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Decarbamylation of Acetylcholinesterases is Markedly Slowed as Carbamoyl Groups Increase in Size

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Abstract

Carbamates are esters of substituted carbamic acids that react with acetylcholinesterase (AChE) by initially transferring the carbamoyl group to a serine residue in the enzyme active site accompanied by loss of the carbamate leaving group followed by hydrolysis of the carbamoyl enzyme. This hydrolysis, or decarbamylation, is relatively slow, and half-lives of carbamoylated AChEs range from 4 min to more than 30 days. Therefore, carbamates are effective AChE inhibitors that have been developed as insecticides and as therapeutic agents. We show here, in contrast to a previous report, that decarbamylation rate constants are independent of the leaving group for a series of carbamates with the same carbamoyl group. When the alkyl substituents on the carbamoyl group increased in size from *N*-monomethyl- to *N,N*-dimethyl-, *N*-ethyl-*N*-methyl-, or *N,N*-diethyl-, the decarbamylation rate constants decreased by 4-, 70-, and 800-fold, respectively. We suggest that this relationship arises as a result of active site distortion, particularly in the acyl pocket of the active site. Furthermore, solvent deuterium oxide isotope effects for decarbamylation decreased from 2.8 for *N*-monomethylcarbamoyl AChE to 1.1 for *N,N*-diethylcarbamoyl AChE, indicating a shift in the rate-limiting step from general acid-base catalysis to a likely conformational change in the distorted active site.

Keywords

Acetylcholinesterase; Decarbamylation; Alzheimer's disease

1. Introduction

Acetylcholinesterase (AChE) (1) catalyzes the hydrolysis of the neurotransmitter acetylcholine, and rapid hydrolysis of this ester is essential for normal cholinergic synaptic

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transmission. At neuromuscular junctions, acetylcholine is released by fusion of intracellular vesicles with the presynaptic membrane and hydrolyzed in the synaptic cleft in less than 100 μ sec (3). Ligand binding studies (4) and X-ray crystallography (5) of AChE have revealed a narrow active site gorge some 20 Å deep with two separate ligand binding sites. At the base of the gorge is the acylation or A-site where a tryptophan residue binds the trimethylammonium group of acetylcholine and a catalytic triad of glutamate, histidine, and serine residues participate in a triad that catalyzes the transient acylation and deacylation of the serine during each substrate turnover. The peripheral or P-site near the mouth of the gorge is the site of initial binding of acetylcholine in the catalytic pathway (6).

Acetylcholine hydrolysis proceeds by transfer of the acetyl group to the active site serine of AChE followed by hydrolysis of the acetyl enzyme. Carbamates are esters of substituted carbamic acids that also react with AChE in a two-step process, with initial transfer of the carbamoyl group to the serine of AChE followed by hydrolysis of the carbamoyl enzyme. This hydrolysis is relatively slow, and half-lives of carbamoylated AChEs range from 4 min to more than 30 days ((7) and data in Table 1 below). Because carbamates are poor, slowly reversible AChE substrates, they are effective AChE inhibitors that have been widely developed as insecticides (8).

Alzheimer's disease (AD) is a form of dementia characterized by the presence of amyloid and tau deposits which lead to the loss of cholinergic neurons in the brain. Partial inhibition of AChE preserves residual released acetylcholine and restores some of the cholinergic function lost in AD patients. A small number of AChE inhibitors have been approved by the FDA for this symptomatic treatment of AD, and one of these is rivastigmine (marketed as Exelon), the 3-[(1-dimethylamino) ethyl] phenyl ester of *N*-ethyl-*N*-methylcarbamic acid (see Figure 1 below) (9–11). Of particular interest here, rivastigmine and the smaller *N*-ethyl-*N*-methylcarbamoyl chloride (EMCC) form identical carbamoyl AChE intermediates and yet the decarbamoylation rate was reported to be two orders magnitude slower for rivastigmine (12). The crystal structure of rivastigmine-bound TcAChE suggested a structural explanation for this unexpected difference. It revealed that His440 (2) in the catalytic triad had moved out of hydrogen bonding distance to Glu327 and that the *N*-ethyl-*N*-methylcarbamoyl group attached to Ser200 and the leaving group cleaved from rivastigmine, (–)-*S*-3-[1-(dimethylamino)ethyl]phenol (NAP, see Figure 1 below), were trapped inside the distorted A-site (12). One explanation for the survival of this structure during crystallization was that NAP and the *N*-ethyl-*N*-methylcarbamoyl group mutually blocked the other's release from the distorted active site (12). To test this proposed novel mechanism involving synergistic inhibition, we conducted a systematic study of the decarbamoylation rates for a series of carbamates wherein we varied both the *N*-alkyl-groups on the carbamate and the ester-linked leaving groups. We report here the results of our study.

¹Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; EMCC, *N*-ethyl-*N*-methylcarbamoyl chloride; HI-6, 1-(2-hydroxy-iminomethylpyridinium)-1-(4-carboxyamino)-pyridinium dimethylether dichloride; hAChE, human acetylcholinesterase; mAChE, mouse acetylcholinesterase; TcAChE, *Torpedo californica* acetylcholinesterase; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); HTMA, 3-hydroxy-*N,N,N*-trimethylanilinium; M7H, 7-hydroxy-1-methylquinolinium; NAP, 3-[1-(dimethylamino)ethyl]phenol; 2-PAM, 2-pyridinealdoxime methiodide;

²Throughout this paper we number amino acid residues according to both the hAChE and the TcAChE sequences, with the species designation clearly indicated.

