Nitrogen Isotope Effects on Acetylcholinesterase-Catalyzed Hydrolysis of *o*-Nitroacetanilide

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Abstract: The nitrogen-15 isotope effect on V/K for *Electrophorus electricus* acetylcholinesterase-catalyzed hydrolysis of *o*-nitroacetanilide has been determined by isotope ratio mass spectrometry. The effect determined in buffered H_2O (0.1 M sodium phosphate, 0.1 N NaCl, pH 7.3, 25 °C) is $^{15}V/K = 1.0119 \pm 0.0005$. A small though palpable decrease of the isotope effect is observed when the reaction is run in equivalently buffered D_2O (pD = 7.7), $^{15}V/K = 1.0106 \pm 0.0002$. The corresponding solvent isotope effect is $^{D}V/K = 1.56 \pm 0.03$. The solvent isotope effect on the nitrogen isotope effect is interpreted in terms of a mechanism in which successive transition states for induced fit and for formation and decomposition of a uninegative tetrahedral intermediate contribute to rate determination of V/K. Numerical modeling allows relatively narrow limits to be placed on the isotope effects for the chemical steps. The solvent and substrate isotope effects for the formation of the tetrahedral intermediate are $^{D}k_5 = 2.6$–3.7 and $^{15}k_5 = 1.000$–1.009, respectively. The corresponding isotope effects for the decomposition of the intermediate are $^{D}k_7 = 1.0$–1.5 and $^{15}k_7' = {}^{15}k_5{}^{15}k_7/{}^{15}k_6 = 1.027$–$1.053$. The value of $^{15}k_7'$ is consistent with a transition state for decomposition of the tetrahedral intermediate in which C–N bond breaking is occurring.

The mechanism of acetylcholinesterase (AChE[1]) catalysis[2–4] bears a formal similarity to that of the serine proteases.[5–8] Both classes of enzymes hydrolyze substrates via an acyl enzyme mechanism, as outlined in Scheme I:[2–4,9]

Scheme I

$$E + A \underset{k_2}{\overset{k_1}{\rightleftarrows}} EA \xrightarrow{k_a} F + P \xrightarrow[H_2O]{k_d} E + Q$$

EA is the Michaelis complex and F the acyl enzyme intermediate, and accordingly k_a and k_d are the respective rate constants for acylation and deacylation. For this mechanism, V/K provides information on the acylation stage of catalysis only, as illustrated by eq 1:

$$V/K = k_{cat}[E]_T/K_m = \frac{k_1 k_a [E]_T}{k_2 + k_a} \quad (1)$$

Therefore, mechanistic probes of AChE function, such as kinetic isotope effects, provide a window on acylation transition-state structure and reaction dynamics when V/K is measured.

The physiological function of AChE is the hydrolytic destruction of the neurotransmitter ACh in the central and peripheral nervous systems. The second-order acylation rate constant k_{cat}/K_m for ACh turnover is rate-limited by diffusion (i.e., $k_{cat}/K_m = k_1$, cf. eq 1),[2,10,11] and therefore physiological AChE catalytic power is highly evolved.

An unfortunate sequela of AChE catalytic power is that transition states in the acylation stage of ACh turnover that involve covalent bond rearrangements are transparent to kinetic probes. Fortunately, AChE has a broad substrate specificity.[2] Alternate substrates include thioesters, amides, aryl esters, and anilides. For example, though k_{cat}/K_m for hydrolysis of *o*-nitroacetanilide (ONAC) is 3 orders of magnitude lower than that for ACh,[12] the catalytic accelerations of the two reactions are comparable.[13] This comparison suggests that alternate substrates provide a viable window on the chemical transition states of acylation and thence on the sources of AChE catalytic power. This opportunity is exploited herein to characterize the acylation transition state for AChE-catalyzed hydrolysis of ONAC.

Materials and Methods

Materials. *o*-Nitroacetanilide was synthesized as previously described.[12] Just prior to $^{15}V/K$ determinations, ONAC was recrystallized

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(1) Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; ATCh, acetylthiocholine; ONA, *o*-nitroaniline; ONAC, *o*-nitroacetanilide; HPLC, high-performance liquid chromatography; S200, serine at position 200 in the AChE sequence; H440, histidine at position 440 in the AChE sequence; TLC, thin-layer chromatography; $^{D}V/K$, solvent deuterium isotope effect on V/K; $^{15}V/K_H$, N-15 isotope effect on V/K, measured in buffered H_2O; $^{15}V/K_D$, N-15 isotope effect on V/K, measured in D_2O.

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(13) The catalytic accelerations for AChE-catalyzed hydrolyses of ACh and ONAC are comparable because the 10^3-fold ratio of k_{cat}/K_m values for these substrates[2] is similar to that for their nonenzymic hydrolyses. For example, the respective rate constants for base-catalyzed hydrolyses of ACh[34] and ONAC are 2.2 and 0.01 M^{-1} s^{-1} (Quinn, D.; States, M.; Viering, M., unpublished observations), and thus their ratio is 220.

