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Catalysis by acetylcholinesterase: Evidence that the rate-limiting step for acylation with certain substrates precedes general acid-base catalysis

(enzyme mechanism/diffusion control/induced-fit conformational change/pH dependence/deuterium oxide isotope effects)

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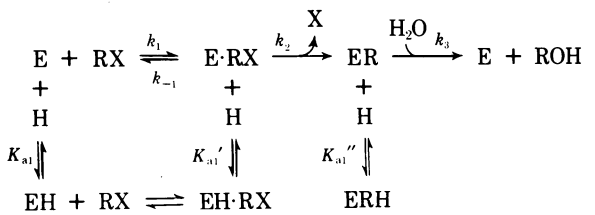
ABSTRACT **Inferences about the catalytic mechanism of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) are frequently made on the basis of a presumed analogy with chymotrypsin, EC 3.4.21.1. Although both enzymes are serine hydrolases, several differences in the steady-state kinetic properties 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 nonlinear inhibition by $[H^+]$. Deuterium oxide isotope effects for 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.**

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) is classified as a serine hydrolase along with other esterases and peptidases which show essentially irreversible active-site phosphorylation (see ref. 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 (EC 3.4.21.1), 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 (see ref. 3).

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



The proposed intermediates include the initial Michaelis complex E·RX and the acyl enzyme ER, for which evidence has long been obtained (5, 6, 1). 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. Furthermore, Hammett relationships with positive rho values are found with chymotrypsin both for deacylation (7) and acylation (8) reactions and indicate rate-limiting general base catalysis. Deacylation rates are typically reduced in deuterium oxide by factors of 2 to 3 (9), in agreement with this indication. Partial protonation generally accompanies loss of the leaving group during acylation (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.

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 (see refs. 1, 12, and 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. 11S acetylcholinesterase is a tetramer of essentially identical catalytic subunits, each with a molecular weight of about 75,000 (14). The active site includes an anionic group which aids in the binding of cationic substrates (see refs. 1, 12, and 13). It is also an *esterase* as opposed to a *peptidase;* acetylacetylcholinesterase is hydrolyzed some 10^6 times faster than acetylchymotrypsin (ref. 15, and see Table 1). Although chymotrypsin is rapidly acylated by specific ester substrates (e.g., ref. 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).

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 have been examined in this paper. 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 by Eq. 1, where v is velocity.

$$v = k_{cat}E_{tot}/(1 + K_{app}/[RX]) \qquad [1]$$

The pH dependence of k_{cat}/K_{app} (the second-order rate) and k_{cat} (the first-order rate) have long been formulated for

Table 1. Apparent pK_a values associated with acetic acid ester substrates of acetylcholinesterase

Substrate	k_{cat}/K_{app}		k_{cat}	
	pK_{a1}	pK_{a2}	pK_{a1}''	pK_{a2}''
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 *Materials and Methods*.

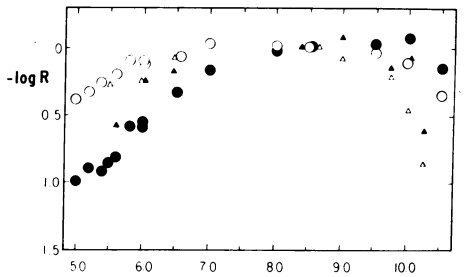


FIG. 1. The pH dependence of phenyl acetate hydrolysis by acetylcholinesterase. *R* values were calculated as outlined in *Materials and Methods* for the following parameters: (○) k_{cat}/K_{app} in 0.1 M NaCl; (△) k_{cat}/K_{app} in 1.0 M NaCl; (●) k_{cat} in 0.1 M NaCl; (▲) k_{cat} in 1.0 M NaCl.

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; the apparent pK_a of k_{cat} is a weighted average of the pK_{a1}' and pK_{a1}'' values for this group in E·RX and ER (see ref. 12). 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 (9, 19); a similar pK_{a1}' is obtained for most E·RX species and a pK_{a1}'' of 7.0–7.2 is seen with acetylchymotrypsin ER (9). In contrast, apparent pK_{a1} values which vary between 5.5 and 6.3 have been reported by Krupka (20, 21) for partially purified bovine erythrocyte acetylcholinesterase. A pK_{a1}' of 5.2–5.5 for E·RX was inferred from all substrates for which acetylation is rate-determining, while deacetylation-limited substrates indicated a pK_{a1}'' of 6.3 for the acetyl enzyme ER. Of particular interest in these acetylcholinesterase studies is the suggestion that protonation of *two* groups in the free enzyme can affect activity (21).

