorus electricus* was purified as an 11 S species free of detectable protein contaminants as described previously[14]. Acetic acid ester substrates were commercially available reagent grades; phenyl acetate and isoamyl acetate were redistilled and *p*-nitrophenyl acetate was recrystallized before use. *p*-Nitrophenyldiethylphosphate (Paraoxon) and deuterium oxide were from Sigma. Diisopropylfluorophosphate (DFP) was a gift from Dr. Sara Ginsburg. Steady-state kinetic investigations at 25 °C and computer analyses of the resultant $1/v$ *versus* $1/[RX]$ plots were carried out essentially as described previously[23]. The reciprocal of the slope of such plots gives the relative k_{cat}/K_{app}, and the reciprocal of the intercept, the relative k_{cat}. Reaction rates were measured either titrimetrically or spectrophotometrically as described elsewhere[22].

The second order phosphorylation rates for *p*-nitrophenyldiethylphosphate and diisopropylfluorophosphate were determined by monitoring the simultaneous hydrolysis of either methyl acetate or *p*-nitrophenyl acetate. Conditions were defined such that the ratio of the velocity of hydrolysis of the acetic acid ester (at a concentration far below its K_{app}) to the *free* enzyme normality was effectively constant during the phosphorylation reaction; this permitted direct continuous determination of the phosphorylation rate by a technique previously applied to carbamoylating agents[13,23,24].

The effects of pH or high ionic strength on the kinetic parameters k_{cat}/K_{app} and k_{cat} were assessed in terms of R. For pH studies, R is defined as the ratio of the value of the kinetic parameter at a given pH to that at pH=8.5. For ionic strength variations, R is the ratio at a given ionic strength to that at 0.1 M NaCl. Values of the apparent inhibition constants K_{a_1} and K''_{a1} were estimated visually from the initial slopes of plots[23] of $1/R$ versus $[H^+]$. Curvature in these plots precluded the computer analysis described previously.

RESULTS

General effect of pH on the hydrolysis of acetic acid esters

The pH dependence of phenyl acetate hydrolysis is shown in Fig. 1. Values of pK_a and pK''_{a1} estimated from these data are shown in Table I. A clear distinction of approximately one pK unit between pK_{a_1} and pK''_{a1} for phenyl acetate in 0.1 M NaCl is observed. Values of pK_{a_1} and pK''_{a1} for acetylcholine are also shown in Table I, and a much smaller difference is observed. The agreement of pK''_{a1} for both substrates is expected, because k_{cat} for both is thought to reflect the deacylation rate k_3 (refs. 1 and 12 and note similarity of k_{cat} in Table II). The difference in pK_{a_1} between phenyl acetate and acetylcholine is consistent with the observations of Krupka on the erythrocyte enzyme[20]; slight differences in assay solvent prevent a precise quantitative comparison of the two enzymes.

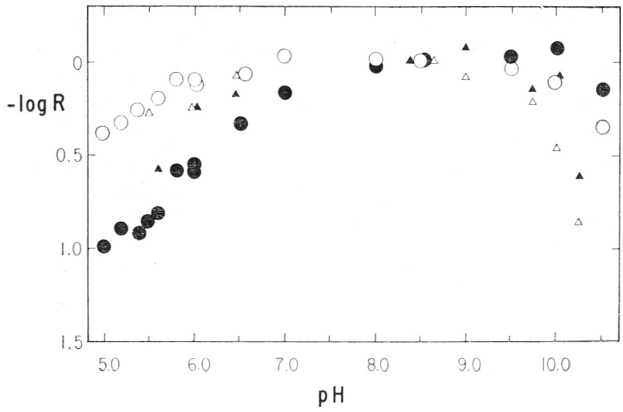

Fig. 1. The pH dependence of phenyl acetate hydrolysis by acetylcholinesterase. R values were calculated as outlined in the Methods for the following parameters:: (○) k_{cat}/K_{app} in k_{cat} 0.1 M NaCl; (△) k_{cat}/K_{app} in 1.0 M NaCl; (●) k_{cat} in 0.1 M NaCl; (▲) k_{cat} in 1.0 M NaCl.

The requirement of an acidic group with a pK_a of 9 to 10.5 for enzyme activity has been noted by several investigators[1]. The data in Table I with 0.1 M NaCl indicate that such a group has higher pK_{a_2} and pK''_{a2} values than in some previous estimates. No decrease in the apparent k_{cat}/K_{app} for acetylcholine is observed up to pH = 10.25, in contrast to a previous report[25]. Only when assays are conducted in 1.0 M NaCl do the pK_{a_2} and pK''_{a2} values for this group become clearly discernible.