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from H_2O:EtOH (4:1 or 3:2 v/v) to afford white needles that showed single spots on TLC in benzene and CH_2Cl_2 mobile phases (respective R_f values are 0.07 and 0.17); *o*-nitroaniline (ONA, R_f = 0.24 and 0.43 in the respective mobile phases) was not found in recrystallized ONAC. Evaluation of ONAC on a Beckman Instruments HPLC (C_{18} column, flow rate 2 mL min^{-1}, 7:3 MeOH:H_2O mobile phase (v/v)) showed that the recrystallized sample contained <0.5% ONA. *Electrophorus electricus* AChE (type V-S, 4 mg of protein, purchased from Sigma Chemical Co.) was dissolved in 2.0 mL of 0.1 M sodium phosphate buffer, pH 7.3, that contained 0.1 N NaCl and was dialyzed for 18 h at 5 °C against three changes of 500 mL each of the same buffer. Buffer salts were purchased from Sigma Chemical Co. and used as received. H_2O was distilled and then deionized by passage through a mixed-bed ion-exchange column (Barnstead, Sybron Corp.). D_2O (99.8% D) was purchased from Sigma Chemical Co. and was used as received. The NH_3 content of H_2O and D_2O used in buffer preparations was determined by the Berthelot alkali phenol method[14] and was less than 1 μM. HPLC grade benzene was purchased from EM Science. Silica gel that contains neither binder nor fluorescent indicator was purchased from ICN Biochemicals.

Enzyme Kinetics. Reactions were conducted at pH 7.3 in H_2O or pD 7.7 in D_2O and at 25.0 ± 0.1 °C in 0.1 M sodium phosphate buffer that contained 0.1 N NaCl and 0.1 mM ONAC. Production of *o*-nitroaniline was followed at 412 nm on a Beckman DU7 UV–visible spectrophotometer. The AChE-catalyzed hydrolysis of acetylthiocholine (ATCh) was followed at 412 nm on an HP8452A diode array spectrophotometer by the coupled assay procedure described by Ellman et al.[15] Reaction conditions, including buffer compositions, for determining the pH–V/K profile for ATCh hydrolysis are described in the legend of Figure 2. V/K values were calculated by least-squares fitting[16] of time courses to eq 2:

$$A = (A_0 - A_{inf})e^{-(V/K)t} + A_{inf} \quad (2)$$

A, A_0, and A_{inf} are absorbances at times t, 0, and infinity, respectively. Solvent isotope effects were determined by measuring V/K values under the above conditions in equivalently buffered D_2O.[17-19] A Corning Model 125 pH meter, equipped with a glass combination electrode, was used to measure the pL (L = H, D) of isotopic buffers; pD values were calculated by adding 0.4 to the pH meter reading.[20]

pH–V/K profiles were fit to eq 3, which describes activity as depending on the basic form of a single active-site amino acid:

$$V/K = \frac{(V/K)_{lim}K_a}{[H^+] + K_a} \quad (3)$$

$(V/K)_{lim}$ is the value of V/K at high pH, and K_a is the apparent acid dissociation constant of the active-site residue.

$^{15}V/K$ Determinations. For each determination, ~1 mmol of ONAC was dissolved in 250 mL of buffer, and the sample was thermostated at 25.0 ± 0.1 °C in a refrigerated, circulating water bath that was also used to control the temperature in the spectrophotometer. During incubations as long as 30 min, no nonenzyme hydrolysis of ONAC was detected. The reaction was initiated by adding 0.4 mg of dialyzed AChE, and the progress of the reaction was determined by monitoring 1 mL of the sample at 440 nm. Duplicate reactions were run in H_2O to 7% conversion, and triplicate reactions were run to 10% and 13% conversions. Duplicate reactions in D_2O were run to 10% conversion. At the desired fractional conversion to product, the reaction was terminated by adding sufficient HCl to drop the pH or pD to 3.0 or 3.4, respectively. Spectrophotometric observation of the acidified sample showed that AChE does not catalyze the hydrolysis of ONAC, which accords with the reported pL–rate behavior of the enzyme,[12] nor does nonenzymatic hydrolysis occur. ONAC and ONA were removed from the reaction mixture by 3 or 4 successive extractions into 250 mL of HPLC grade benzene. Control experiments showed that each extraction removed >90% of ONAC and ONA, and therefore the efficiency of the successive extractions is >99%. The extraction efficiency of ONA was verified by measuring A_{412} values of the residual reaction mixture. The benzene extracts were combined, reduced in volume to ~3 mL on a rotary evaporator, and applied to a preparative TLC plate that was subsequently developed in a benzene mobile phase. The progress of the TLC separation could be followed visually, since ONA, the higher mobility component, is yellow. The ONA band was scraped from the plate, the gel was transferred to a small column, and the ONA was eluted with benzene. Evaporation of the benzene eluate afforded crystalline ONA whose properties (NMR and UV spectra, melting point, TLC elution) match those of a commercial preparation of ONA. The overall recovery of ONA after TLC separation, estimated by measuring A_{230}, A_{278}, and A_{404} values in MeOH, was ≥96%. Therefore, contributions to $^{15}V/K$ from isotopic fractionation during sample workup are negligible.