2. Materials and methods

2.1. hAChE

Recombinant hAChE was expressed as a secreted, disulfide-linked dimer in *Drosophila* S2 cells and purified by affinity chromatography as outlined previously (13). In some experiments, purified hAChE was reductively radiomethylated with [³H]formaldehyde and sodium cyanoborohydride (14). This process methylates primary amino groups on the N-terminus and lysine residues but has essentially no effect on AChE activity.

2.2. Carbamates

N,N-Dimethyl- and *N,N*-diethylcarbamoyl chlorides were commercial samples from Sigma-Aldrich Chemical Co (St. Louis, MO). Rivastigmine and EMCC were gifts from Dr. Albert Enz, Novartis Institutes for Biomedical Research, Basel, Switzerland. *N*-methylcarbamoyl chloride was synthesized from *N*-methylformamide. Other carbamates were synthesized by reacting a slight excess of the appropriate carbamoyl chloride with the appropriate phenol in the presence of either triethylamine or sodium hydride. Synthesis of *N*-methylcarbamates utilized triethylamine as a base in dichloromethane at room temperature, while synthesis of the other carbamates employed sodium hydride for generation of the phenolate nucleophile in dimethylformamide at 0 °C. Some carbamates were converted to their quaternary ammonium iodides by allowing them to react with methyl iodide overnight. The salts were filtered and recrystallized from an appropriate solvent. The carbamoyl fluorides were prepared by stirring the corresponding chlorides with anhydrous KF in 18-crown-6 ether at room temperature for 24–48 hr. The fluoride was distilled out of the reaction mixture, and the structure was confirmed by ¹⁹F NMR. The proton NMR spectra of the synthesized carbamates were consistent with their structures.

2.3. Assay of substrate hydrolysis

Hydrolysis rates ν for the substrate acetylthiocholine were measured in a coupled Ellman reaction in which thiocholine generated in the presence of the indicated concentration of DTNB was determined by the formation of the thiolate dianion of DTNB at 412 nm ($\epsilon_{412\text{ nm}} = 14,150\text{ M}^{-1}\text{cm}^{-1}$) (15). Total AChE concentrations (E_{tot}) were calculated assuming 450 units/nmol (2) ³ 3. Assays were conducted at 25° C in 20 mM sodium phosphate, 60 mM NaCl, and 0.02% Triton X100 (pH 7.0) unless otherwise noted.

2.4. Carbamate reaction with hAChE

With carbamate substrates, both the formation and hydrolysis of the carbamoylated enzyme (*EC*), are slow enough to allow equilibrium assumptions. A general approach is given in Scheme 1 (7, 16–18). In this scheme, k_{12} and k_{21} are overall carbamoylation and decarbamoylation rate constants that incorporate intrinsic rate constants as described below. The leaving group *P* and carbamic acid *COH* are the products released during the carbamoylation and decarbamoylation reactions, respectively. The terms $E \cdot M$ and $EC \cdot N$

³One unit of AChE activity corresponds to 1 μmol of acetylthiocholine hydrolyzed/min under standard pH-stat assay conditions at pH 8 (1, 2). Our conventional spectrophotometric assay at 412 nm is conducted in pH 7.0 buffer. With wild type hAChE and 0.5 mM acetylthiocholine, this assay results in 4.8 $\Delta 412\text{ nm}/\text{min}$ with 1 nM AChE or about 76 % of the pH stat assay standard.

represent all species in equilibrium with E and EC , respectively (i.e., $[E] \cdot M + [EC] \cdot N = [E]_{\text{tot}}$, where $[E]$ and $[EC]$ are the respective concentrations of E and EC , and E_{tot} is the total concentration of enzyme active sites), and M and N are sums of terms (involving equilibrium constants and ligand concentrations) that depend on the details of a selected reaction scheme. The rate equation corresponding to Scheme 1 can be solved explicitly if all ligand concentrations remain essentially constant over the reaction time course, as noted previously (16). For example, the more explicit Scheme 2 is sufficient to analyze the data we present here. In this scheme, carbamate (CX) reaction is coupled to acetylthiocholine (S) hydrolysis. Since a detailed analysis of the carbamoylation reaction is not conducted here, M is simplified to $M = 1 + [CX]/K_D + [S]/K_{\text{app}}$, while the decarbamoylation term N more explicitly includes additional species that can modify the decarbamoylation rate: $N = 1 + [CX]/K_C + [S]/K_{\text{app}}$. The corresponding rate constants k_{12} and k_{21} are given in Eqs. 1a and 1b:

$$k_{12} = \frac{k_2 \frac{[CX]}{K_D}}{1 + \frac{[CX]}{K_D} + \frac{[S]}{K_{\text{app}}}} \quad k_{21} = \frac{k_3 \left(1 + \frac{b_1 [CX]}{K_C} + \frac{b_2 [S]}{K_S} \right)}{1 + \frac{[CX]}{K_C} + \frac{[S]}{K_S}} \quad (\text{Eqs. 1a \& b})$$

In the experiments that were analyzed with Eq. 1a here, K_{app} was assigned as 0.08 mM (19).