In 1.0 M NaCl the apparent values of pK''_{a1} , pK_{a_2} and pK''_{a2} are reduced by some 0.5 pK units. A similar observation has been made for the pK_2 values associated with the dephosphorylation of acetylcholinesterase[26]. In 1.0 M NaCl significant binding of Na^+ to the catalytic anionic site may be inferred from its competitive inhibition of acetylcholine hydrolysis. The R value at pH = 8.5 for k_{cat}/K_{app} with acetylcholine in 1.0 M NaCl relative to 0.1 M NaCl is 0.26. This Na^+ binding however blocks neither phenyl acetate binding (the corresponding R is 1.47) nor that of isoamyl acetate or methyl acetate (T. L. Rosenberry and E. Bock, unpublished observations); it also has only a slight effect on deacylation (R values at pH = 8.5 for k_{cat} with acetylcholine and phenyl acetate in 1.0 M NaCl relative to 0.1 M NaCl are 0.90 and 0.85, respectively).

Detailed analysis of the pH *dependence of* k_{cat}/K_{app} *for neutral acylating agents*

Although the apparent pK_{a_1} values of 5.5 for phenyl acetate and 6.3 for acetylcholine in Table I coincide with the general assignments for neutral and cationic acetic acid ester substrates by Krupka[20], a detailed investigation in our laboratory with a series of neutral acetic acid esters and with *p*-nitrophenyldiethylphosphate indicates that such a classification does not hold[22]. Methyl acetate, *p*-nitrophenyl acetate and *p*-nitrophenyl phosphate all give pK_{a_1} estimates significantly greater than the 5.5 value for phenyl acetate and approach the 6.3 values for acetylcholine. The data for phenyl acetate and

p-nitrophenyl phosphate are shown in Fig. 2. The pH dependence of k_{cat}/K_{app} for methyl acetate and *p*-nitrophenyl acetate closely follow the pH dependence of second-order phosphorylation with *p*-nitrophenyl phosphate[22]. The precise pK_{a_1} values are difficult to determine because the appropriate $1/R$ values do not increase linearly with $[H^+]$. Instead, the values of k_{cat}/K_{app} at low pH for all substrates studies are higher than those anticipated for linear inhibition by $[H^+]$, an observation also made previously[20]. Estimates of pK_{a_1} from the initial slopes of $1/R$ *versus* $[H^+]$ are included in Table II.

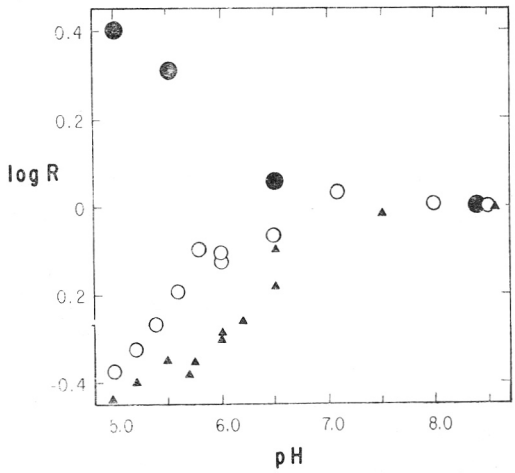

Fig. 2. The pH dependence of second-order acylation of acetylcholinesterase by phenyl acetate and by two phosphorylating agents. R values for k_{cat}/K_{app} for phenyl acetate in 0.1 M NaCl are transferred from Fig. 1 (○). R values for second-order phosphorylation rates in 0.1 M NaCl with either *p*-nitrophenyldiethylphosphate (▲) or diisopropylfluorophosphate (●) were obtained as outlined in the Methods. For 18 μM *p*-nitrophenyldiethylphosphate the second-order phosphorylation rate was observed to be 1.8×10^3 M^{-1} s^{-1}; 120 μM diisopropylfluorophosphate gave a second-order phosphorylation rate of 1.3×10^2 M^{-1} s^{-1}. Phosphorylation data were obtained with both methyl acetate and *p*-nitrophenylacetate for *p*-nitrophenyldiethylphosphate and with methyl acetate for diisopropylfluorophosphate.

The pH dependence of second-order phosphorylation with diisopropylfluorophosphate differs qualitatively from that of all other acylating agents. The rate increases on decreasing the pH from 6 to 5.

Deuterium Oxide Effects on k_{cat}/K_{app} and k_{cat}

The values of k_{cat}/K_{app} and k_{cat} in D_2O relative to the corresponding values in H_2O are also given in Table II. Because of the broad pH maximum shown by acetylcholinesterase, the deuterium oxide effects on the kinetic parameters observed at pH = 8.5 should be little affected by deuterium isotope effects on the pK_a values of enzyme catalytic groups. A deuterium oxide effect on k_{cat} of somewhat greater than 2 is observed both for acetylcholine and phenyl acetate for which $k_{cat} \approx k_3$, and for substrates for which $k_{cat} < k_3$. The observed effect on k_{cat} for phenyl acetate is consistent with a previous report[8] of 2.3. In contrast, deuterium oxide effects for k_{cat}/K_{app} vary from 1.1 for acetylcholine to 1.9 for *p*-nitrophenyl acetate. To our knowledge, only one other example of a deuterium isotope as low as 1.1 for either k_{cat} or k_{cat}/K_{app} for a serine hydrolase has been reported, and that concerns a series of very poor substrates for

TABLE II.