The $^{15}N/^{14}N$ ratios of ONA product samples and of unreacted ONAC starting material were measured by using a Finnigan Delta S isotope ratio mass spectrometer combined on-line with a Heraeus elemental analyzer. For each of the reactions described in the preceding paragraph, two or three measurements of the isotope ratio were made. Observed $^{15}V/K$ values were calculated by using eq 4:

$$^{15}V/K_{obs} = \ln(1 - f)/\ln(1 - fR_{ONA}/R_{ONAC}) \quad (4)$$

R_{ONAC} and R_{ONA} are the respective isotope ratios of ONAC before reaction with AChE (i.e., 0% hydrolysis) and of ONA after a known fractional hydrolysis f.

Results and Discussion

V/K values for AChE-catalyzed hydrolysis of ONAC were measured in triplicate in H_2O and D_2O buffers, and the resulting solvent isotope effect is $^{D_2O}V/K$ = 1.56 ± 0.03. This effect is identical to that previously reported for AChE-catalyzed hydrolysis of ONAC in the same buffer system as used herein but which also contained 2% MeCN (v/v).[12] The small solvent isotope effect on V/K has been described in terms of a mechanism in which an isotopically insensitive induced-fit step (i.e., k_3) follows substrate binding but precedes the isotope-dependent k_5 step,[12] as outlined in Scheme II.

Scheme II

$$E + A \underset{k_2}{\overset{k_1}{\rightleftarrows}} EA \underset{k_4}{\overset{k_3}{\rightleftarrows}} EA' \xrightarrow{k_5} F + P \xrightarrow{k_7} E + Q$$

The equations for V/K and $^DV/K$ for this mechanism follow:[12,21]

$$V/K = \frac{k_1k_3k_5[E]_T}{k_2(k_4 + k_5)} \quad (5)$$

$$^DV/K = \frac{^Dk_5 + k_5/k_4}{1 + k_5/k_4} \quad (6)$$

The observed effect, $^DV/K$ = 1.56 ± 0.03, arises because the intrinsic effect, $^Dk_5$ = 2.4 ± 0.2, is partially masked by the commitment k_5/k_4 = 1.4 ± 0.2.[12] Several observations suggest that reactions of AChE with neutral substrates are partially rate-limited by an induced fit step:[2,4,12,22-25] (1) solvent isotope effects on V for hydrolysis of anilides and esters are ≳2, while those for V/K are ~1.6 or less, and (2) pK_a values determined from dependences of V/K on pH are less than the intrinsic pK_a of H440 of the active site, on whose basic form AChE activity

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Table I. Nitrogen Isotope Effects on V/K of Acetylcholinesterase-Catalyzed Hydrolysis of *o*-Nitroacetanilide[a]

		observed isotope effects[d]		calculated isotope effects[d]	
f[b]	N[c]	$^{15}V/K_H$	$^{15}V/K_D$	$^{15}V/K_H$	$^{15}V/K_D$
0.07	3	1.006 13 ± 0.000 02		1.012 34 ± 0.000 03	
0.07	3	1.005 92 ± 0.000 05		1.011 91 ± 0.00007	
0.10	3	1.006 47 ± 0.000 09		1.0130 ± 0.0001	
0.10	2	1.005 93 ± 0.000 04		1.011 93 ± 0.000 06	
0.10	2	1.005 03 ± 0.000 04		1.010 11 ± 0.000 06	
0.10	3		1.0055 ± 0.0002		1.0111 ± 0.0003
0.10	3		1.005 22 ± 0.000 07		1.0105 ± 0.0001
0.13	3	1.0058 ± 0.0003		1.0118 ± 0.0004	
0.13	3	1.005 90 ± 0.000 04		1.011 87 ± 0.000 06	
0.13	3	1.0060 ± 0.0001		1.0121 ± 0.0001	
				1.0119 ± 0.0005	**1.0106 ± 0.0002**

[a] Reactions were run at 25.0 ± 0.1 °C, pH 7.3 in H_2O or pD 7.7 in D_2O, in 0.1 M sodium phosphate buffers that contained 0.1 N NaCl, $[ONAC]_0$ = 4 mM, and [AChE] = 1.6 μg/mL. Additional procedures are described in Materials and Methods (cf. $^{15}V/K$ Determinations). [b] Fractional conversion of substrate to product; cf. eq 4. [c] Number of determinations. [d] For the relationship between observed and calculated isotope effects, see eq 7 and the accompanying discussion. Error limits are standard errors of the mean, except when two determinations were made, in which case error limits are ± one-half the range of the determinations. Boldface values are weighted means of the isotope effect determinations, calculated as described by Palmer and Jencks.[33]

depends. The effect of the acylation reaction dynamics outlined in Scheme II on the expression of the ^{15}N isotope effect is discussed below.

