One-Proton Catalysis in the Deacetylation of Acetylcholinesterase[1]

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Abstract: Proton inventories (determination of kinetic parameters in binary mixtures of protium and deuterium oxides) for the action of acetylcholinesterases on phenyl acetate indicate that the overall solvent isotope effect of 2.42 ± 0.03 (electric-eel enzyme), or 2.28 ± 0.11 (erythrocyte enzyme), on k_{cat} arises from a single site in the transition state. This parameter is strongly dominated by the deacetylation rate. Deacetylation, the physiological reaction of these enzymes, thus experiences catalysis by a single protonic site, rather then multiple protonic sites as in the serine proteases when they act upon their natural substrates or close analogues. The mechanistic analogy between acetylcholinesterase action and serine-protease action therefore does not extend to properties of the acid–base catalytic entity of the enzymes. The solvent isotope effects on k_{cat}/K_m (1.46 for eel enzyme and 1.61 for erythrocyte enzyme) may also arise from a single site.

Acetylcholinesterase is an enzyme of the nervous system, which serves to catalyze the hydrolyze of the neurotransmitter acetylcholine (Scheme I, eq 1).[2] The enzymes acts through a double-displacement mechanism (Scheme I, eq 2), first forming an acetyl-enzyme, which bears the acetyl group on a serine residue of the active site; in a second stage, the acetyl-enzyme hydrolyzes. The two kinetic parameters V and V/K (equal to the total enzyme concentration times k_{cat} and k_{cat}/K_m, respectively) are related as shown in eq 3 and 4 of Scheme I to the kinetic constants of the mechanistic steps.

The structure of acetylcholinesterase is complex, and it has not been possible yet to obtain a crystallographic analysis.[2] Since the enzyme is a serine hydrolase, analogies to the known active-site structures of the serine proteases have been used to establish expectations for the still unknown active-site structure of acetylcholinesterase.[3]

One such feature is the acid–base catalytic entity of the active site. The serine proteases have an evolutionarily conserved "catalytic triad" of serine, histidine, and aspartate, originally called the charge-relay system by Blow, Birktoft, and Hartley.[4] Various evidence now indicates that this entity does not "relay charge" in resting states of the enzyme, accessible to crystallographic and NMR spectroscopic study, nor in complexes of the enzyme stable enough for such examinations.[5]

However, kinetic studies—capable of probing the situation in the transition state—indicate that, when a sufficient level of enzyme–substrate interaction is present in the catalytic transition state, a multisite acid–base catalytic entity functions; when an insufficient level of interaction is present, a single protonic site is employed in catalysis.[5] For example, with trypsin[6] or elastase,[7] oligopeptide analogues exhibit proton inventories which are nonlinear (i.e., plots of rate vs. atom fraction of deuterium in mixtures of protium and deuterium oxides are curved). The rates depend on the second power of the atom fraction of deuterium and this suggests the participation of two (perhaps more) centers in generation of the isotope effect. With the same enzymes, simple acetyl substrates, incapable of the level of enzyme–substrate interaction achieved by the oligopeptide analogues, produce linear proton-inventory plots, indicating that a single site acts in catalysis.

Our view[5] is that the numerous interactions between enzyme and substrate in the oligopeptide transition states activate a multisite catalytic entity (presumably the charge-relay system, although this cannot be deduced from the isotope effects). When those interactions are absent, the entity is not activated, only the nearest base (the histidine) acts and one-site catalysis is observed.

This leaves us with a difficulty if a multiproton entity is available in acetylcholinesterase: in the deacetylation transition state, there is very little opportunity for formation of activating enzyme–substrate interactions. The acetyl group is too small and nonfunctionalized. This suggests that acetylcholinesterase achieves its catalysis differently from the serine proteases: either it has no multisite catalytic entity or, if it does, it must become activated in a different way. We therefore carried out proton inventories on the "natural" deacetylation reaction of two enzymes, one from electric eel and one from erythrocytes.