*Deuterium Isotope Effects on k_{cat}/K_{app} and pK_i of k_{cat}/K_{app} for Several Substrates of Acetylcholinesterase**

Substrate	rel. k_{cat}	$k_{cat}(H_2O)/k_{cat}(D_2O)$	log k_{cat}/K_{app}	$k_{cat}/K_{app}(H_2O)/k_{cat}/K_{app}(D_2O)$	pK_{a_1} of k_{cat}/K_{app}
Acetylcholine	100	2.34 ± .15 2.43 ± .43[a]	8.2	1.07 ± .10 1.23 ± .20[a]	6.3
Phenyl acetate	107	2.04 ± .28[b] 2.32 ± .23[c]	6.9	1.23 ± .17[b] 1.46 ± .10[c]	5.5
Isoamyl acetate	13	2.84 ± .70	5.8	1.26 ± .28	5.6
p-Nitrophenyl acetate	8	2[d]	5.4	1.93 ± .07	6.2
Methyl acetate	—	—	3.1	2[d]	6.0
p-Nitrophenyl diethylphosphate	—	—	—	—	6.1

* Unless otherwise noted, values of k_{cat} were observed at pH = 8.5, 0.1 M NaCl, and are defined relative to an arbitrary value of 100 for the k_{cat} of acetylcholine. Values of k_{cat}/K_{app} are the observed second order rate constants (M^{-1} s^{-1}) at pH = 8.5, 0.1 M NaCl, assuming an actual value of k_{cat} of acetylcholine of 1.6×10^4 s^{-1} (ref. 13). Values of pK_{a_1} were obtained as outlined in the Methods. The error values for the isotope effects are the square root of the estimated variance, determined for the ratio as outlined previously.[22]

[a]Solvent contained 1.0 M NaCl at pH = 8.5. [b]pH stat assay. [c]Spectrophotometric assay. [d]An insufficient number of observations were made to statistically analyze these values.

chymotrypsin[26]. While the number of substrates analyzed here is small, it is noteworthy that neutral substrates with deuterium isotope effects for k_{cat}/K_{app} of less than 1.4 are associated with the lowest apparent pK_{a_1} values for k_{cat}/K_{app}.

DISCUSSION

The investigation of the mechanism of catalysis by acetylcholinesterase described in this report is limited primarily to the properties of the second-order acylation rate constant. For substrates which achieve a steady-state hydrolysis rate, this constant is defined as k_{cat}/K_{app} according to eq. 1; however, the second-order acylation rate given by virtually irreversible phosphorylating agents has an identical mechanistic formulation[13], and the acylation properties of both classes of enzyme reagents are considered here.

The pH dependence of k_{cat}/K_{app} for virtually all substrates of chymotrypsin (at pH $\leqslant$ 9) is in accord with the model in Scheme 1[8,19], and k_{cat}/K_{app} for these substrates generally shows a deuterium isotope effect which further indicates that the rate-limiting step k_2 involves general-base catalysis[8,27]. In contrast, the properties of the second-order acylation rates with acetylcholinesterase suggest a mechanism more complex than Scheme 1. The apparent pK_{a_1} for k_{cat}/K_{app} varies among a variety of acetylcholinesterase substrates; a value of 5.5 observed for phenyl acetate is clearly distinct from the 6.3 value obtained with acetylcholine, and a continuum of pK_{a_1} values between 5.5 and 6.3 is observed for several other acylating agents. In addition the inhibition by $[H^+]$ which defines this pK_a is non-linear; that is, K_{app}/k_{cat} does not increase as a linear function of $[H^+]$.

Two formal ways of extending the model in Scheme 1 may be considered to account for the observations on k_{cat}/K_{app} for acetylcholinesterase substrates[22]. In the first protonation of either of two distinct groups in the free enzyme can affect the activity. The proposal of two histidine residues by Krupka[21] was noted in the Introduction, but formally equivalent is the alternative postulate that the two residues represent a single histidine residue and the carboxylate residue which defines the anionic site within the catalytic site. The details of these proposals are given elsewhere[22]. Either of these proposals would explain the observed pH dependence of k_{cat}/K_{app}, but only with several assumptions. Thus, the variation in the apparent pK_{a_1} values among neutral substrates can be explained if the protonation of an apparent $pK_{a_1} = 6.3$ group reduces the binding of some, but not all, neutral substrates; and non-linear inhibition by H^+ could arise from a perturbation of the actual pK_{a_1} values of these groups either by mutual interaction between them or by interaction with other groups at the catalytic site[20]. However, these proposals cannot account for the low deuterium oxide effects observed for k_{cat}/K_{app} for several acetylcholinesterase substrates (Table II).