$^{15}V/K_{obs}$ measurements for AChE-catalyzed hydrolysis of ONAC are listed in Table I. Because the isotope effects were determined with natural ^{15}N abundance ONAC and there are two nitrogen atoms in the substrate, $^{15}V/K_{obs}$ contains contributions from each of the nitrogen sites:

$$\frac{1}{^{15}V/K_{obs}} = \frac{(^{15}V/K_{amido})^{-1} + (^{15}V/K_{nitro})^{-1}}{2} \qquad (7)$$

The derivation of this equation is detailed in the Appendix. $^{15}V/K_{amido}$ and $^{15}V/K_{nitro}$ are the isotope effect contributions from the amido and nitro functions of ONAC, respectively. The secondary isotope effect from the nitro function is expected to be negligible, and hence $^{15}V/K_{nitro} = 1$.[26]

The value of $^{15}V/K_H$ calculated in Table I is 1.0119 ± 0.0005 for the reaction run in buffered H_2O. If the transition states that produce the solvent isotope effect and the nitrogen isotope effect are the same, one can calculate the intrinsic nitrogen effect by using an expression equivalent to eq 6, albeit transformed as follows:

$$^{15}V/K_H - 1 = \frac{^{15}k_5 - 1}{1 + C} \qquad (8)$$

In this equation, $C = k_5/k_4$. The effect calculated from eq 8 is $^{15}k_5 = 1.029 \pm 0.003$. The commitment for the reaction run in D_2O is $C_D = C/^Dk_5 = 0.6 \pm 0.1$. Substitution of this value into an expression that is equivalent to eq 8 gives a predicted $^{15}V/K_D = 1.018 \pm 0.002$, clearly larger than the effect of 1.0106 ± 0.0002 measured for the reaction run in buffered D_2O. Moreover, the direction of change of the isotope effect calculated from eq 8 is opposite the observed direction of change (cf. Table I). Therefore, a model in which the nitrogen isotope effect and the solvent isotope effect arise from a single transition state cannot account for the measured effects.

The fact that the N-15 effect is smaller in D_2O than in H_2O requires that there are serial isotope-sensitive steps. If the second of these steps has a larger N^{-5} isotope effect than the first, then the larger solvent isotope effect must reside in the first step. A mechanistic model which can account for the observed, though small, solvent isotope effect on the N-15 effect is given in Scheme III:

Scheme III

$$E + A \underset{k_2}{\overset{k_1}{\rightleftarrows}} EA \underset{k_4}{\overset{k_3}{\rightleftarrows}} EA' \underset{k_6}{\overset{k_5}{\rightleftarrows}} T \xrightarrow{k_7} F + P \xrightarrow{k_9} E + Q$$

In this mechanism, an additional intermediate (T) is proposed in the acylation stage of catalysis. The most straightforward, precedented identify for this intermediate is a uninegative tetrahedral intermediate that forms on the reversible addition of S200 to the carbonyl carbon of the substrate. V/K for this mechanism is given by eq 9:

$$V/K = \frac{k_1k_3k_5k_7[E]_T}{k_2(k_4k_6 + k_4k_7 + k_5k_7)} \qquad (9)$$

From eq 9 one derives the following set of equations for the solvent isotope effect and the dependence of $^{15}V/K$ on solvent isotope:[29]

$$^DV/K = \frac{^Dk_7 + b^Dk_5 + ab}{1 + b + ab} \qquad (10)$$

$$^{15}V/K_H = \frac{^{15}k_7' + b^{15}k_5 + ab}{1 + b + ab} \qquad (11)$$

$$^{15}V/K_D = \frac{^Dk_7{}^{15}k_7' + {}^Dk_5b^{15}k_5 + ab}{^Dk_7 + {}^Dk_5b + ab} \qquad (12)$$

In eqs 10–12 $^Dk_5$, $^Dk_7$, and $^{15}k_5$ are the respective solvent deuterium

(26) The following considerations suggest that the contribution from the nitro function can be ignored: (1) Hengge and Cleland measured $^{15}K_a$ = 1.0023 for acid ionization of *p*-nitrophenol and ascribed this equilibrium secondary nitrogen isotope effect to quininoid resonance interaction betwe en the phenoxy oxygen and the nitro function.[27] This equilibrium isotope effect is the maximum $^{15}k_{sec}$ effect expected for a transition state in which the bond to the phenoxy leaving group is completely broken. (2) Because the ρ values for ionization of para-substituted phenols and anilines are similar (2.11 and 2.77, respectively[28]), one can reasonably expect a maximum value for $^{15}V/K_{nitro}$ that is similar to $^{15}k_{sec}$ = 1.0023 for *p*-nitrophenol. Moving the nitro substituent from the para to the ortho position attenuates the resonance interaction with the amido group, since the coplanar arrangement of the nitro group and the aromatic ring required for resonance interaction is sterically impeded by the *o*-acetamido function. This steric attenuation of resonance will decrease the maximum $^{15}k_{sec}$. (3) The measured nitrogen isotope effects in Table I are not intrinsic effects. An isotopically insensitive induced-fit step contributes to rate determination of V/K.[12] Therefore, even if the intrinsic $^{15}V/K_{nitro}$ is 1.0023, the corresponding contribution to $^{15}V/K_{obs}$ is less. If the contribution to $^{15}V/K_{obs}$ from $^{15}V/K_{nitro}$ is 1.0023, one calculates from eq 7 the following $^{15}V/K_{amido}$ values for the reactions in H_2O and D_2O, respectively: $^{15}V/K_H = 1.0096 \pm 0.0005$ and $^{15}V/K_D = 1.0082 \pm 0.0001$. Therefore, the salient observation described herein, that the N-15 effect is decreased when measured in D_2O versus H_2O, remains intact.