In this report a variability in the pK_{a1} of k_{cat}/K_{app} for several substrates of highly purified eel 11S acetylcholinesterase is confirmed. These pK_{a1} values and observed deuterium oxide effects provide new information about the acylation of acetylcholinesterase.

MATERIALS AND METHODS

Acetylcholinesterase from electric organs of the eel *Electrophorus electricus* was purified as an 11S 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. Steady-state kinetic investigations at 25° and computer analyses of the resultant $1/v$ versus $1/[RX]$ plots were carried out essentially as described previously (22). 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 volumes with the pH stat were either 5 or 10 ml, and standardized 0.005–0.10 N NaOH was the titrant. The solvent was 0.1 M NaCl unless otherwise noted, and the pH was adjusted with standard buffers. Spectrophotometric assays were also used with phenyl acetate at pH 8.0 (0.1 M sodium phosphate, $\Delta\epsilon_{270}$ 1400) and with *p*-nitrophenyl acetate both at pH 8.5 (0.1 M NaCl, 0.05 M Tris·HCl, $\Delta\epsilon_{400}$ 16,000) and at pH 5.0–8.0 at the *p*-nitrophenol–*p*-nitrophenoxide isosbestic point (0.1 M NaCl, 0.05 M sodium phosphate, or 0.05 M sodium acetate, $\Delta\epsilon_{347}$ 5000). Rates were corrected for the nonstoichiometric release of protons for acetate at low pH and for phenol and *p*-nitrophenol at high pH, both in H_2O and D_2O.

The second order phosphorylation rate for *p*-nitrophenyldiethylphosphate was 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 (23, 22; see ref. 13).

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_{a1} and K_{a1}'' were estimated visually from the initial slopes of plots of $1/R$ versus $[H^+]$ (22). Curvature in these plots precluded the computer analysis used previously (22).

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_a'' estimated from these data are shown in Table 1. A clear distinction of approximately one pK unit between pK_{a1} and pK_{a1}'' for phenyl acetate in 0.1 M NaCl is observed. Values of pK_{a1} and pK_{a1}'' for acetylcholine are also shown in Table 1, 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 (see refs. 1, 12, and note similarity of k_{cat} in Table 2). The difference in pK_{a1} 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.

The requirement of an acidic group with a pK_a of 9–10.5 for enzyme activity has been noted by several investigators (see ref. 1). The data in Table 1 with 0.1 M NaCl indicate that such a group has higher pK_{a2} and pK_{a2}'' values than 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 (24). Only when assays are conducted in 1.0 M NaCl do the pK_{a2} and pK_{a2}'' values for this group become clearly discernible.

In 1.0 M NaCl the apparent values of pK_{a1}'', pK_{a2}, and pK_{a2}'' are reduced by some 0.5 pK units. A similar observa-

Table 2. Deuterium oxide effects on k_{cat} and k_{cat}/K_{app} and pK_1 of k_{cat}/K_{app} for several substrates of acetylcholinesterase

Substrate	rel k_{cat}	$\frac{k_{cat}(H_2O)}{k_{cat}(D_2O)}$	$\log \frac{k_{cat}}{K_{app}}$	$\frac{k_{cat}/K_{app}(H_2O)}{k_{cat}/K_{app}(D_2O)}$	pK_1 of k_{cat}/K_{app}
Acetylcholine	100	2.34 ± 0.15 2.43 ± 0.42[a]	8.2	1.07 ± 0.10 1.23 ± 0.20[a]	6.3
Phenyl acetate	107	2.04 ± 0.28[b] 2.32 ± 0.23[c]	6.9	1.23 ± 0.17[b] 1.46 ± 0.10[c]	5.5
Isoamyl acetate	13	2.84 ± 0.70	5.8	1.26 ± 0.28	5.6
p-Nitrophenyl acetate	8	2[d]	5.4	1.93 ± 0.07	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} sec^{-1}) at pH 8.5, 0.1 M NaCl, assuming an actual value of k_{cat} of acetylcholine of 1.6×10^4 sec^{-1} (see ref.13). Values of pK_{a1} were obtained in H_2O as outlined in the *Materials and Methods*. The error value for the D_2O effect is 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.