It is of interest to consider the formulation of an alternative explanation in which the influence of kinetic rate constants both alters the actual pK_a value of a single catalytic group of $pK_a = 6.3$ in the free enzyme and decreases the deuterium oxide effect on k_{cat}/K_{app}. For Scheme 1 only one type of pK_a perturbation appears possible. If virtual equilibrium does not obtain ($k_2 > k_{-1}$), $k_{cat}/K_{app} = k_1$ (refs. 13, 28); the bimolecular rate constant k_1 becomes rate-limiting rather than the general base catalysis step k_2 at low substrate concentrations. As shown by Renard and Fersht[28], in this case the apparent pK_{a_1} for a basic group required for k_2 decreases by an amount directly related to k_2/k_{-1}. Such a mechanism accounts nicely for the low deuterium oxide effect on k_{cat}/K_{app} for acetylcholine. The extremely high value of k_{cat}/K_{app} for acetylcholine (Table II) is discussed elsewhere and suggests that $k_{cat}/K_{app} \approx k_1$ does hold for acetylcholine and other rapidly hydrolyzed cationic substrates of acetylcholinesterase[13]. The bimolecular reaction rate would be expected to have little or no deuterium oxide effect. Since protonation of the $pK_{a_1} = 6.3$ group appears to block the *binding* of cationic substrates and inhibitors[1,20], no kinetic perturbation of this pK_a is observed.

Certain neutral substrates, in particular, phenyl acetate and isoamyl acetate, give apparent pK_{a_1} values below 6.3 in the present data. However, a kinetic perturbation of pK_a arising from $k_2 > k_{-1}$ seems most unlikely in these cases. The value of k_{cat}/K_{app} for isoamyl acetate in Table II appears far too small for a bimolecular reaction. Furthermore, neutral and cationic carbamoylating agents, for which k_{cat}/K_{app} values are some 10^4 times smaller than those for acetic acid esters, show a discrepancy in pK_{a_1} values similar to that for acetic acid esters[29]. It would appear that kinetic contributions to pK_{a_1} would have to occur within a mechanism more complex than Scheme 1, one which permits virtual equilibrium of at least the initial Michaelis complex. Such a mechanism is presented in Scheme 2.

$$
\begin{array}{lllllll}
E & + RX \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot RX & \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} (E & \cdot & RX)' & \xrightarrow{-X,\ k_2} ER & \xrightarrow{+H_2O,\ k_3} E + ROH \\
+ & + & + & & & & \\
H & H & H & & & & \\
\Downarrow & \Downarrow & \Downarrow & & & & \\
EH + RH & \rightleftharpoons EH \cdot RH & \underset{k'_{-01}}{\overset{k'_{01}}{\rightleftharpoons}} (EH & \cdot & RH)' & &
\end{array}
$$

Scheme 2, which focuses on enzyme acylation, extends the simpler mechanism in Scheme 1 by introducing the intermediate $(E \cdot RX)'$. In particular, it may be shown[22] that if general base catalysis by a group with an actual pK_a of 6.3 occurs at k_2 subsequent to the rate-limiting formation of $(E \cdot RX)'$ (*i. e.* $k_2 > k'_{-1}$), then the apparent pK_a for k_{cat}/K_{app} is less than 6.3. In this case $(E \cdot RX)'$ is a second enzyme-substrate intermediate formed by a conformational change of the initial Michaelis complex and is identical to the induced-fit complex proposed by Koshland[30]. Induced-fit as a pre-equilibrium process has been considered likely for a number of enzymes[30] including acetylcholinesterase[13,31,32]; but this proposal of *rate-limiting induced-fit*, in which the rate of the limiting conformational change is substrate dependent, appears to be novel.

The equations which have been derived for the pH dependence of k_{cat}/K_{app} arising from Scheme 2 assume equilibria both among E, EH, $E \cdot RX$ and $EH \cdot RX$ and between $(E \cdot RX)'$ and $(EH \cdot RX)'$[13,22]. These equations have a form similar to those derived by Renard and Fersht for the simpler non-equilibrated formulation of Scheme 1[28]. This formulation of Scheme 2 can account for most of the current observations on k_{cat}/K_{app} for acetic acid esters. The apparent pK_a for k_{cat}/K_{app} is a function of rate constants which may be expected to vary among acylating agents, in agreement with the data in Table II.