Scheme I. Kinetics and Mechanism of Acetylcholinesterase Action

$$CH_3COOR + H_2O \rightarrow CH_3CO_2^- + ROH + H^+ \quad (1)$$

$$E + AcOR \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \{E,AcOR\} \xrightarrow[-ROH]{k_3} E\text{-}Ac \xrightarrow{k_5} E + AcO^- + H^+ \quad (2)$$

$$V = ek_3k_5/(k_3 + k_5) \qquad e = \text{total enzyme concentration} \quad (3)$$

$$V/K = ek_1k_3/(k_2 + k_3) \quad (4)$$

Table I. Kinetic Parameters[a] in Mixed Isotopic Waters[b] for Hydrolysis of 0.8–8.2 mM Phenyl Acetate by Acetylcholinesterases at pH 7.60 and Equivalent[c] at 25.00 ± 0.05 °C

	electric eel[e]		red blood cell[f]	
atom fraction of deuterium, n	10^8V, M s^{-1}	$10^5V/K$, s^{-1}	10^7V, M s^{-1}	$10^4V/K$, s^{-1}
0.000	403 (3)[g]	168 (2)	181 (6)	152 (7)
0.238	350 (6)	153 (4)	155 (3)	142 (3)
0.475	293 (4)	139 (3)	134 (3)	122 (5)
0.713	232 (2)	130 (3)	104 (1)	110 (2)
0.950	180 (2)	117 (3)	86 (0.5)	101 (2)

[a] Maximal velocity V and its ratio to substrate concentration at half-maximal velocity V/K, determined by extrapolation from data at no less than three substrate concentrations, each velocity reproduced at least three times. Errors in parentheses are standard deviations. [b] Containing 1% by volume of methanol. [c] All solutions contained 0.0434 M K_2HPO_4 and 0.0066 M KH_2PO_4. [e] Sigma V-S, 10 units/L. [f] Sigma XIII, further purified, 12.5 units/L. [g] Enzyme active-site concentration 1.7×10^{-10} M by modified Ellman assay,[11] yielding $k_{cat} = 2.4 \times 10^4$ s^{-1}.

Results

Phenyl acetate was employed as substrate, its hydrolysis generating a convenient spectrophotometric signal. Although the

(1) This research was supported by the U.S. Army Medical Research and Development Command, Contract No. DAMD 17-83-C-3199.

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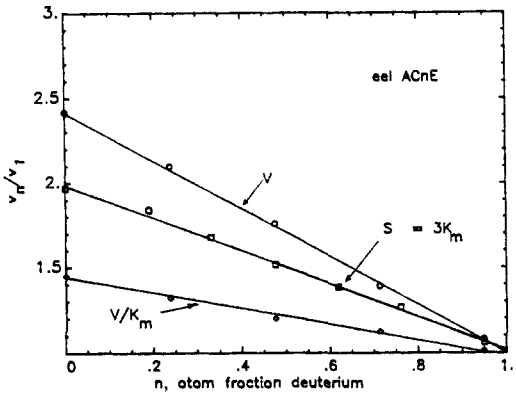

Figure 1. Proton-inventory plots (v_n/v_1 vs. n) for extrapolated values of V (upper plot) and V/K (lower plot) for hydrolysis of phenyl acetate by electric-eel acetylcholinesterase at 25 °C, pH 7.6 and equivalent. The middle line gives experimental points for [PhOAc] = 3 K_m. The lines are least-squares fits to a linear equation.