Experimental traces of carbamoylation and decarbamoylation reactions coupled to acetylthiocholine hydrolysis were obtained, and the equation for the general solution used to fit these traces to Schemes 1 and 2 is given in Eq. 2. Data were fitted to Eq. 2 by unweighted nonlinear

$$A_{412} = \left(\frac{v_0}{k_{12} + k_{21}} \right) \left[k_{21} (t - t_0) + \left(\frac{k_{12}}{k_{12} + k_{21}} - R \right) \left(1 - e^{-(k_{12} + k_{21})(t - t_0)} \right) \right] + A_{412(t_0)} \quad (\text{Eq. 2})$$

regression with SigmaPlot (vers. 12.0). In Eq. 2, A_{412} is the continuously monitored absorbance at 412 nm, t is the time of the reaction, t_0 is the time at which carbamate and enzyme were mixed, v_0 is the initial substrate hydrolysis rate at t_0 , R is the fraction of total enzyme that is carbamoylated at the reaction initiation, and $A_{412(t_0)}$ is the blank absorbance in the absence of enzyme. We previously reported a similar version of Eq. 2 (7). To maximize accuracy in the stopped flow experiments here, mixing was triggered slightly after the trace time was initiated. This necessitated addition of t_0 to the equation.

2.5. Measurements of the decarbamoylation rate constant k_{21}

Two protocols were employed to measure the rate constant k_{21} . One used stopped-flow methods to accurately measure the approach to the carbamoylation steady state following mixing of enzyme and carbamate. A Hi-Tech SFA 20 stopped-flow apparatus thermostatted at 25 °C was used to rapidly mix equal volumes (300 μ L) of AChE in one syringe and acetylthiocholine, DTNB, and carbamate in the other. Final concentrations of acetylthiocholine and DTNB were 0.25 mM and 2.0 mM, respectively. This higher than

conventional concentration of DTNB was employed to insure that DTNB reaction with thiocholine was not rate limiting. Absorbance at 412 nm was recorded on a Varian Cary 3A spectrophotometer at fixed intervals of 33 msec.

The second protocol for obtaining k_{21} measured the increase in enzyme activity as carbamoylated AChE slowly decarbamoylated. Since half times for decarbamoylation here were as long as weeks, the key to accurate measurements was precise determination of the fully decarbamoylated enzyme activity. The AChE was radiolabeled to enable this determination. A calibration ratio of radiomethylated AChE activity monitored spectrophotometrically with 0.5 mM acetylthiocholine to radioactivity determined by scintillation counting was established for stock AChE solutions. In a typical assay an AChE sample was inactivated with a carbamate for at least an hour (overnight with *N,N*-diethylcarbamates), and unreacted carbamate was removed by adding 100 μ L of the mix to a 1.5 ml G-50 Sephadex spin column. The columns were prepared freshly and were equilibrated with phosphate buffer prior to addition of the mix. Recovery of enzyme from the columns was monitored by scintillation counting, and an aliquot (50 μ L) of the fraction with the highest enzyme concentration was added to 3.0 mL of assay buffer containing 0.1 to 1.0 mM acetylthiocholine. Typically, 0.2 – 8 h of reactivation showed some curvature in the Ellman trace and allowed fitting with Eq. 2, although some reactivation reactions with *N,N*-diethylcarbamates were extended to 24 h. Data fitting was conducted with k_{12} and t_0 in Eq. 2 set to zero and $v_f = v_0 k_{21}/(k_{12} + k_{21})$ fixed as the predicted final velocity determined from scintillation counting and the calibration ratio. Values listed for k_{21} are means of n measurements with the standard error of the mean.

2.6 Solvent isotope effects

Decarbamoylation reactions in D₂O were paired with reactions in H₂O to maximize precision in comparing rates. Ellman assays were conducted at 25° C in 20 mM sodium phosphate and 0.02% Triton X-100 in D₂O or H₂O, and both buffers were adjusted to pH 7.0. Final assay concentrations of acetylthiocholine and DTNB were 0.25 mM and 0.33 mM DTNB, respectively.

3. Results

3.1. Measurement of decarbamoylation rate constants k_{21}

The focus of this study was the measurement of the overall decarbamoylation rate constant k_{21} for a series of carbamates (CX in Scheme 2) with different leaving groups (X) and with carbamoyl groups (C) of various size (Figure 1). Two protocols were used to measure k_{21} . The first is illustrated in Figure 2, where the approach to the steady state after mixing carbamate and enzyme was monitored. The carbamate in Figure 2a was *N*-monomethyl neostigmine, one of the most reactive carbamoylating agents of AChE. Although analysis of k_{12} was given only minor emphasis in this report, the data in Figure 2a combined with additional k_{12} measurements at higher *N*-monomethyl neostigmine concentrations and fitted to Eq. 1a gave $k_2 = 51 \pm 2 \text{ min}^{-1}$; $K_D = 3.2 \pm 0.4 \text{ }\mu\text{M}$; and $k_2/K_D = 16 \pm 2 \text{ min}^{-1}\mu\text{M}^{-1}$. The steady state in Figure 2a was reached in about one minute, and subsequent substrate turnover was high enough to give an accurate measurement of $k_{21} = 0.013 \text{ min}^{-1}$. Rivastigmine was