tion has been made for the pK values associated with the dephosphorylation of acetylcholinesterase (25). 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_{a1} values of 5.5 for phenyl acetate and 6.3 for acetylcholine in Table 1 coincide with general assignments for neutral and cationic acetic acid ester substrates by Krupka (20), a detailed investigation with a series of neutral acetic acid esters and with *p*-nitrophenyldiethylphosphate shows that such a dichotomized classification does not hold. In Fig. 2 the pH dependence of k_{cat}/K_{app} in the region of pK_1 for phenyl acetate and isoamyl acetate differs significantly from that of methyl acetate and *p*-nitrophenyldiethylphosphate. The precise pK_{a1} 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_{a1} from the initial slopes of $1/R$ versus $[H^+]$ are included in Table 2. Methyl acetate, *p*-nitrophenyl acetate, and *p*-nitrophenylphosphate all give pK_{a1} estimates significantly greater than the 5.5 value for phenyl acetate and approach the 6.3 value for acetylcholine.

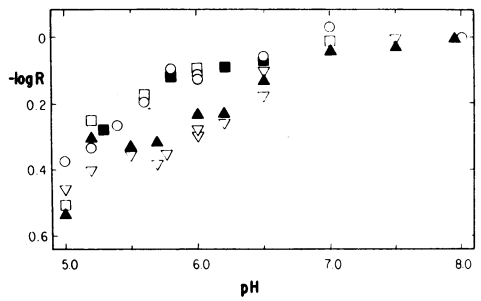


FIG. 2. The pH dependence of k_{cat}/K_{app} for several neutral acetic acid esters and for *p*-nitrophenyldiethylphosphate with acetylcholinesterase. R values for k_{cat}/K_{app} in 0.1 M NaCl were calculated for (○) phenylacetate; (□,■) isoamyl acetate; and (▲) methyl acetate. Open symbols with these substrates represent R values from reciprocal plot slopes (see *Materials and Methods*), while filled symbols represent individual paired observations at a single substrate concentration far below K_{app}. (▽) R values for second-order phosphorylation rates in 0.1 M NaCl with *p*-nitrophenyldiethylphosphate obtained as outlined in the *Materials and Methods*. The second order phosphorylation rate was 1.8×10^3 M^{-1} sec^{-1} from data at 18 μM *p*-nitrophenyldiethylphosphate. Phosphorylation data obtained both with methyl acetate and with *p*-nitrophenyl acetate are included.

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 2. These values for acetylcholine as a function of pH are shown in Fig. 3. 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 $k_{cat} \cong k_3$, and for substrates for which $k_{cat} < k_3$. The observed effect on k_{cat} for phenyl acetate is consistent with a previous report of 2.3 (9). 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

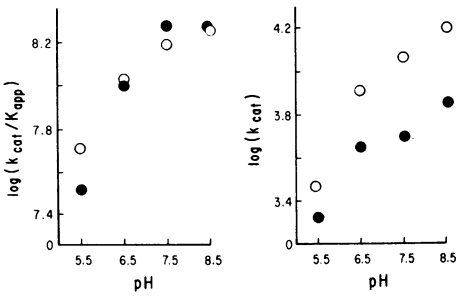


FIG. 3. Deuterium oxide effects on k_{cat}/K_{app} and k_{cat} for acetylcholine with acetylcholinesterase. Values were obtained as outlined in the *Materials and Methods*. At the time of the assay the solvent contained at least 98% D_2O in all cases. The pH values were read directly from the pH meter, standardized in the usual way with buffers in H_2O. (○), H_2O; (●), D_2O.

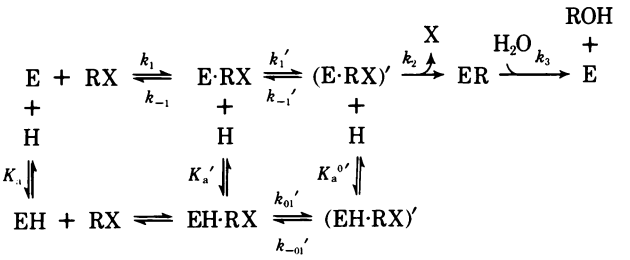
other example of a deuterium oxide effect as low as 1.1 for either k_{cat} or k_{cat}/K_{app} for a serine hydrolase has been reported, and that concerns a series of very poor substrates for chymotrypsin (see ref. 26). While the number of substrates analyzed here is small, it is noteworthy that neutral substrates with deuterium oxide effects for k_{cat}/K_{app} of less than 1.4 are associated with the lowest apparent pK_{a1} values for k_{cat}/K_{app}.