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(29) Equations 10–12 are based on the assumption that the equilibrium solvent isotope effect for formation of T is $^Dk_5/^Dk_6 = 1.0$. This is a reasonable assumption since the exchangeable protic positions of the active-site residues that are involved in catalysis (i.e., S200 and H440) and the NH function of ONAC in the reactant and tetrahedral intermediate should have fractionation factors ~1.[17-19]

isotope effects on the k_5 and k_7 steps and the N-15 effect on the k_5 step; ${}^{15}k_7' = {}^{15}k_5{}^{15}k_7/{}^{15}k_6$ is the product of the N-15 effect on the k_7 step and the equilibrium N-15 effect for the formation of T. These isotope effects and the commitments $a = k_5/k_4$ and $b = k_7/k_6$ give six unknowns on which the observed N-15 and solvent isotope effects depend, a seeming hopelessly underdetermined situation. However, reasonably narrow limits can be placed on the isotope effects and commitments. The ratio of the respective N-15 effects minus one is given by eq 13:

$$\frac{{}^{15}V/K_H - 1}{{}^{15}V/K_D - 1} = {}^{D}V/K\frac{{}^{15}k_7' - 1 + b({}^{15}k_5 - 1)}{{}^{D}k_7({}^{15}k_7' - 1) + {}^{D}k_5 b({}^{15}k_5 - 1)} \quad (13)$$

Solving this equation for ${}^{D}k_7$ gives eq 14, in which $R = {}^{D}V/K({}^{15}V/K_D - 1)/({}^{15}V/K_H - 1)$:

$${}^{D}k_7 = R + \frac{b(R - {}^{D}k_5)({}^{15}k_5 - 1)}{{}^{15}k_7' - 1} \quad (14)$$

Since ${}^{D}V/K = 1.56 \pm 0.03$, $R = 1.38 \pm 0.08$. Accordingly, if ${}^{15}k_5 = 1$ (a reasonable lower limit for the expected small normal effect for tetrahedral intermediate formation), then ${}^{D}k_7 = R$. Moreover, since ${}^{D}k_5$ is larger than ${}^{D}k_7$, ${}^{D}k_5$ must be larger than ${}^{D}V/K$. Hence, $R - {}^{D}k_5$ is less than 1, and the ratio on the right hand side of eq 14 must be negative, which necessitates that ${}^{D}k_7 \leq R$. A lower limit ${}^{D}k_7 = 1$ is set because one expects that expulsion of *o*-nitroanilide from T will be catalyzed by proton bridging to the amide nitrogen, which cannot generate an inverse contribution to the solvent isotope effect. These considerations place ${}^{D}k_7$ in the range 1.0–1.5; note that the upper limit is almost 2 standard deviations greater than R.

Given the above-placed limits on ${}^{D}k_7$, one turns to the published proton inventory to calculate values for a, b, and ${}^{D}k_5$. If one imagines that ${}^{D}k_5$ arises from a single transition-state proton transfer, then $k_{5,n} = k_5(1 - n + n\phi_5^T)$, where n is the atom fraction of deuterium in isotope mixtures of H_2O and D_2O. This and a similar expression for $k_{7,n}$ are used to derive the following from eq 9:

$$\frac{(V/K)_0}{(V/K)_n} = \frac{1}{1 + b + ab}\left(ab + \frac{b}{1 - n + n\phi_5^T} + \frac{1}{1 - n + n\phi_7^T}\right) \quad (15)$$

In this equation, ϕ_5^T (= $1/{}^{D}k_5$) and ϕ_7^T (= $1/{}^{D}k_7$) are the respective isotopic fractionation factors for the proton transfers that stabilize the transition states of the k_5 and k_7 steps. The value of the commitment b was constrained at 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0, and at each of these ${}^{D}k_7$ was constrained at the values 1.0, 1.1, 1.2, 1.3, 1.4, and 1.5. For each pair of constrained values of b and ${}^{D}k_7$, the proton inventory was fit to eq 15 by least-squares procedures[16] to determine a, ${}^{D}k_5$, and $(V/K)_0$. An example fit is shown in Figure 1.

The procedure outlined in the preceding paragraph generated 84 combinations of the commitments and intrinsic solvent isotope effects. Of these, 19 had values of $a < 0$ and were therefore discarded. For the remaining combinations, values of the N-15 effects were calculated according to eqs 16 and 17:

$${}^{15}k_5 = [{}^{15}V/K_D({}^{D}k_7 + {}^{D}k_5 b + ab) - {}^{D}k_7{}^{15}V/K_H(1 + b) - ab\{1 + {}^{D}k_7({}^{15}V/K_H - 1)\}]/b({}^{D}k_5 - {}^{D}k_7) \quad (16)$$