the carbamate examined in Figure 2b, and it reacted more slowly with AChE. The data in Figure 2b and additional k_{12} measurements at various rivastigmine concentrations were fitted to Eq. 1a to give $k_2 = 1.5 \pm 0.5 \text{ min}^{-1}$; $K_D = 130 \pm 80 \text{ }\mu\text{M}$; and $k_2/K_D = 12 \pm 4 \text{ min}^{-1}\text{mM}^{-1}$. This value of k_2/K_D was about 1000-fold lower than that for *N*-monomethyl neostigmine. An estimate of $k_{21} = 0.003 \text{ min}^{-1}$ was also obtained from Figure 2b. However, because all data in Figure 2b were fitted simultaneously, the fitted final slope is higher than the observed final slope. The corresponding fitted value of k_{21} is more than an order of magnitude larger than the more accurate measurements described below. To further compound the problem, any residual nonenzymatic substrate hydrolysis will increase the fitted k_{21} , and for some carbamates the value of b_1 in Eq. 1b is greater than 1 (see Discussion). Although the protocol in Figure 2 is useful because it can give simultaneous information about k_{12} and k_{21} , we conclude that its estimates of k_{21} values less than 0.01 min^{-1} are unreliable.

The second protocol for determining the decarbamylation rate constant k_{21} is shown in Figure 3. This protocol involves radiolabeling the AChE by reductive methylation to allow calculation of the total enzyme concentration and hence the final substrate turnover rate v_f that is expected when the enzyme is completely decarbamoylated. Since the half lives of some of the carbamoylated AChEs examined here are on the order of weeks, it is not practical to measure v_f directly from an extended reaction trace. The decarbamylation of AChE after reaction with *N*-monomethyl neostigmine is shown in Figure 3a. A $k_{21} = 0.013 \text{ min}^{-1}$ was calculated from these data, in good agreement with the value obtained from Figure 2a. Decarbamylation of *N*-ethyl-*N*-methylcarbamoyl AChE generated from rivastigmine is shown in Figure 3b. The calculated $k_{21} = 0.00016 \text{ min}^{-1}$ is nearly 100-fold lower than that for *N*-monomethylcarbamoyl AChE in Figure 3a and also considerably lower than the k_{21} determined for the same carbamoylated enzyme in Figure 2b, as noted above. Reaction traces similar to those in Figure 3 were obtained when assays were conducted in 0.1, 0.5, and 1.0 mM acetylthiocholine, and no significant differences in k_{21} values were observed. A similar lack of effect of acetylthiocholine concentration on k_{21} was observed for carbamoyl AChE produced by carbamoylcholine at acetylthiocholine concentrations up to 30 mM (7). Therefore, we concluded that any binding of acetylthiocholine to the carbamoylated enzymes had no effect on k_{21} and that, according to Eq. 1b, with this decarbamylation protocol $k_{21} = k_3$ (however, see Discussion).

Additional decarbamylation rate constants k_{21} measured with the second protocol are listed in Table 1. Carbamates with one of four carbamoyl groups and one of five leaving groups were employed, and Figure 1 gives the structure of these leaving groups. Table 1 shows the carbamoyl group and the leaving group for each carbamate examined. The average number of measurements n of each rate constant are also listed, and in most cases this number is at least four. Values of k_{21} became progressively smaller as the carbamoyl groups increased in size, but for a given carbamoyl group there were no significant differences in k_{21} among the five examined leaving groups. Overall mean values of k_{21} for the *N*-monomethyl-, *N,N*-dimethyl-, and *N*-ethyl-*N*-methylcarbamoyl groups were 12,100-, 3300-, and $170 \times 10^{-6} \text{ min}^{-1}$, respectively. These values were in good agreement with previously reported decarbamylation rate constants, as noted in the Discussion. Values of k_{21} for carbamates with *N,N*-diethylcarbamoyl groups have not been reported previously. The overall mean

value of k_{21} for these carbamates in Table 1 was $15 \times 10^{-6} \text{ min}^{-1}$, more than an order of magnitude lower than the corresponding k_{21} for carbamates with *N*-ethyl-*N*-methylcarbamoyl groups in Table 1.

3.2. Solvent kinetic isotope effects

Analyses of solvent kinetic isotope effects in D_2O relative to H_2O have been useful in clarifying details of the AChE reaction pathway with a variety of substrates (20, 21), but we've found no reports of these isotope effects on the decarbamylation of AChE. We examined D_2O kinetic isotope effects on k_{21} for series of carbamoylated AChEs generated from HTMAs with carbamoyl substituents of various size (Table 2). Since the simplest interpretation of Scheme 2 and Eq. 1b under our experimental conditions is that $k_{21} = k_3$, we anticipated isotope effects of 2 – 3 because proton transfer should be rate limiting for k_3 (see Discussion). A value in this range was observed for *N*-monomethylcarbamoylated AChE, but isotope effects for larger carbamoylated AChEs were progressively smaller with an isotope effect of 1.1 for *N,N*-diethylcarbamoylated AChE (Table 2). The basis for this decrease is considered in the Discussion.