DISCUSSION

The variation in the apparent pK_{a1} from 5.5 to 6.3 among several acetic acid ester substrates is inconsistent with a formulation of Scheme 1 in which E and E·RX are in virtual equilibrium. Two formal ways of extending Scheme 1 to account for this variation may be proposed. In the first, protonation of either of two distinct groups in the free enzyme is postulated to affect activity by preventing substrate binding and/or blocking general acid-base catalysis. The affinity of cationic inhibitors for either the free or the acetyl enzyme is decreased by more than an order of magnitude with decreasing pH (20, 27). The apparent pK_{a1} and pK_{a1}'' corresponding to these decreases are 6.2–6.3, the same as that for the pH dependence of k_3. From the coincidence of these pK_a values plus further data on the effects of certain cationic ligands on deacetylation at low pH (20), Krupka (21) has proposed that an imidazole of pK_a 5.2–5.5 acts as a general base during acetylation and that a second imidazole of pK_a 6.3 both acts as a general base during deacetylation and, when protonated, blocks cation binding at the catalytic site. Alternatively, one could postulate a single general base catalyst of pK_a 5.2–5.5 in the free enzyme and pK_a'' 6.3 in the acetyl enzyme; the pK_{a1} of 6.3 in the free enzyme would correspond to the carboxylate group which defines the anionic site within the catalytic site. The pH dependence of betaine binding disputes this alternative postulate (20).

Proposals which invoke two distinct groups in the free enzyme have difficulty in explaining the current data. The variation in the apparent pK_{a1} values among neutral substrates could be explained by postulating that protonation of the pK_a 6.3 group reduces the binding of some, but not all, neutral substrates. Low deuterium oxide isotope effects observed for k_{cat}/K_{app} with certain substrates also would require a postulate of a variable deuterium oxide effect on substrate binding.

In view of these rather arbitrary postulates, it is of interest to consider a second formal extension of Scheme 1 in which the influence of kinetic rate constants alters the actual pK_a value of a single catalytic group of pK_a 6.3 in the free enzyme. If virtual equilibrium of E and E·RX in Scheme 1 does not obtain ($k_2 > k_{-1}$), then $k_{cat}/K_{app} \cong k_1$ (see refs. 28 and 13). Under this condition the bimolecular rate constant k_1, not the general base catalysis step k_2, becomes rate-limiting at low substrate concentrations. As shown by Renard and Fersht (28), in this case the apparent pK_{a1} of a basic group required for k_2 decreases by an amount directly related to k_2/k_{-1}. Furthermore, if k_1 becomes rate-limiting, little or no deuterium oxide effect would be expected. Although this formulation can account nicely for the low deuterium oxide effect observed here for acetylcholine, for which the high k_{cat}/K_{app} value (Table 2) already suggests that k_1 is rate-limiting (13), it fails to explain similar observations in other substrates with much lower k_{cat}/K_{app} values. Phenyl acetate and isoamyl acetate give apparent pK_{a1} values below 6.3 and show low deuterium oxide effects on k_{cat}/K_{app}; and carbamoylating agents, for which k_{cat}/K_{app} values are some 10^4 times smaller than those for acetic acid esters, show an analogous variation in pK_{a1} values (29). It would appear that kinetic perturbations of pK_{a1} 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.



Scheme 2, which focuses on enzyme acylation, extends the simpler mechanism in Scheme 1 by introducing the intermediate (E·RX)'. In particular, it may be shown (see *Appendix*) 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·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·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 (31, 32; see ref. 13), but the author is not aware of other proposals of *rate-limiting induced-fit* in which the rate of the limiting conformational change is substrate dependent.

According to Eq. 2A derived from the rate-limiting induced-fit model, the apparent pK_a for k_{cat}/K_{app} is a function of rate constants which may be expected to vary for each acylating agent. This prediction is consistent with observations in Table 2. Furthermore, if $k_1' \neq k_{01}'$, the inhibition of k_{cat}/K_{app} may be nonlinear in $[H^+]$, again as observed. Variations in this apparent pK_a have been reported previously for neutral acetylating (27), carbamoylating (29), and phosphorylating agents (see ref. 13), all of which appear to utilize the same catalytic pathway (12). Phosphorylating agents are of particular interest in that, unlike other substrates and acylating agents, second-order enzyme phosphorylation rates depend significantly on the quality of the leaving group and display positive rho values (see ref. 32). Consequently the rate-limiting step according to 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 should be the actual pK_a of the catalytic group. The pK_a for second-order phosphorylation for one phosphorylating agent, *p*-nitrophenyldiethylphosphate, was observed to be 6.1 (Fig. 2, Table 2). This pK_a approaches the highest pK_a values observed with acetic acid esters and thus supports the idea that apparent pK_a values below 6 are kinetically perturbed. However, the inhibition is again non-linear in $[H^+]$, a complication not anticipated by Scheme 2 for an agent for which k_2 is presumably rate-limiting at all pH values. Such non-linearity may arise from the protonation of a second active site group which interacts with the pK_a 6.3 group or from the intervention of an acid-catalyzed pathway at low pH, complexities beyond the scope of Scheme 2.