$${}^{15}k_7' = {}^{15}V/K_H(1 + b + ab) - b({}^{15}k_5 - a) \quad (17)$$

The range of ${}^{15}k_5$ values so generated is 0.993–1.009. However, an inverse value for ${}^{15}k_5$ is not reasonable. The partial double bond character of the amide C–N bond of ONAC will be lost on formation of the tetrahedral intermediate, which will decrease the stiffness of the C–N bond and generate a normal isotope effect. An upper limit for ${}^{15}k_7'$ of 1.053 was calculated by multiplying the Streitwieser limit[30] of 1.044 for breaking a C–N single bond by 1.009, the upper limit for ${}^{15}k_5$ and therefore the expected upper limit for ${}^{15}k_5/{}^{15}k_6$. Of the remaining 65 combinations of commitments and intrinsic isotope effects, 30 either exceed the upper limit for ${}^{15}k_7'$ or have inverse values of ${}^{15}k_5$ and are therefore discarded. This procedure gives values that fall in the following ranges: a = 0–2.6, b = 0.05–2.0, ${}^{D}k_5$ = 2.6–7.6, ${}^{D}k_7$ = 1.0–1.5, ${}^{15}k_5$ = 1.000–1.009, and ${}^{15}k_7'$ = 1.012–1.053.

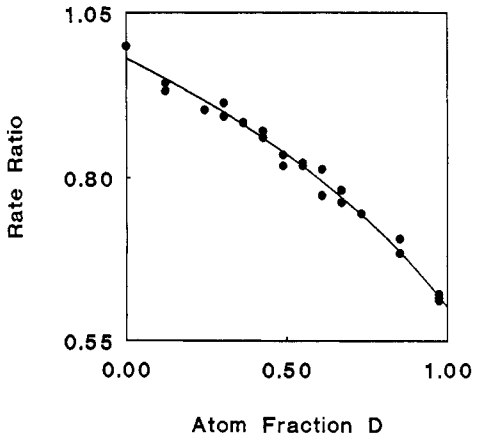
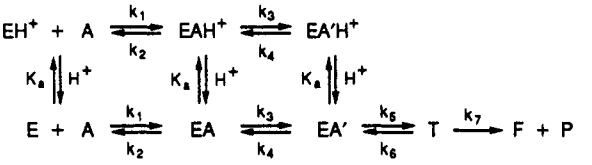


Figure 1. Proton inventory for V/K of AChE-catalyzed hydrolysis of *o*-nitroacetanilide. The displayed values were calculated from data in ref 12 and are plotted as V_n/V_0 versus the atom fraction of deuterium. The nonlinear line was generated by least-squares fitting[16] of the data to eq 15. The values of the constrained parameters are b = 1.5 and ${}^{D}k_7$ = 1.2. The parameters determined by least-squares analysis[16] are a = 1.4 ± 0.3, ${}^{D}k_5$ = 2.8 ± 0.2, and $(V/K)_0 = (1.63 \pm 0.01) \times 10^{-3}$ s^{-1}.

The ranges can be narrowed further by considering the pH–V/K profile for AChE-catalyzed hydrolysis of ONAC[12] shown in Figure 2. Also shown in the figure is the pH–V/K profile for hydrolysis of ATCh, which provides a measure of the intrinsic pK_a of the active-site general acid–base catalyst, H440.[2-4,31] The respective pK_a values for the ONAC and ATCh reactions are 5.63 ± 0.03 and 6.22 ± 0.03, and thus the difference between them is 0.59 ± 0.06. This pK_a suppression arises due to partial rate limitation of the ONAC reaction by the pH-insensitive induced fit step, as outlined in Scheme IV:

Scheme IV



Equation 18, in which $k_{7E} = k_1k_3k_5k_7/k_2k_4k_6$ is the rate constant for conversion of the E + A reactant state to the transition state of the k_7 step, gives the dependence of V/K on pH for this mechanism:

$$(V/K)/[E]_T = \frac{k_{7E}K_a}{(1 + b)[H^+] + K_a(1 + b + ab)} \quad (18)$$

From this equation one can show that the difference between intrinsic and observed pK_a values is

(30) Huskey, W. P. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL; pp 37–72. See in particular Table 1 on p 49 of this reference.

(31) Krupka showed that binding of quaternary ammonium ligands to AChE is prevented when an active-site residue that has a pK_a = 6.2 is protonated. Krupka's experiments thus provide an equilibrium measure of the intrinsic pK_a of H440: (a) Krupka, R. M. *Biochemistry* **1966**, *5*, 1983–1988. (b) Krupka, R. M. *Biochemistry* **1966**, *5*, 1988–1998.