4. Discussion

4.1. Kinetics of decarbamylation

Our initial goals in this study were 1) to measure the decarbamylation rate constants k_{21} for carbamoylated AChEs generated by a series of carbamic acid esters; and 2) to determine whether the ester leaving group had any effect on these rate constants. As discussed below, this second point arose from a report that *N*-ethyl-*N*-methylcarbamoylated AChE formed with *N*-ethyl-*N*-methylcarbamoyl chloride (EMCC) decarbamoylates more rapidly than that formed with rivastigmine (12). It is clear from our data in Table 1 that the decarbamylation rate constant is independent of the leaving group for a given carbamoyl group. Regardless of the leaving group, the decarbamylation rate constants decreased by factors of 4-, 70-, and 800 –fold relative *N*-monomethylcarbamoylated AChE, respectively, as the size of the *N*-alkyl- groups in the carbamoyl moiety increased to *N,N*-dimethyl, *N*-ethyl-*N*-methyl and finally to *N,N*-diethyl. This trend can be extended further by noting that k_{21} for a carbamoyl AChE with no *N*-alkyl groups generated from carbamoyl choline is 0.13 min^{-1} (7), 10-fold larger than that for *N*-monomethylcarbamoylated AChE. Consistent with the data in Table 1, a k_{21} value of $2 \times 10^{-2} \text{ min}^{-1}$ was reported for the *N*-monomethylcarbamoylated AChE formed by physostigmine (22), and a 20–30 fold decrease in k_{21} was reported in going from the *N,N*-dimethyl- to the *N*-ethyl-*N*-methylcarbamoylated AChE generated by rivastigmine (23).

Clearly, and to our disappointment (15), our work does not support the previous suggestion that the *N*-ethyl-*N*-methylcarbamoylated AChE formed with EMCC decarbamoylates more rapidly than that formed with rivastigmine (12). We suspect that this discrepancy results from the fact that aqueous solutions of EMCC are very unstable and carbamoylate AChE poorly. When chloride in EMCC was converted to fluoride (another small leaving group but one that forms a more stable carbamate), the carbamylation of AChE was more effective. While carbamates with larger carbamoyl groups did decarbamoylate more slowly, we saw no

evidence that this involved trapping of the leaving group. In fact, carbamoylation with the *N,N*-diethylcarbamate of M7H resulted in the immediate release of the fluorescent leaving group at a rate several orders of magnitude larger than k_{21} for the *N,N*-diethylcarbamoylated AChE (data not shown).

One novel feature of our report is our data on the largest carbamic acids whose esters were examined here, the *N,N*-diethylcarbamic acids. The rate constants for decarbamoylation of AChE modified with this carbamoyl group are low and can be difficult to measure. An early effort failed to detect formation of *N,N*-diethylcarbamoylated AChE from the corresponding *N,N*-diethylcarbamoyl fluoride or *N,N*-diethylcarbamoyl choline (24); but, with greater sensitivity and longer time courses for the four *N,N*-diethylcarbamic acid esters in Table 1, we were able to generate *N,N*-diethylcarbamoylated AChE and measure its decarbamoylation rate constant. The accuracy of our rate constant measurements was improved by radiolabeling the AChE. We chose reductive radiomethylation for the labeling, as this modification results in dimethylation of only the N-terminal α -amino and lysine ϵ -amino groups. These groups are distant from the AChE active site, and the modification has essentially no effect on AChE activity. From the ratio of radioactivity to enzyme activity in the initial AChE preparation, it was possible to calculate the AChE concentration following carbamoylation and removal of excess carbamate by gel filtration and fix the corresponding activity value (v_f) in the fitting program. Complete removal of excess carbamate by gel filtration is essential for accurate determination of v_f , as simple dilution of the carbamoylation reaction is not sufficient to eliminate continued carbamoylation in the final steady state. We chose to use a continuous spectrophotometric assay of decarbamoylation in the presence of acetylthiocholine substrate (Figure 3). This assay can be justified because we saw no difference in k_{21} values as the acetylthiocholine concentrations in the assay were varied from 0.1 to 1.0 mM. Furthermore, we previously showed that acetylthiocholine at concentrations up to 30 mM had no effect on the decarbamoylation rate constant k_{21} for carbamoylated AChE generated from carbamoyl choline (Figure 8b in (7)). This lack of effect indicated that $b_2 \approx 1$ for any acetylthiocholine complex in Scheme 2 and Eq. 1b. We occasionally used the alternative spectrophotometric decarbamoylation assay in Figure 2. This assay is conducted in the presence of the carbamate and has the advantage that it provides estimates of both k_{12} and k_{21} , but it becomes insensitive to k_{21} when the k_{21} value is small. Furthermore, the measured k_{21} , given in Eq. 1b, cannot be converted to k_3 unless b in Eq. 1b is known. The measured value of b for carbamoyl choline is 0.14 (7), suggesting that the presence of a carbamate may alter the intrinsic decarbamoylation rate constant k_3 .

