Biochemical Evaluation of Photolabile Precursors of Choline and of Carbamylcholine for Potential Time-Resolved Crystallographic Studies on Cholinesterases[†]

Ling Peng,[‡] Israel Silman,[§] Joel Sussman,[‖,⊥] and Maurice Goeldner*,[‡]

Laboratoire de Chimie Bio-organique, URA 1386 CNRS, Faculté de Pharmacie, Université Louis Pasteur Strasbourg, BP 24, F-67401 Illkirch Cédex, France, Departments of Neurobiology and Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel, and Protein Data Bank, Department of Biology, Brookhaven National Laboratory, Upton, New York 11973

ABSTRACT: Acetylcholinesterase and butyrylcholinesterase both rapidly hydrolyze the neurotransmitter acetylcholine. The unusual three-dimensional structure of acetylcholinesterase, in which the active site is located at the bottom of a deep and narrow gorge, raises cogent questions concerning traffic of the substrate, acetylcholine, and the products, choline and acetate, to and from the active site. Time-resolved crystallography offers a promising experimental approach to investigate this issue but requires a suitable triggering mechanism to ensure efficient and synchronized initiation of the dynamic process being monitored. Here we characterize the properties of two photolabile triggers which may serve as tools in time-resolved crystallographic studies of the cholinesterases. These compounds are 2-nitrobenzyl derivatives of choline and of carbamylcholine, which generate choline and carbamylcholine, respectively, upon photochemical fragmentation. Both photolabile compounds are reversible inhibitors, which bind at the active sites of acetylcholinesterase and butyrylcholinesterase with inhibition constants in the micromolar range, and both photofragmentation processes occur rapidly and with a high quantum yield, without substantial photochemical damage to the enzymes. Photolysis both of acetylcholinesterase and of butyrylcholinesterase, complexed with a 2-nitrobenzyl derivative of choline, resulted in regeneration of enzymic activity. Photolysis of acetylcholinesterase complexed with the 2-nitrobenzyl derivative of carbamylcholine led to time-dependent inactivation, resulting from carbamylation of acetylcholinesterase, which could be reversed upon dilution, due to decarbamylation. Both sets of experiments demonstrated release of choline within the active site. In the former case, choline was produced photochemically at the active site. In the latter case, choline was generated enzymatically, within the active site, concomitantly with carbamylation of the acetylcholinesterase. The two photolabile compounds may thus serve as complementary probes for time-resolved studies of the route of product release from the active sites of the cholinesterases.

Any study of fast conformational changes in enzymes and other proteins requires the development of appropriate time-resolved experimental approaches. The emergent methodology of time-resolved macromolecular crystallography (Cruickshank et al., 1992; Hajdu & Anderson, 1993; Bolduc et al., 1995; Farber, 1995) offers the possibility of following such conformational changes at the atomic level, provided efficient and synchronized initiation of the dynamic process can be achieved within the crystals of the protein investigated. While a pH jump was used to trigger deacylation of a transiently

stable (*p*-guanidinobenzoyl)trypsin (Singer et al., 1993), several groups have developed photoregulatory approaches based either upon photochemical release of the desired effector or upon photochemical unblocking of enzymic activity (Porter et al., 1993). Photochemical triggering methods are suitable for investigating fast enzymatic reactions, provided that photochemical release of the effector is fast and efficient. Examples include triggering of GTP hydrolysis upon Ha-*ras* p21 by photorelease of GTP from a photolabile 2-nitrobenzyl derivative (Schlichting et al., 1990), and the deacylation of a conjugate of chymotrypsin by photoisomerization of an inert *trans*-acyl enzyme derivative (Stoddart et al., 1991).

Hydrolysis of the neurotransmitter acetylcholine (ACh)[1] by the cholinesterases acetycholinesterase (AChE) and butyrylcholinesterase (BuChE) is a particularly fast enzymatic process (Chatonnet & Lockridge, 1989). AChE operates at a rate which is almost diffusion-limited, with a turnover number approaching $20\,000\ \mathrm{s}^{-1}$ (Bazelyansky et al., 1986;

[1] Abbreviations: AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; ChEs, cholinesterases; ACh, acetylcholine; CCh, carbamylcholine; ATCh, acetylthiocholine; BuTCh, butyrylthiocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); BzCh, benzoylcholine.