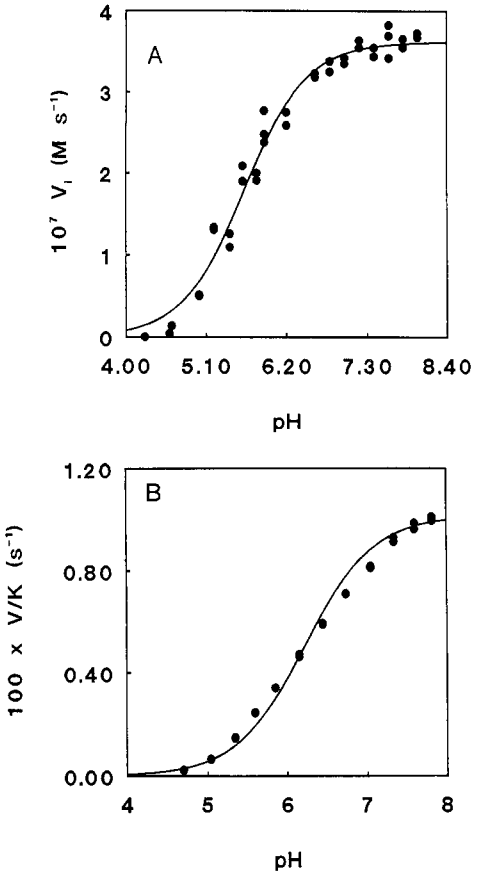


Figure 2. pH–V/K profiles for AChE-catalyzed reactions; pK_a values were determined by fitting the profiles to eq 3 by least-squares procedures.[16] (A) pH–V/K profile for hydrolysis of *o*-nitroacetanilide, reprinted with permission from ref 12. The fit gives $pK_a = 5.63 \pm 0.03$. (B) pH–V/K profile for AChE-catalyzed hydrolysis of acetylthiocholine (ATCh). Reactions were conducted at 25.0 ● 0.1 °C in sodium acetate buffers (pH ≤ 5.6) or sodium phosphate buffers (pH > 5.6) that contained 74.7 ng of AChE, 0.3 mM DTNB, and 0.01 mM ATCh ($K_m/10$). The reaction volume was 1.00 mL, buffer concentration was 0.05 M, and ionic strength was adjusted to 0.2 with NaCl. The fit gives $pK_a = 6.22 \pm 0.03$.

$$\Delta pK_a = pK_a^{int} - pK_a^{obs} = \log \frac{1 + b + ab}{1 + b} \qquad (19)$$

Finally, from eq 19 one can derive an expression for the fraction of rate limitation by the pH-insensitive induced fit step k_3:

$$f_3 = \frac{10^{\Delta pK_a} - 1}{10^{\Delta pK_a}} \qquad (20)$$

The value of f_3 so calculated is 0.74 ± 0.17. Since $f_3 = ab/(1 + b + ab)$, the value of f_3 calculated according to eq 20 from the measured ΔpK_a can be used to further constrain the combinations of commitment factors a and b that satisfy both the proton inventory and the pH–V/K behavior of AChE-catalyzed hydrolysis of ONAC. All pairs of a and b that give calculated f_3 values that fall in the range 0.4–1.0 (i.e., within 2 standard deviations of the experimental f_3) are, conservatively, considered acceptable. From the acceptable set of proton inventory fits described earlier, one can therefore narrow the ranges of intrinsic isotope effects. This procedure gives the following final ranges for commitments and intrinsic isotope effects: $a = 0.9–2.6$, $b = 0.35–2.5$, $^Dk_5 = 2.6–3.7$, $^Dk_7 = 1.0–1.5$, $^{15}k_5 = 1.000–1.009$, $^{15}k_7' = 1.027–1.053$.

The picture that emerges is that the serial transition states for induced fit and formation and decomposition of the tetrahedral intermediate have approximately the same free energies and thus contribute comparably to rate determination. Therefore, as realized before,[2] the acylation stage of AChE-catalyzed hydrolysis of anilides is marked by matched internal thermodynamics. Formation of the tetrahedral intermediate is subject to a large solvent isotope effect, as one expects for general base catalysis by H440 of S200 attack on the carbonyl carbon of ONAC. This step generates a relatively dimunitive N-15 effect, which results from the loss of amide resonance and the addition of a fourth ligand to the carbonyl carbon. The breakdown of the tetrahedral intermediate, on the other hand, is subject to a small solvent isotope effect and a sizeable N-15 effect. If one uses the upper end of the calculated range of $^{15}k_5$ (i.e., 1.009) as a measure of the largest possible equilibrium isotope effect on k_5/k_6, the range for $^{15}k_7$ is 1.018–1.044. The N-15 effects for papain-catalyzed hydrolysis of *N*-benzoyl-L-argininamide are in the range 1.021–1.024 at pH 4.0–8.0 and are interpreted in terms of C–N bond breaking during rate-limiting decomposition of a tetrahedral intermediate.[32] These papain isotope effects fall at the lower end of the range of $^{15}k_7$ values calculated herein and thus support a model for the transition state of the k_7 step of AChE-catalyzed hydrolysis of ONAC in which appreciable breaking of the C–N bond has taken place. This model may also explain the small $^Dk_7$ value, since the development of a highly basic amide leaving group should be stabilized by general acid assistance from the erstwhile-formed imidazolium ion form of H440, albeit via an asymmetric proton transfer.

Acknowledgment. We thank Professor Jim Gloer for checking the purity of recrystallized *o*-nitroacetanilide by HPLC. This work was supported by NIH Grants NS21334 to D.M.Q. and GM43043 to M.H.O.