Values of k_{21} for *N,N*-diethylcarbamoylated AChE in Table 1 are around $1 - 2 \times 10^{-5} \text{ min}^{-1}$, among the lowest decarbamoylation rate constants yet reported. This value of k_{21} corresponds to a half time for the *N,N*-diethylcarbamoylated AChE of nearly 800 hr, about an order of magnitude larger than that for the *N*-ethyl-*N*-methylcarbamoylated AChE generated by rivastigmine. We found one previous estimate of this half life from a study that examined the recovery of AChE in rat brain following in vivo treatment with *N,N*-diethylcarbamoyl fluoride (25). The reported recovery half time of 182 hr is in agreement with our values considering the higher 37 °C in vivo temperature. This agreement, however, may be coincidental, as the half time for AChE itself in the steady state in rat brain is only 68 hr (26). Other carbamates have also been reported to have very low k_{21} values,

particularly *N*-monoalkyl- or *N*-alkyl-*N*-methylcarbamates in which the alkyl group is large (22, 23). The k_{21} for eptastigmine, an *N*-monoalkylcarbamate with an *N*-heptyl group, after carbamoylation of hAChE was reported to be $4 \times 10^{-5} \text{ min}^{-1}$, and that for MF268, another *N*-monoalkylcarbamate with an *N*-8-(2,6-dimethylmorpholino)octyl group, was too small to be measured (22). Examination of a series of *N*-alkyl-*N*-methylcarbamates clarified this trend (23). We noted earlier that this laboratory reported a 20–30 fold decrease in k_{21} in going from the *N,N*-dimethyl- to the *N*-ethyl-*N*-methylcarbamoylated AChE. However, k_{21} did not decrease further as the carbamate was modified from *N*-ethyl-*N*-methyl- to *N*-hexyl-*N*-methyl-. The trend for hBChE was slightly different (23). The value of k_{21} for the *N,N*-dimethylcarbamoylated BChE was similar to that for hAChE, and it decreased only 2–3 fold in going from the *N,N*-dimethyl to the *N*-ethyl-*N*-methyl- and about 2-fold more to the *N*-hexyl-*N*-methylcarbamoylated BChE.

4.2. Crystal structures of carbamoylated AChEs

Several crystal structures of carbamoylated AChEs have been reported. We noted in the Introduction the structure of rivastigmine-bound TcAChE, with both its *N*-ethyl-*N*-methylcarbamoyl moiety and separated leaving group NAP in the A-site (12). In this structure, the *N*-ethyl and the *N*-methyl substituents are in contact with the acyl pocket residues F288 and F290, respectively, although hydrogen bonding in the catalytic triad is disrupted. In addition to this structure, two structures of *N*-monoalkylcarbamates have been obtained (27, 28). The alkyl groups in these carbamates are large, corresponding to 2-ethylphenyl- in ganstigmine (28) and 8-(*cis*-2,6dimethylmorpholino)octyl- in MF268 (27). As noted in the previous section, the decarbamoylation rate constants k_{21} for *N*-monoalkylcarbamoyl AChEs with large alkyl groups are relatively small. In both structures no substantial conformational changes relative to the native AChE structures are apparent, and after carbamoylation only the carbamoyl group is observed in the active site and no leaving group is evident. The carbamoyl alkyl group in both cases extends along the active site gorge toward the P-site. To rationalize the low decarbamoylation rate constants k_{21} for these carbamoylated AChEs, despite the absence of significant A-site distortion, the authors noted the relatively short 2.83 Å distance observed between the N atom of the N-H,N-2-ethylphenylcarbamoyl moiety (H donor) and Ne2 of His440 (H acceptor) (2.83 Å) following carbamoylation. This distance corresponds to a strong hydrogen bond, and it could help to explain the small decarbamoylation rate constant if Ne2 is no longer available to a neighboring water molecule that is essential in the decarbamoylation step. This rationalization breaks down, however, when it is noted that the *N*-alkyl-*N*-methylcarbamoylated AChEs, for which this strong hydrogen bond cannot form, also have low values of k_{21} (23). These authors suggest that the increased resistance to reactivation of the conjugates obtained by such compounds may be associated with a substantial change in the orientation of the triad His440 as proposed for rivastigmine (12).

The pronounced decrease in k_{21} for *N,N*-diethylcarbamoylated AChE seen in Table 1 suggests even greater distortion of the A-site arising from the shape of this carbamoyl group. In this context, it is of interest to examine the X-ray crystal structure of diethoxyphosphorylated hAChE generated from paraoxon (29), in which the phosphoryl group has some shape similarity to the carbamoyl group in *N,N*-diethylcarbamoylated

AChE. Franklin *et al.* note that, in this structure, one ethoxy group faces the cationic site (W86) and the other faces the acyl pocket (F295, F297, and F338). Hydrogen bonding within the catalytic triad remains intact (Ser203 O γ to H477 N ϵ 2 and H477 N δ 1 to E334 O ϵ 1), but the adjacent acyl pocket residues and acyl loop (residue positions 280–297) are significantly perturbed relative to their positions in the ligand-free hAChE structure. Due to its relatively crowded local environment, a side-chain rotation of F295 is insufficient to accommodate the adduct, and instead movements in residue backbone positions are necessary to avert steric clash. The C α and the sidechain of R296 shift by 4.9 Å and 14.9 Å[°], respectively, and the sidechain guanidinium group is displaced to a position within the P-site where it is stabilized by π -stacking against the hydroxyphenyl ring of Y72. Large acyl loop backbone rearrangements have also been seen in mAChE and TcAChE inhibited by the organophosphate diisopropylfluorophosphate (30, 31). However, stereoselective inhibition of AChE by organophosphate nerve agents that place a methyl group into the acyl pocket does not affect the acyl loop in the same manner (29, 30, 32).