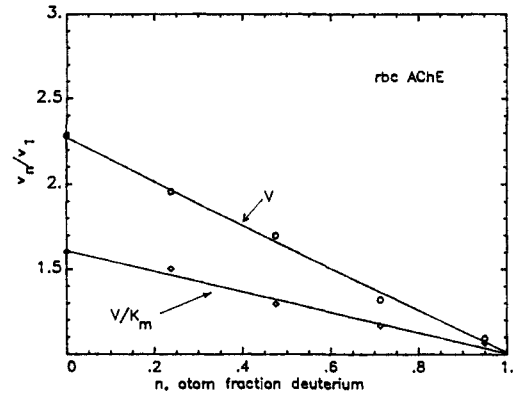

Figure 2. Proton-inventory plots for V (upper plot) and V/K (lower plot) for hydrolysis of phenyl acetate by erythrocyte acetylcholinesterase at 25 °C, pH 7.6 and equivalent. The lines are least-squares fits to a linear equation.

acetylation reaction is "unnatural" with this substrate, the acetyl-enzyme generated is the same as that from acetylcholine. The deacetylation reaction is therefore the "natural" reaction of the enzyme. Rates in protium oxide and four mixtures with deuterium oxide were measured as a function of substrate concentration and the rates were extrapolated by the Lineweaver–Burk expression to zero concentration (V/K) and infinite concentration (V). The data are shown in Table I for the electric-eel and the erythrocyte enzymes. Figures 1 and 2 show the proton-inventory plots.

The overall solvent isotope effects are

$$k^{HOH}_{cat}/k^{DOD}_{cat} = 2.42 \pm 0.03 \text{ for the electric-eel enzyme}$$
$$= 2.28 \pm 0.11 \text{ for the erythrocyte enzyme}$$

$$(k_{cat}/K_m)^{HOH}/(k_{cat}/k_m)^{DOD} = 1.46 \pm 0.07 \text{ for the electric-eel enzyme}$$
$$= 1.61 \pm 0.11 \text{ for the erythrocyte enzyme}$$

Discussion

The proton-inventory data in Figures 1 and 2 give the appearance at linearity and thus of one-site catalysis throughout. This is substantiated by statistical treatment. None of the plots can support a second-order term in n at a confidence level above 90%.

For the V/K effects, which are around 1.5, it might well be true that contributions from other sites could be concealed in the noise of the fit. Thus one cannot strongly exclude multisite contributions to these isotope effects. For acetylcholine itself, some event prior to chemical catalysis appears to limit the V/K rate,[8] and this would be consistent with a small or absent solvent isotope effect for it.[3]

The V effects are more easily interpreted, especially that for the eel enzyme. The straightforward interpretation is that one protonic site and one site only is involved in catalysis. The real test as to whether the data can, on the other hand, accomodate a second-site contribution to the isotope effect is to obtain a best-fit set of parameters (fractionation factors) to the two-site form of eq 5 in Scheme II.

$$k_n/k_0 = (1 - n + n\varnothing_1)(1 + n + n\varnothing_2)$$

When this expression was used to fit the data for the electric-eel enzyme, the values obtained for the parameters were $\varnothing_1 = 1.03 \pm 0.04$ and $\varnothing_2 = 0.40 \pm 0.02$. Since one of these values is unity, the isotope effect is in fact, even on a two-site model, arising from a single site. A similar fit for the erythrocyte-enzyme data yields values of 0.87 ± 0.15 and 0.50 ± 0.10. Again, one factor is within experimental error of unity and the effect comes essentially wholly from the other site. The data thus indicate no concealment of a second-site contribution within the noise of the observed linear dependence. Rather they favor one-site catalysis.

It is necessary, however, to proceed somewhat more carefully. The treatment just given is wholly applicable and correct *if* a single step limits the rate for the parameter under consideration. Otherwise, a more complex approach is required (Scheme II, eq 6)[5].