For phenyl acetate and isoamyl acetate, substrates for which the apparent pK_a deviates farthest below 6.3, the relationship $k_2 > k'_{-1}$ obtains; at high pH the maximal $k_{cat}/K_{app} \cong k'_1/K_s$ (*cf.* eq. 3). Since neither k'_1 nor the initial binding constant K_s is expected to have as large a deuterium oxide effect as k_2, the lower deuterium oxide effects observed for k_{cat}/K_{app} with these substrates also are consistent with this catalytic model.

This formulation of Scheme 2 in principle can also account for non-linear inhibition of second-order acylation by H^+. If $K'_1 \neq k'_{01}$, a plot of K_{app}/K_{cat} versus $[H^+]$ is not linear but shows inflection points; if $k'_1 > k'_{01}$, the initial slope reflects the actual pK_a of the catalytic group. A graphical illustration of this point is given by Renard and Fersht[28], and this formalism can fit quite precisely the pH dependence of second-order methyl acetate and *p*-nitrophenyl acetate hydrolysis[22] (*cf.* Fig. 2). Other data, however, indicate that this is not the major source of non-linear inhibition by H^+. The deuterium oxide isotope effects on k_{cat}/K_{app} for methyl acetate and *p*-nitrophenyl acetate are approximately 2, significantly higher than those for the other substrates noted above and in the range expected for rate-limiting general-base catalysis. Furthermore, the pH dependence of k_{cat}/K_{app} for these substrates closely follows that for second-order phosphorylation with *p*-nitrophenyldiethylphosphate, an acyl-

ating agent for which, for reasons given in the following paragraph, general acid-base catalysis is thought to be rate-limiting.

Phosphorylating agents are of particular relevance to Scheme 2 in that, unlike other substrates and acylating agents, enzyme phosphorylation rates depend significantly on the quality of the leaving group and display positive rho values[32]. Consequently the rate-limiting step in Scheme 2 would appear to be the general base-catalyzed step k_2, and any induced-fit step would occur in a prior equilibrium. In this case, the apparent pK_a for second order phosphorylation should be the actual pK_a of the catalytic group. The correspondence of the pH dependence for phosphorylation with *p*-nitrophenyldiethylphosphate (Fig. 2) with that for k_{cat}/K_{app} for the two substrates which have a relatively large deuterium isotope effect associated with k_{cat}/K_{app} suggests that this pH dependence reflects the proton titration curve of the general acid-base catalyst in the free enzyme, presumably an imidazole residue. Although the pH dependence of k_{cat}/K_{app} for acetylcholine has not been investigated as thoroughly as those for neutral substrates, this pH dependence appears to be within experimental error of the same titration curve[22]. If this is the case, then protonation of the general acid-base catalytic residue appears to block cation binding to the free enzyme, a conclusion reached previously for the acetyl enzyme[20]. The presence of inflection points in this titration curve, as indicated by non-linear H^+ inhibition, cannot be explained by Scheme 2 and may reflect pK_a perturbations resulting from electrostatic interactions between this catalytic residue and other titratable groups at the catalytic site.

The pH dependence of a second phosphorylating agent, diisopropylfluorophosphate, is shown in Fig. 2 to involve an *increase* in rate on decreasing the pH from 6 to 5. No previous reports of a similar pH dependence for any acetylcholinesterase acylating agent have appeared. It is tempting to speculate that this pH dependence arises from interaction of the fluoride moeity of diisopropylfluorophosphate with a protonated group at the catalytic site. Fluoride ion is an inhibitor of acetylcholinesterase, and the apparent affinity of fluoride ion for the catalytic site increases dramatically below pH = 6[33,34]. No identification of the protonated group in the enzyme responsible for fluoride in binding has been made. Analysis of the pH dependence of acylation by diisopropylfluorophosphate requires a separation of the apparent dissociation constant K_{app} from the first order acylation constant k_a (ref. 13), probably by stopped-flow techniques. The observation is difficult to explain regardless of which parameter is responsible for the rate increase at low pH. If only the binding is increased at low pH, then this increased binding must reflect a productive binding interaction; non-productive substrate binding does not affect second-order acylation rates[35]. An increase in the rate constant k_a would imply acid catalysis of the phosphorylation, a notion difficult to reconcile with the evidence that k_2 is rate-limiting for phosphorylation for either Scheme 1 or Scheme 2. It is possible that fluoride is such a good leaving group that the induced-fit step k'_1 preceding k_2 in Scheme 2 is rate-limiting for diisopropylfluorophosphate (note curvature in the Hammett plot in reference 36). In this case an apparent acid catalysis of k_a could occur if $k'_1 < k'_{01}$.