In Fig. 4 a schematic visualization is offered as a summary of the induced-fit model in Scheme 2. Rate-limiting induced-fit obtains at low substrate concentrations with substrates for which $k_2 > k_{-1}'$. Under this condition at high pH, $k_{cat}/K_{app} \cong k_1'/K_s$ (Eq. 2A); although k_1' is not well de-

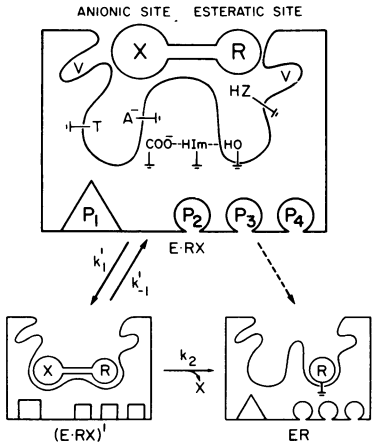


FIG. 4. Diagrammatic model of the acetylcholinesterase catalytic site based on the induced-fit mechanism in Scheme 2. The initial enzyme–substrate complex is E·RX; the induced-fit complex, (E·RX)'; and the acyl enzyme, ER. Residues identified or presumed at or near the catalytic site include the charge-relay complex COO^- –HIm –HO (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 P_1 or exclusively to peripheral sites P_2, P_3, P_4 may affect the rate constants k_1' and k_{-1}'. (See ref. 13 for further details.)

fined, both it and K_s seem likely to involve smaller deuterium oxide effects than a step involving general acid-base catalysis. Phenyl acetate and isoamyl acetate show both this reduced deuterium oxide effect as well as the greatest deviation in apparent pK_{a1} below 6.3; they thus satisfy two important criteria for rate-limiting induced-fit. In contrast, corresponding deuterium oxide effects of about 2 are observed for substrates which have a pH dependence of k_{cat}/K_{app} similar to that for second-order phosphorylation with *p*-nitrophenyldiethylphosphate. The rate-limiting step for these substrates thus is consistent with general acid-base catalysis.

It is noteworthy that substrates rate-limited by induced-fit at low substrate concentrations may still be rate-limited by general base catalysis at substrate saturation if $k_1' > k_2$ in Scheme 2 (13). Indeed, k_{cat} for isoamyl acetate shows a large deuterium oxide effect consistent with this formulation.

APPENDIX

According to Scheme 2 with the assumption of rapid equilibrium both among E, EH, E·RX and EH·RX and between (E·RX)' and (EH·RX)', k_{cat}/K_{app} is given by Eq. 2A.

$$\frac{k_{cat}}{K_{app}} = \frac{k_2 k_1'\left(1 + \frac{k_{01}[H^+]}{k_1' K_a'}\right)}{\left(1 + \frac{[H^+]}{K_a}\right)\left(k_2 + k_{-1}'\left[1 + \frac{k_{01}[H^+]}{k_1' K_a'}\right]\right)K_s} \quad [2A]$$

In Eq. 2A, $K_s = k_{-1}/k_1$. Eq. 2A is formally analogous to that arising from the simpler nonequilibrium mechanism considered by Renard and Fersht (28). An illustrative simplification of Eq. 2A considered by these authors assumed that $k_1' = k_{01}'$. If it is further assumed here that $K_a = K_a'$, Eq. 3A obtains.

$$\frac{k_{cat}}{K_{app}} = \frac{k_2 k_1'}{\left(k_{-1}' + k_2 + k_{-1}'\frac{[H^+]}{K_a}\right)K_s} \quad [3A]$$

The observed K_a in the case of Eq. 3A is given by Eq. 4A.

$$K_a(\text{observed}) = (1 + k_2/k_{-1}')K_a \quad [4A]$$

Hence the actual K_a is perturbed by kinetic rate constants to the extent that $k_2 > k_{-1}'$. A qualitatively similar conclusion can be drawn from the general case in Eq. 2A in which $(k_{cat}/K_{app})^{-1}$ is not necessarily linear in $[H^+]$.

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