We know from the work of Forede and Wilson[9] that k_{cat} for the electric-eel enzyme is not wholly determined by one step but rather that both acylation and deacylation (k_3 and k_5, respectively, in Scheme I) contribute. The weighting factors (eq 6c of Scheme II) are 0.26 for acylation and 0.74 for deacylation (thus deacylation is "74% rate-limiting"), as calculated from $k_5 = 1.5 \times 10^4$ s^{-1} (Froede and Wilson[9]) and $k_{cat} = 1.1 \times 10^4$ s^{-1} (calculated from $k_{cat} = 1.0 \times 10^4$ s^{-1} for acetylcholine under the conditions of Froede and Wilson[9] and the fact[2] that k_{cat} for phenyl acetate is 10% greater than that for acetylcholine under the same conditions; our larger value of k_{cat} is presumably a reflection of enzyme purity [cf. Table I, footnote *g*]). Properly therefore we are obliged to employ the expression of eq 7, together with some model of the proton inventories for acylation (P_a) and deacylation (P_d), such as are given by eq 8.

These three models correspond to three cases of concern. Model 1 imagines that there is no isotope effect on acylation and a one-site isotope effect on deacylation. Model 2 takes the more reasonable view that the isotope effect on acylation is equal to the observed effect on V/K, which includes the acylation step, and it agains takes a one-site isotope effect to account for deacylation. Model 3 assumes again that the V/K isotope effect holds for acylation but postulates a two-site origin for the deacylation isotope effect.

The data for the eel enzyme are compared with these three models in Figure 3. It is apparent that the only satisfactory fit is to model 2. A critical result is that not only does model 2 fit but that model 3 does not. Model 2 postulates one-site catalysis is the "natural" deacylation reaction and fits the data. Model 3

Scheme II. Proton Inventory of k_{cat} in Acetylcholinsterase Action

$$k_n/k_0 = \prod_i^{tran}(1 - n + n\phi_i^T)/\prod_j^{reac}(1 - n + n\phi_j^R) \quad (5)$$

$$k_{cat}^{-1} = k_3^{-1} + k_5^{-1} \quad (6a)$$

$$\frac{k_{cat}^0}{k_{cat}^n} = \frac{k_{cat}^0}{k_3^0}\frac{1}{(k_3^n/k_3^0)} + \frac{k_{cat}^0}{k_5^0}\frac{1}{(k_5^n/k_5^0)} \quad (6b)$$

$$w_3 = k_{cat}^0/k_3^0;\ w_5 = 1 - w_3 = k_{cat}^0/k_5^0 \quad (6c)$$

$$k_{cat}^0/k_{cat}^n = w_3/P_a + w_5/P_d \quad (7)$$

Model 1: $P_a = 1$; $P_d = 1 - n + n/2.92$ (8a)

Model 2: $P_a = 1 - n + n/1.46$; $P_d = 1 - n + n/2.76$ (8b)

Model 3: $P_a = 1 - n + n/1.46$; $P_d = (1 - n + n/1.66)^2$ (8c)

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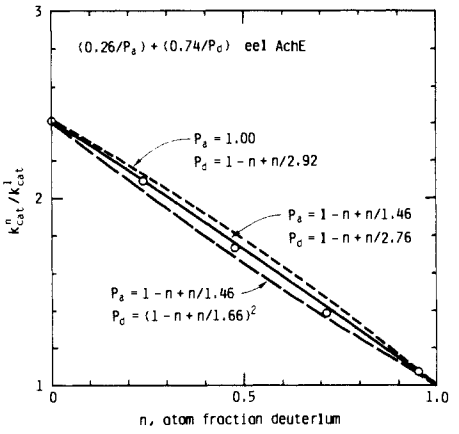

Figure 3. Comparison of the proton-inventory data for the eel enzyme with models 1 (upper), 2 (middle), and 3 (lower) (models as given in Scheme II, eq 8).

postulates two-site catalysis and does not fit.

Can the non-fitting models be adapted to fit? Model 1 should not be pursued because it contains the unreasonable hypothesis of no isotope effect on the acylation reaction. This reaction is chemically similar to deacylation and ought therefore to have a roughly comparable isotope effect. Furthermore the parameter V/K, which is determined in part by acylation, does exhibit an isotope effect in the correct direction.