In Fig. 3 a schematic visualization is offered as a summary of the induced-fit model in Scheme 2. This kinetic scheme allows several possible rate-limiting steps, and evidence detailed above indicates that the rate-limiting step differs

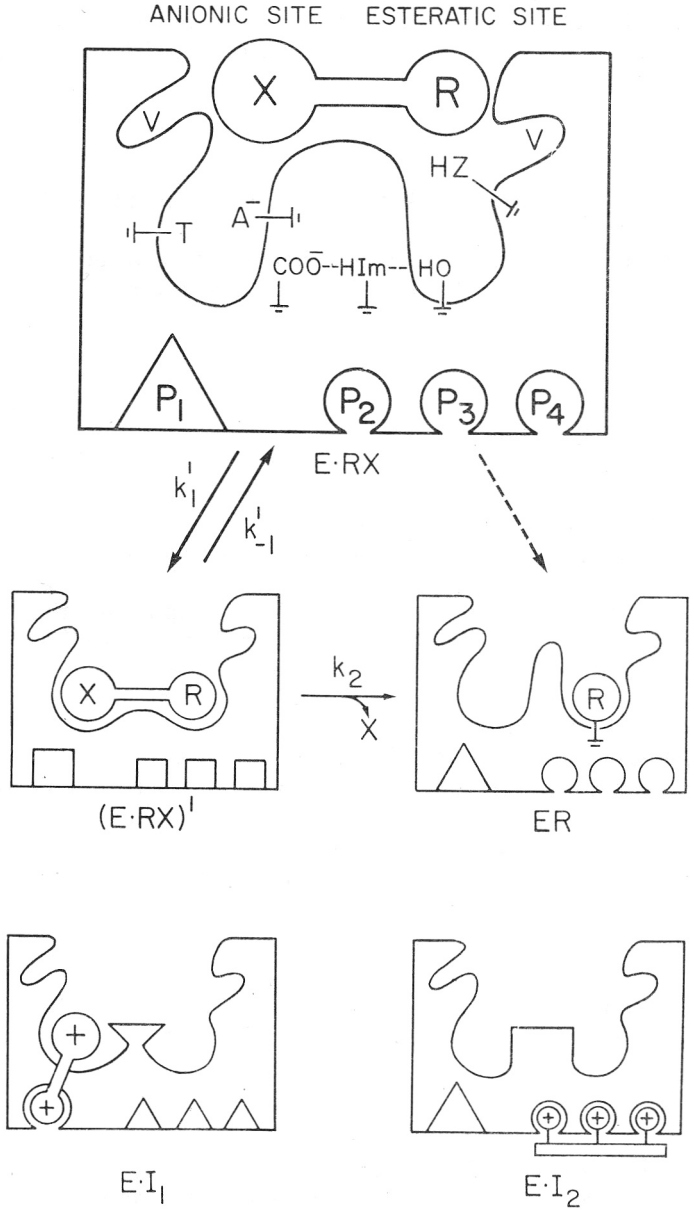

Fig. 3. Diagrammatic model of the acetylcholinesterase catalytic site based on the induced-fit mechanism in Scheme 2. The initial enzyme-substrate complex is E · RX; the induced-fit complex, (E · RX)'; and the acyl enzyme, ER. Residues identified or presumed at or near the catalytic site include the charge-relay complex COO^- - - HIm - - HO (ref. 4); an acidic group HZ; the anionic group A^- which defines the anionic site; a tryptophan residue T near the anionic site; and adjacent hydrophobic areas V. The binding of multivalent cationic ligands either to both the catalytic site and a peripheral site as in E · I_1 or exclusively to peripheral sites as in E · I_2 may affect the rate constants k_1' and k_{-1}' (See ref. 13 for further details.)

both among substrates and with the reaction conditions. For acetylcholine the formation of Michaelis complex E · RX appears to be rate-limiting at low substrate concentrations ($k_{-1} < k_2$ in Scheme 1 or $k_{-1} < k'_1$ in Scheme 2) such that eq. 2 obtains.

$$\frac{k_{cat}}{K_{app}} \approx k_1 \tag{2}$$

At high substrate concentrations $k_{cat} \cong k_3$ for acetylcholine, and deacetylation appears to be rate-limiting under these conditions.

The second-order acylation rates of other agents with acetylcholinesterase can be adequately accounted for by eq. 3, a formula derived for Scheme 2 at high pH.

$$\frac{k_{cat}}{K_{app}} = \frac{k_2 k_1'}{(k'_{-1} + k_2) K_s} \tag{3}$$

In eq. 3, $K_s = k_{-1}/k_1$. Thus, for phenyl acetate and isoamyl acetate, it was argued earlier that $k_2 > k'_{-1}$, implying that $k_{cat}/K_{app} \approx k'_1/K_s$ and that the induced-fit step is rate-limiting at low substrate concentrations. With other acetic acid esters the pH dependence and the deuterium isotope effect both suggest that general-base catalysis remains rate-limiting under all conditions. In this case $k_2 < k'_{-1}$; from eq. 3, $k_{cat}/K_{app} = k_2 k'_1/k'_{-1} K_s$, and any induced-fit complex occurs in equilibrium prior to k_2. Fig. 3 emphasizes that not all substrates need pass through the induced-fit complex (E · RX)′ to react. Phosphorylating agents, for example may react directly from the Michaelis complex; such a bypass of part of the catalytic machinery could explain the positive rho values indicative of unassisted general-base catalysis which distinguishes phosphorylation from other acylation reactions.