Additional structures in Franklin *et al.* (29) indicate that these shifts in the acyl pocket residues and acyl loop in diethoxyphosphorylated hAChE can be largely reversed by cationic ligand binding to the active site. The interaction of cationic oximes with organophosphorylated AChEs is of intense interest because oximes have been shown to reactivate these AChEs by displacing the serine hydroxyl and releasing the organophosphorylated oxime (33, 34). The structure of the complex of diethoxyphosphorylated hAChE with the cationic oxime 2-PAM reveals two bound 2-PAM molecules, one in the P-site and the other stacked against the sidechain of W86, while that with the dicationic HI-6 shows HI-6 in the P-site. In both of these ternary complexes, the backbone conformation of the acyl loop resembles that of the ligand-free state and appears to be stabilized by oxime binding. Therefore, diffusion of these oximes into the crystals of diethoxyphosphorylated hAChE promoted a restoration of the acyl pocket residues and acyl loop to their locations in the unmodified hAChE structure. These observations raise the question of whether the crystal structure of rivastigmine with TcAChE reflects a restoration of a “normal active site” with bound NAP even with the carbamoyl group present.

4.3. Kinetic isotope effects on decarbamylation rate constants k_{21} in deuterium oxide

The turnover number k_{cat} for serine hydrolases is a combination of k_2 for the acylation step and k_3 for the deacylation step. These steps, shown in Scheme 2, typically involve general acid-base catalysis with proton transfer, and as a consequence these rate constants show a D₂O kinetic isotope effect (a decrease of 2- to 3-fold when D₂O replaces H₂O as the solvent) (35). An isotope effect of 2.8 in Table 2 for decarbamylation of carbamoylated AChE generated by *N*-monomethyl neostigmine is in this range, suggesting that for this reaction k_{21} can be equated with k_3 . The *N*-monomethylcarbamoylated AChEs also show the highest k_{21} values in Table 1. However, as the alkyl groups on carbamoylated AChEs increase in size, not only do the k_{21} values in Table 1 decrease dramatically but also the D₂O kinetic isotope effects in Table 2 decrease to the point where the isotope effect on k_{21} for *N,N*-diethylcarbamoylated AChE is only slightly above 1. The large size of this carbamoyl group is likely to be an important factor in this shift away from proton transfer in the rate-limiting step for k_{21} . The X-ray crystal structures noted above indicate that AChEs

carbamoylated with large carbamoyl groups or phosphorylated with large phosphoryl groups show significant distortion of the acyl pocket as well as the larger active site gorge. One possibility is that the active site is so highly distorted that this *N,N*-diethylcarbamoylated enzyme is reactivating itself by spontaneous water hydrolysis without any help from the catalytic groups on the enzyme. However, this possibility seems unlikely, as extrapolated rate constants for the hydrolysis of *N,N*-dialkylcarbamates at neutral pH appear much lower than the average k_{21} of $1.5 \times 10^{-5} \text{ min}^{-1}$ observed for *N,N*-diethylcarbamoylated AChEs in Table 1 (36).

A second possibility is that distortion of the active site in AChEs carbamoylated with larger *N,N*-dialkylcarbamoyl groups results in a change to an enzymatic rate-limiting step for k_{21} that no longer involves proton transfer. In this regard, it is noteworthy that a similar drop in D_2O kinetic isotope effects from 2.4 on k_{cat} to 1.1 – 1.2 on $k_{\text{cat}}/K_{\text{app}}$ was observed for good substrates of AChE like acetylcholine. To interpret this drop, we proposed that proton transfer in the acylation step k_2 was rate-limiting for k_{cat} but that an earlier step like substrate binding or an induced-fit conformational change that does not involve proton transfer was rate-limiting for the second order rate constant $k_{\text{cat}}/K_{\text{app}}$ (20). This proposal has gained wide acceptance in the cholinesterase field (21). A similar interpretation here is that an earlier, rate-limiting step that does not involve proton transfer should be incorporated into k_{21} for AChEs carbamoylated with large carbamoyl groups or phosphorylated with large phosphoryl groups. For decarbamoylation or dephosphorylation of these AChEs to occur, it may be necessary to invoke a transient conformational change that includes a brief conversion to the unmodified or apo AChE active site and its aligned catalytic triad where the k_3 hydrolysis reaction can take place. It may be necessary to explore molecular modeling to examine the array of conformational variants available in AChEs with large carbamoyl or phosphoryl groups to obtain support for this proposal. However, it should be emphasized that for this conformational step to be rate limiting, it cannot be equilibrated with the subsequent hydrolysis reaction.

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Carbamoyl Group

HTMA

NAP

M7H

1-naphthol

fluoride

Figure 1. Molecular structures of carbamoyl and leaving groups in carbamates examined in this report.

Figure 2.