Chart 1: Photolabile Precursors of Choline (Compound **A**) and of Carbamylcholine (Compounds **B** and **C**)

A

B: R = CH₃

C: R = COOH
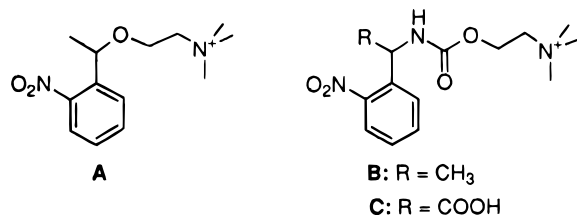
Quinn, 1987). The description of the 3-D structure of AChE (Sussman et al., 1991), of several AChE−inhibitor complexes (Harel et al., 1993, 1995, 1996; Bourne et al., 1995), and of a model of BuChE, based on the 3-D structure of AChE (Harel et al., 1992), has permitted a better understanding of structure−function relationships in the cholinesterases. It has, however, also raised cogent new questions concerning the traffic of substrate and products to and from the active site. Thus, the description of a deep and narrow gorge, with the catalytic triad located near its bottom, appears to be inconsistent with the high turnover rate of AChE already noted. The unusual electrostatic characteristics of AChE, *viz.*, a large dipole moment which may serve to attract the cationic substrate toward the active site (Ripoll et al., 1993), raised the possibility that alternative routes to the active site might exist through the walls of the gorge and be involved in movement of water and/or clearance of products (Gilson et al., 1994). Site-directed mutagenesis was employed to challenge both the role of the electric field and the so-called "back door" hypothesis (Shafferman et al., 1994; Kronman et al., 1994), and the topic has aroused considerable controversy (Antosiewicz et al., 1995a,b; Ripoll et al., 1995).

Time-resolved crystallography would present an ideal approach to monitoring the clearance of choline from the active site of AChE, provided that suitable probes were available. A recently synthesized photolabile precursor of choline, compound **A** (Chart 1; Peng & Goeldner, 1996), and the photolabile precursors of carbamylcholine (CCh), compounds **B** and **C** (Chart 1), synthesized and characterized earlier for time-resolved studies on the nicotinic acetylcholine receptor [Walker et al., 1986; Milburn et al., 1989; reviewed by Hess (1993)] are 2-nitrobenzyl derivatives of the ligands which we are interested in generating. Being highly light-sensitive (Pillai, 1980), 2-nitrobenzyl derivatives are susceptible to rapid photochemical fragmentation [reviewed by McCray and Trentham (1989) and Corrie and Trentham (1993)]. Laser pulse photolysis of these probes permits rapid release of either the reaction product, choline, in the case of **A**, or of a substrate of both AChE and BuChE, namely CCh, in the case of **B** and **C**. In the following, we characterize the interaction of these probes with AChE and BuChE, as well as their relevant photochemical characteristics, and assess their potential use in time-resolved crystallographic studies of the two enzymes.

MATERIALS AND METHODS

Materials

AChE was purified from electric organ tissue of *Torpedo marmorata* (Ehret-Sabatier et al., 1992), and BuChE from human plasma (Lockridge & La Du, 1978). *O*-[1-(2-Nitrophenyl)ethyl]choline iodide (**A**) was synthesized as described (Peng & Goeldner, 1996), while *O*-[1-(2-nitrophenyl)ethyl]carbamylcholine iodide (**B**) and *O*-(α-carboxy-2-nitrobenzyl)carbamylcholine trifluoroacetate (**C**) were purchased from Molecular Probes (Junction City, OR). Carbamylcholine chloride (CCh), acetylthiocholine iodide (ATCh), butyrylthiocholine chloride (BuTCh), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), benzoylcholine chloride (BzCh), *p*-nitrophenyl acetate, propidium iodide, and edrophonium chloride were from Sigma (St. Louis, MO).

Methods

Assays of Enzymic Activity. Unless otherwise indicated, the activity of both AChE and BuChE was monitored spectrophotometrically by the Ellman method (Ellman et al., 1961), at 412 nm, using ATCh and BuTCh, respectively, as substrate. The procedure was as follows: Appropriate enzyme aliquots were added to a cuvette containing 1 mL of the assay mixture, *viz.*, 0.5 mM ATCh (or BuTCh)/0.1 M NaCl/50 mM phosphate, at pH 7.2, containing 1 mg of DTNB/mL. Activity was monitored in a Uvikon 860 spectrophotometer at 20 °C.

Determination of Inhibition Constants. Inhibition of cholinesterases by compounds **A**, **B**, and **C** was measured by the Ellman procedure at six substrate concentrations (20 μM−100 μM) and three inhibitor concentrations, and the data were used to construct Lineweaver−Burk plots.