It is noteworthy that a substrate for which induced-fit is rate-limiting at low substrate concentration may show rate-limiting general base catalysis at high substrate concentration. This is apparent from eq. 4, where the expression for the first-order rate constant k_{cat} derived from Scheme 2 at high pH is given.

$$k_{cat} = \frac{k_1' k_2 k_3}{k_3 (k_1' + k_{-1}' + k_2) + k_2 k_1'} \tag{4}$$

For acylation-limited substrates ($k_3 > k_2$) for which rate-limiting induced-fit is suggested at low substrate concentrations ($k_2 > k'_{-1}$), eq. 4 simplifies to eq. 5:

$$k_{cat} = \frac{k_1' k_2}{k_1' + k_2} \tag{5}$$

Thus if $k'_1 > k_2$, the general base catalysis step k_2 may become rate-limiting at high substrate concentrations. The only substrate example to which eq. 5 applies in the current data is isoamyl acetate. The observation that k_{cat} for this substrate has a large deuterium oxide effect is consistent with rate-limiting k_2 at substrate saturation.

While Fig. 3 and Scheme 2 do not explicitly consider enzyme deacylation, it is of course necessary that all proposed intermediates on the acylation pathway also occur during deacylation. The deuterium oxide isotope effects observed for deacetylation (Table II) suggest that general-base catalysis is rate-limiting.

Any induced-fit acetylenzyme species thus must occur in an equilibrium prior to this rate-limiting step, and any subsequent product complexes formed must break down faster than this step.

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DISCUSSION

E. Reiner:

How are the kinetic properties of the enzyme related to its molecular structure?

T. L. Rosenberry:

The enzyme is, of course, designed to be an efficient catalyst of ester hydrolysis. Despite the fact that the enzyme is a tetramer with a molecular weight of 300 000, we have obtained no evidence for subunit cooperativity during catalysis. The kinetic properties I have been concerned with in this report relate to specific and probably small conformational changes at an individual catalytic site. If the enzyme-substrate complex can undergo an induced-fit conformational change, the specificity and the catalytic efficiency of the enzyme may be increased as emphasized originally by Koshland. If the efficiency of the general-base catalysis step is thus increased to the extent that reaction steps either prior to or subsequent to general-base catalysis become rate-limiting, unusual kinetic properties may be observed. It is in fact the rather novel pH dependencies and deuterium oxide isotope effects observed for acetylcholinesterase that have led to our proposal.

W. N. Aldridge:

Could you explain how your model can explain how some substrates for acetylcholinesterase increase its rate of reaction with methanesulfonyl fluoride?

T. L. Rosenberry:

Acetylcholinesterase has long been known to possess esteratic and anionic portions within a single catalytic site, and there are numerous examples in which both sites may be occupied simultaneously. If such a ternary complex involving the binding of methanesulfonyl fluoride at the esteratic site and an appropriate cationic substrate at the anionic site can form, the local conformation of the esteratic site may be altered so as to increase the acylation rate. Such an observation is quite nicely accounted for by an induced-fit model in which binding of cationic ligand at the anionic site induces a more active conformation at the esteratic site.

E. A. Barnard:

Can you distinguish your suggestion for the additional step, which is in fact an isomerization of the enzyme-substrate complex, from the alternative type of mechanism discussed by others in the past for other enzymes, i. e., a prior isomerization of the active free enzyme? Cannot your data on variations in the pH value derived from the pH dependencies of k_{cat}/K_{app} be alternatively interpreted on that basis.

T. L. Rosenberry:

As long as one assumes that the bimolecular reactions between substrate and any enzyme isomer are not rate-limiting, the answer is no. A general scheme which includes a potential isomerization of either the free enzyme or the enzyme-substrate complex is given in Scheme 3.

$$\begin{array}{ccc} \mathrm{E} + \mathrm{RX} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & \mathrm{E} \cdot \mathrm{RX} \\ k_o \uparrow\downarrow k_{-o} & & k'_1 \uparrow\downarrow k'_{-1} \\ \mathrm{E}' + \mathrm{RX} & \underset{k_{-p}}{\overset{k_p}{\rightleftharpoons}} & \mathrm{E} \cdot \mathrm{RX}' \xrightarrow{k_2} \mathrm{E} + \text{products} \end{array}$$

This scheme ignores proton equilibria and extends Scheme 2 by introducing the »induced« free enzyme species E′. We now want to consider all conditions under which k_{cat}/K_{app} is *not* proportional to k_2, *i. e.*, where k_2 is not rate-limiting.