Appendix. Kinetic Isotope Effects Determined in Competitive Experiments Using Substrates Singly Labeled in Multiple Sites

The possible outcome of an experiment involving a competition between substrates that are labeled with an isotope at two distinct sites are shown in eq A1. The substrate, S_{ij} (i and j denote distinct

$$\begin{aligned} S_{11} &\xrightarrow{k_{11}} P_{11} & S_{11} &\xrightarrow{k_{11}} (A_1 + B_1) = P_{11} \\ S_{12} &\xrightarrow{k_{12}} P_{12} & S_{12} &\xrightarrow{k_{12}} (A_1 + B_2) = P_{12} \\ S_{21} &\xrightarrow{k_{21}} P_{21} & S_{21} &\xrightarrow{k_{21}} (A_2 + B_1) = P_{21} \\ S_{22} &\xrightarrow{k_{22}} P_{22} & S_{22} &\xrightarrow{k_{22}} (A_2 + B_2) = P_{22} \end{aligned} \qquad (A1)$$

sites of isotopic substitution by isotopes 1 and 2), may form a product in which both isotopic sites are retained in a single molecule (P_{ij})[35] or it may react to form products in which the isotopic sites are split into two products (A_i and B_j). In cases where A_1/A_2 and B_1/B_2 can be measured or where the isotopic ratios of individual sites in P_{ij} (or in S_{ij}) can be distinguished, the individual isotope effects for both sites (k_{11}/k_{12} and k_{12}/k_{21}) can be determined if the initial isotopic distribution in the substrate is known. In cases where the isotopic sites of the product (or substrate) are not distinguished experimentally, the observed isotope effect will be a composite of the isotope effects corresponding to the individual sites. The remainder of this appendix

(32) O'Leary, M. H.; Urberg, M.; Young, A. P. *Biochemistry* **1974**, *13*, 2077–2081.
(33) Palmer, J. L.; Jencks, W. P. *J. Am. Chem. Soc.* **1980**, *102*, 6472.
(34) (a) Schowen, R. L. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; pp 77–114. (b) Wright, M. R. *J. Chem. Soc. B* **1968**, 545–547.
(35) In the experiments of Quinn et al.,[36] the substrate bearing ^{14}N at both the amido and the nitro sites is S_{11}, while the respective substrates that contain ^{15}N at the amido and nitro sites are S_{21} and S_{12}. The ^{15}N isotope effects at the amido and nitro sites are k_{11}/k_{21} and k_{11}/k_{12}, respectively (these are V/K isotope effects).
(36) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15–76.

demonstrates that the observed isotope effect is the harmonic mean of the individual isotope effects when one isotope is present at tracer levels and measurements are made at early fractions of reaction.

When the isotopic sites cannot be distinguished, it is the total isotope-1 and isotope-2 content of the product (p_1 and p_2) or substrate (s_1 and s_2) that is determined experimentally (eq A2).

$$p_1 = 2[P_{11}] + [P_{12}] + [P_{21}] \qquad p_2 = 2[P_{22}] + [P_{12}] + [P_{21}]$$
$$s_1 = 2[S_{11}] + [S_{12}] + [S_{21}] \qquad s_2 = 2[S_{22}] + [S_{12}] + [S_{21}] \tag{A2}$$

For the scheme in eq A1, the isotopic ratio of product concentrations will be governed by eq A3.

$$\frac{dp_1}{dp_2} = \frac{2k_{11}[S_{11}] + (k_{12}[S_{12}] + k_{21}[S_{21}])}{(2k_{22}[S_{22}]) + k_{12}[S_{12}] + k_{21}[S_{21}]} \tag{A3}$$

If isotope 2 is present at tracer levels, the terms in parentheses in eq A3 can be omitted ($[S_{11}] \gg [S_{12}] + [S_{21}] \gg [S_{22}]$). Under these conditions, the total isotope-1 content of the substrate is $s_1 = 2[S_{11}]$, and the isotope-2 content of the substrate is $s_2 = [S_{12}] + [S_{21}]$. When $[S_{12}] \sim [S_{21}]$ (a condition that is most likely to be realized early in the reaction) and with the tracer-level approximation, eq A3 simplifies to eq A4 (s_{10} and s_{20} are the initial isotope contents of the substrate).

$$\frac{dp_1}{dp_2} = \frac{k_{11}}{(k_{12} + k_{21})} \frac{s_1}{s_2} = I_{obs} \frac{s_1}{s_2} = I_{obs} \frac{s_{10} - p_1}{s_{20} - p_2} \tag{A4}$$

Equation A4 is identical to the differential form of the equation commonly used to evaluate isotope effects in competitive experiments.[36,37] The integrated form of this equation is eq 4 shown in the Materials and Methods section. A conventional competitive analysis is thus expected to be acceptable (as judged by I_{obs} being independent of the fraction of reaction) in experiments using tracer-level labels in two sites, when the analysis is limited to small fractions of reaction. The observed isotope effect, I_{obs}, will be the harmonic mean of the isotope effects for the individual sites (eq A5 and eq 7 shown in the Results and Discussion section). When the isotope effects are near unity, the harmonic mean will

$$I_{obs}^{-1} = {}^1/_2[(k_{11}/k_{12})^{-1} + (k_{11}/k_{21})^{-1}] \tag{A5}$$

be approximated by the simple arithmetic mean of the individual isotope effects.

(37) Melander, L.; Saunders, W. H., Jr. *Reaction Rates of Isotopic Molecules*; Wiley: New York, 1980; pp 95–102.