Ligand Displacement Measurements. Fluorescence intensity was monitored in a Shimadzu RF−5000 spectrofluorophotometer, using 10-mm path length cuvettes at 20 °C.

AChE (150 μg/mL) was incubated with 30 μM propidium in 5 mM Tris, pH 8.0, 20 °C. Aliquots of **A** were added, and emission was monitored at 625 nm, upon excitation at 510 nm (Taylor et al., 1974).

AChE (30 μg/mL) was incubated with 0.1 μM *N*-methylacridinium in 0.1 M NaCl/20 mM MgCl₂/10 mM phosphate, pH 7.0 at 20 °C. Aliquots of **A** were added, and emission was monitored at 490 nm, upon excitation at 358 nm (Mooser et al., 1972).

Estimation of the Affinities of Reversible Binding of CCh to AChE and BuChE. Aliquots of AChE or BuChE were added to an Ellman reaction mixture containing CCh and either ATCh or BuTCh; enzymic activity was measured immediately by the Ellman method and obtained within 30 s.

For AChE, data were obtained at six ATCh concentrations (20 μM−100 μM) and at three CCh concentrations in order to construct Lineweaver−Burk plots.

For BuChE, data were obtained at 5.0×10^{-4} M BuTCh and at CCh concentrations in the range of 1.0×10^{-7} to 3.3×10^{-2} M.

Enzymic Hydrolysis of CCh by AChE and BuChE. AChE (220 μg/mL) and BuChE (160 μg/mL) were incubated with various concentrations of CCh in 50 mM phosphate buffer, pH 7.2, at 4 °C. At appropriate times, aliquots were withdrawn for measurement of enzymic activity. Regeneration of enzymic activity of carbamylated AChE and BuChE, which had been inactivated by exposure to CCh concentrations of 0.1 and 10 mM, respectively, was achieved by 500-fold dilution into 50 mM phosphate buffer, pH 7.2, at 20 °C.

Table 1: Spectral Properties, Photochemical Reaction Data, and Inhibition Constants of **A**, **B**, and **C**

	A	**B**	**C**
λ_{max} (nm)	261[a]	262[b]	266[c]
ϵ_{max} (M^{-1} cm^{-1})	5300[a]	5200[b]	5200[c]
$t_{1/2}$[d] (μs)	10[a]	24[b]	18[c]
Φ[e]	0.27[a]	0.25[b]	0.80[c]
$K_{i, AChE}$ (μM)	13.0 ± 0.3	44.0 ± 0.9	> 1000
$K_{i, BuChE}$ (μM)	11.1 ± 0.1	78.4 ± 1.5	> 1000

[a] Peng & Goeldner (1996). [b] Walter et al. (1986). [c] Milburn et al. (1989). [d] $t_{1/2}$, half-time of photofragmentation at pH 6.5 and room temperature. [e] Φ, quantum yield.

Protection by Edrophonium of AChE against Carbamylation by CCh. AChE (160 μg/mL) was incubated with edrophonium (2.0×10^{-6} M) and with CCh (4.0×10^{-5} and 1.0×10^{-4} M). At appropriate times, aliquots were withdrawn for measurement of enzymic activity.

Laser Flash Photolysis. Laser pulses of ca. 100 mJ and of ca. 20-ns duration (Peng & Goeldner, 1996) were applied to a quartz cuvette (1 mm × 10 mm × 40 mm) containing 2 μg/mL AChE, together with 5.0×10^{-4} M probe **A** in a total volume of 0.4 mL of 50 mM phosphate buffer, pH 6.5. Enzymic activity was monitored spectrophotometrically, at 400 nm, using 1 mM *p*-nitrophenyl acetate as substrate, in 50 mM phosphate buffer, pH 7.2, 20 °C (Tripathi et al., 1978). BuChE (3 μg/mL) was incubated, in a quartz cuvette (5 mm × 10 mm × 40 mm), with 5.4×10^{-5} M probe **A** in 50 mM phosphate buffer, pH 7.2, in a total volume of 1.2 mL and similarly exposed to 351-nm laser flashes. BuChE activity was monitored spectrophotometrically, at 240 nm, using 5.0×10^{-5} M benzoylcholine as substrate, in 50 mM phosphate buffer, pH 7.2, at 20 °C (Kalow & Lindsay, 1953).