A general solution to Scheme 3 which assumes no rapid equilibrium steps results in rate equations too intractable for interpretation. Furthermore, it is clear from the text that k_2 may not be rate-limiting if $k_2 > k_{-p}$, a condition in which the

bimolecular reaction rate k_p can become rate-limiting. Your suggestion for an alternative mechanism can be directly examined by assuming equilibrated ligand binding (eqs. 6a and 6b).

$$[E \cdot RX] = \frac{k_1 [E] [RX]}{k_{-1}} = \frac{[E] [RX]}{K_s} \tag{6a}$$

$$[E \cdot RX'] = \frac{k_p [E'] [RX]}{k_{-p}} = \frac{[E'] [RX]}{K_p} \tag{6b}$$

If the steady-state approximation is now applied, the rate equation is given by eq. 7.

$$v = k_2 [E \cdot RX'] = \frac{k_2 E_{tot}}{1 + \frac{k'_{-1}}{k'_1} + \frac{K_p}{[RX]}\left(1 + \frac{k_{-o}}{k_o}\right) + \frac{k_2 (K_s + [RX])}{k_o K_s + k'_1 [RX]}} \tag{7}$$

$$\frac{k_{cat}}{K_{app}} = \frac{k_2 k'_1}{[k'_{-1} (1 + \frac{k_o}{k_{-o}}) + k_2] K_s} \tag{8}$$

In this formulation k_{cat}/K_{app} is given by eq. 8. For k_2 not to be rate-limiting, $k_2 > k'_{-1} (1 + k_o/k_{-o})$; in this case, k'_1 becomes rate-limiting.

Equation 8 is similar to eq. 3 in the text, but k'_{-1} has been multiplied by $(1 + k_o/k_{-o})$.

It is easily shown that k_2 is always rate-limiting at low substrate concentration for the reaction flux through E′ when E′ and E · RX′ in Scheme 3 are in equilibrium. Since the effect of increasing k_o/k_{-o} is to increase the reaction pathway through E′ at the expense of the pathway through E · RX, one can see the basis for the difference between eq. 8 and eq. 3. It is also apparent that k_o, the rate of the free enzyme isomerization, never becomes rate-limiting in the k_{cat}/K_{app} parameter.

SAŽETAK

Kataliza acetilkolinesterazom. Stupnjevi ograničenja brzine uključeni u aciliranje acetilkolinesteraze octenom kiselinom i fosforilirajućim spojevima

T. L. Rosenberry

Zaključci o katalitičkom mehanizmu acetilkolinesteraze najčešće su izvedeni na osnovu pretpostavke o postojanju sličnosti s kimotripsinom. Iako su oba enzima serinske hidrolaze, zapaženo je da se razlikuju u nekim kinetičkim svojstvima u stacionarnom stanju. U ovom radu pažnja je usredotočena na konstantu reakcije drugog reda k_{cat}/K_{app}. Dok je za kimotripsin, kao što je poznato, zavisnost te veličine o pH i izotopni učinak deuterija uglavnom u skladu s jednostavnim modelima koji uključuju ograničenje brzine kiselo-baznom katalizom, ovaj rad pokazuje da je stanje s acetilkolinesterazom zamršenije. Prividni pK_a od k_{cat}/K_{app} za acetilkolinesterazu iznosi od 5,5 do 6,3 za neutralne supstrate i uključuje nelinearnu inhibiciju s H^+. Izotopni učinak deuterija na k_{cat}/K_{app} iznosi od 1,1 za acetilkolin do 1,9 za *p*-nitrofenil-acetat. Čini se da brzina bimolekularne reakcije ograničava brzinu za acetilkolin pri niskim koncentracijama, a postavljena je hipoteza da je za vrijednosti prividnog pK_a i slabe izotopne učinke deuterijevog oksida, povezane s niskim koncentracijama fenilacetata i izoamilacetata, odgovorno inducirano pristajanje koje ograničava brzinu. Ostali neutralni esteri octene kiseline daju pri svim koncentracijama vri-

jednosti prividnog pK_a i izotopnog učinka deuterijevog oksida koji su u skladu s ograničenjem brzine kiselo-baznom katalizom. Istražena je također zavisnost pH o reakciji aciliranja drugog reda s dva fosforilirajuća spoja. Jedan od tih spojeva, diizopropilfluorofosfat, pokazao je vrlo neuobičajen porast stupnja aciliranja pri padu pH od 6 na 5.

ODJELI ZA BIOKEMIJU I NEUROLOGIJU,
ŠKOLA ZA LIJEČNIKE I KIRURGE,
SVEUČILIŠTE KOLUMBIJA, NEW YORK, U. S. A.