Reactions of the carbamates a) *N*-monomethyl neostigmine and b) rivastigmine with hAChE. The carbamates or buffer blanks were mixed with hAChE in a stopped-flow apparatus and reactions were monitored at 412 nm with 0.25 mM acetylthiocholine and 2.0 mM DTNB as outlined in the Experimental Procedures (black traces labeled “observed”). The reaction data for carbamates were fitted to Eq. 2 (pink traces labeled “fit”) with R set to zero, either t_0 or $A_{412(0)}$ fixed at a value determined by inspection or from controls without enzyme, and with v_0 , $A_{412(0)}$, k_{12} and k_{21} as the fitted parameters. The reaction data for

blanks without carbamate gave a linear fit in panel A (cyan trace labeled “fit”) or a linear fit in panel B that was calculated by normalizing panel A data to the AChE concentration (cyan trace labeled “calculated”). a) Final concentrations of *N*-monomethyl neostigmine and hAChE after mixing were 5.0 μM and 2.0 nM, respectively. Fitting to Eq. 2 with t_0 fixed at 0.100 min gave $k_{12} = 14 \text{ min}^{-1}$ and $k_{21} = 13 \times 10^{-3} \text{ min}^{-1}$. b) Final concentrations of rivastigmine and hAChE after mixing were 2.0 mM and 0.33 nM, respectively. Fitting to Eq. 2 with $A_{412(t_0)}$ fixed at 0.114 min gave $k_{12} = 1.24 \text{ min}^{-1}$ and $k_{21} = 3.1 \times 10^{-3} \text{ min}^{-1}$. The carbamate trace in panel b was offset by 0.5 min.

Figure 3.

Decarbamoylation reactions following treatment of hAChE with a) *N*-monomethyl neostigmine and b) rivastigmine. AChE was radiomethylated, carbamoylated, isolated on a spin column, and subjected to continuous spectrophotometric assay at 412 nm with 0.25 mM acetylthiocholine and 0.33 mM DTNB as outlined in the Experimental Procedures (black traces labeled “observed”). The reaction data were fitted to Eq. 2 (pink traces labeled “fit”) with k_{12} and t_0 set to zero, v_f fixed at a predetermined value, and $A_{412(0)}$, R , and k_{21} as the fitted parameters. Calculated traces corresponding to v_f are shown in cyan (labeled

“calculated”). a) Concentrations of *N*-monomethyl neostigmine and hAChE in the initial mix were 1.0 mM and 1.8 nM, respectively, and 50 μ L of the spin column recovery was added to 3.0 mL of assay buffer containing 0.25 mM acetylthiocholine. Fitting to Eq. 2 with v_f fixed at 0.143 A/min gave $k_{21} = (12.9 \pm 0.02) \times 10^{-3} \text{ min}^{-1}$ and $R = 0.90$. b) Concentrations of rivastigmine and hAChE in the initial mix were 2.0 mM and 1.8 nM, respectively, and 50 μ L of the spin column recovery was added to 3.0 mL of assay buffer containing 0.25 mM acetylthiocholine. Fitting to Eq. 2 with v_f fixed at 0.192 A/min gave $k_{21} = (155 \pm 0.4) \times 10^{-6} \text{ min}^{-1}$ and $R = 0.996$. The lower value of R in panel a) relative to panel b) reflects faster reactivation of *N*-monomethylcarbamoylated AChE during gel filtration and prior to initiation of the reaction trace. Carbamate traces in panels a) and b) were offset by 2.0 and 5.0 min, respectively.

SCHEME 1.

SCHEME 2.

Table 1.Decarbamylation Rate Constants k_{21} in the Absence of Carbamate at 25 °C ^a

Carbamoyl Group Substituents	Leaving Group ^b	Carbamate Common Name	n^c	$k_{21} \times 10^6 \text{ min}^{-1}$
<i>N</i> -monomethyl				
	HTMA	Monomethyl neostigmine	8	12300 ± 400
	NAP		4	12500 ± 200
	1-Naphthol	carbaryl	7	11300 ± 600
	M7H		10	12900 ± 500
<i>N,N</i> -dimethyl				
	HTMA	neostigmine	15	3600 ± 300
	M7H		6	3000 ± 300
	F		4	3200 ± 200
<i>N</i> -ethyl- <i>N</i> -methyl				
	HTMA		16	150 ± 20
	NAP	rivastigmine	19	160 ± 10
	M7H		4	200 ± 40
<i>N,N</i> -diethyl				
	HTMA		11	16 ± 5
	NAP		4	23 ± 6
	M7H		9	14 ± 4
	F		2	7 ± 1

^aValues of k_{21} obtained with the second protocol described in the Experimental Procedures, as illustrated in Figure 3.^bLeaving group structures are shown in Figure 1.^cNumber of independent measurements.

Table 2.Deuterium Oxide Kinetic Isotope Effects on Decarbamylation Rate Constants k_{21} at 25 °C ^a

Carbamoyl Group Substituents	Leaving Group ^b	<i>n</i> ^c	$k_{21}^{\text{H}_2\text{O}} / k_{21}^{\text{D}_2\text{O}}$ ^c
<i>N</i> -monomethyl	HTMA	1	2.8 ± 0.2
<i>N,N</i> -dimethyl	HTMA	4	2.4 ± 0.2
<i>N</i> -ethyl- <i>N</i> -methyl	HTMA	3	1.8 ± 0.2
<i>N,N</i> -diethyl	HTMA	3	1.1 ± 0.2

^aValues of k_{21} obtained as outlined in the Experimental Procedures and Table 2.^bLeaving group structures are shown in Figure 1.^cPairs of duplicate or triplicate measurements of k_{21} in H₂O or D₂O were collected, and the ratio of the means of k_{21} in H₂O to k_{21} in D₂O ($k_{21}^{\text{H}_2\text{O}} / k_{21}^{\text{D}_2\text{O}}$) in each pair comprised one independent measurement. The means and standard errors of *n* independent measurements are tabulated.