Model 3 might be altered by increasing the number of sites contributing to the isotope effect for acylation, but that would only increase the curvature which is already too large. Either model 2 or model 3 could be altered to make the isotope effects for acylation and deacylation more similar, but when the isotope effects are the same, then the situation is as if a single transition state were under observation. The observed linearity will apply to both steps.

It seems unlikely therefore that a model can be generated which is consistent with two-site catalysis in deacylation and which does not depend upon a fortuitous cancellation of effects to generate the observed linear proton inventory. If the only case were that of the eel enzyme, this argument would lack force, but the linearity also seen for the erythrocyte enzyme suggests that no fortuitous cancellation is at work. We cannot compare the various models with the data for the erythrocyte enzyme without assuming the weighting factors for acylation and deacylation, which have not been measured, but it would not be surprising if they were qualitatively similar to those for the eel enzyme. Deacylation thus appears to involve one-site catalysis.

We conclude that the mechanistic analogies between the serine proteases and acetylcholinesterase do not extend to the acid–base catalytic machinery and its activation by the natural substrate of the enzyme.

Experimental Section

Materials. Inorganic salts, buffer components, and preparative reagents were reagent grade and were used without further purification. Protium oxide was distilled from a copper-bottom still, passed through a Barnstead mixed-bed ion-exchange column, boiled for 20 min, and cooled suddenly. Deuterium oxide (Stohler 99.8 atom % deuterium) was distilled and the 100–102 °C fraction was used. Phenyl acetate (Eastman) was purified by column chromatography and vacuum distillation.

Enzyme. Acetylcholinesterase from *Electrophorus electricus* was obtained with an activity of 1000 micromolar units/mg (Sigma type V-S) lyophilized powder. Acetylcholinesterase from human erythrocytes was obtained with an activity of 1 unit/mg of solid (Sigma XIII) and was further purified by affinity chromatography according to Rosenberry and Scoggins.[10] The progression of enzyme purification was monitored with the modified[11] Ellman assay. The enzyme (purified about 20-fold) was stored in 0.01 M phosphate buffer at pH 7.00 with 0.1% Triton X-100 (peak fractions; 7–10 unit/mL) and kept frozen until use.

AChE Kinetics. Solutions of AChE from the *Electrophorus electricus* were prepared in 0.1 M phosphate buffer at pH 6.9, at 46 ng/mL, by dilution of the Sigma lyophilized preparation and were frozen until use. Rates were determined with a Cary 118 spectrophotometer interfaced to a DEC-Heathkit H-11 microcomputer. Reactions were conducted at enzyme concentrations of 0.005–0.01 unit/mL of AChE from *Electrophorus electricus* and 0.0125 unit/mL of AChE from human erthrocytes based on the Ellman assay. The pH was maintained at 7.60 in protium oxide (0.0066 M KH_2PO_4, 0.0434 M K_2HPO_4) and corresponding pL in H_2O/D_2O mixutres was achieved by use of the same buffer composition throughout. H_2O/D_2O mixtures were made up by volume from buffer stock solutions in the pure isotopic solvents.

For a typical run, 940 μL of buffer was equilibrated at 25.00 ± 0.05 °C (as monitored by a thermistor probe) in a quartz cuvette in the Cary 18 cell compartment, 10 μL of phenyl acetate was delivered from a methanolic stock solution, the solution was mixed and its absorbance recorded, and then the reaction was initiated by the introduction of 50 μL of the AChE stock solution. Initial rates were calculated from the computer-stored data by a linear least-squares method. The nonezymic hydrolysis of phenyl acetate is completely negligible under these conditions. Enzyme kinetic parameters, V and V/K, were obtained by linear-squares fit of the reciprocal rates to the reciprocal of phenyl acetate concentration.

Registry No. AcOPh, 122-79-2; D_2, 7782-39-0; acetylcholinesterase, 9000-81-1.

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