AChE (294 μg/mL) was incubated with 4.2×10^{-3} M probe **B**, in 50 mM phosphate buffer, pH 6.5, total volume 0.4 mL, in a quartz cuvette (1 mm × 10 mm × 40 mm). After exposure to either 1 or 20 351-nm laser pulses, aliquots were withdrawn for assay of enzymic activity both by the Ellman method and by using *p*-nitrophenyl acetate as substrate (Tripathi et al., 1978). Regeneration of the enzymic activity of carbamylated AChE was achieved by 500-fold dilution into 50 mM phosphate buffer, pH 7.2, at 20 °C.

RESULTS

*Compounds **A**, **B**, and **C** Are Reversible Inhibitors of AChE and BuChE.* **A**, **B**, and **C** all act as reversible inhibitors of both *Torpedo* AChE and human plasma BuChE. Inhibition constants are in the micromolar range for **A** and **B**, whereas **C** is a very weak inhibitor of both enzymes (Table 1). Whereas inhibition of AChE by **A** (Figure 1a) and **B** (not shown) is of the mixed type, that of BuChE is purely competitive (Figure 1b). Both mixed-type and competitive inhibition imply that **A** and **B** bind to the anionic subsite of the active site.

*Compound **A** Binds to the Active Site of AChE.* The binding of compound **A** at the active site of AChE was further assessed by ligand displacement experiments. AChE has two binding sites for quaternary ammonium ligands, the anionic subsite of the active site and the peripheral anionic site (Quinn, 1987). The anionic subsite of the active site is adjacent to the catalytic triad, near the bottom of the aromatic gorge (Sussman et al., 1991), while the peripheral site is located at the entrance to the gorge (Harel et al., 1993, 1995;
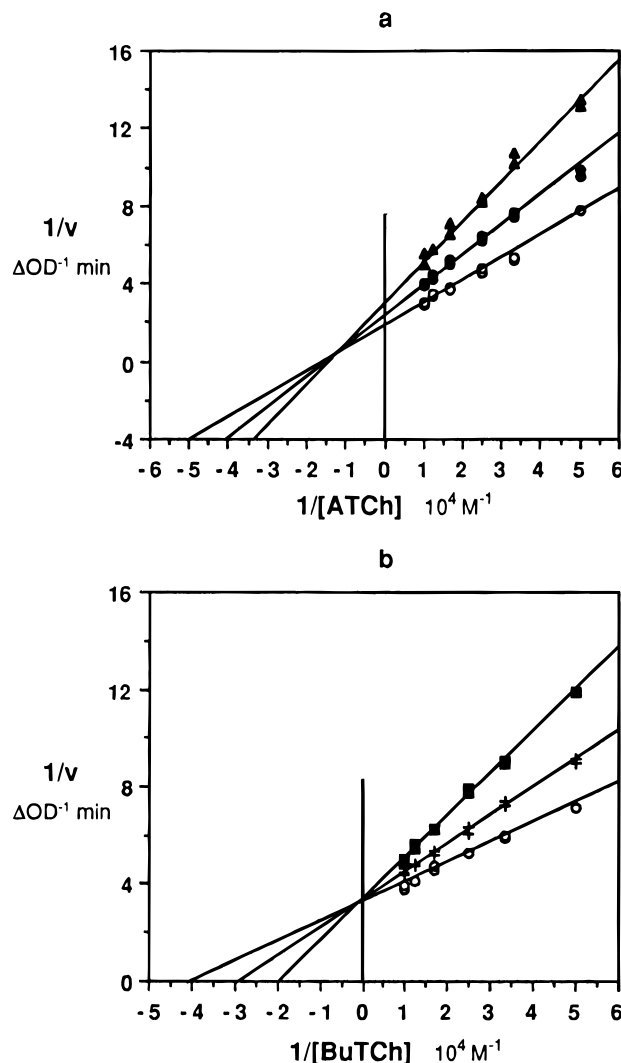

FIGURE 1: Lineweaver−Burk plots of inhibition of AChE (a) and BuChE (b) by compound **A**. (a) (○) no **A**; (●) 3.0×10^{-6} M **A**; (▲) 6.0×10^{-6} M **A**. (b) (○) no **A**; (+) 1.0×10^{-6} M **A**; (■) 2.0×10^{-6} M **A**.

Bourne et al., 1995). The site of interaction of ligands with AChE can be established by ligand displacement experiments using site-specific fluorescent probes. *N*-Methylacridinium is an active-site-specific fluorescent ligand whose fluorescence is quenched upon binding (Mooser et al., 1972). Propidium is a peripheral-site-specific ligand whose fluorescence is enhanced upon binding (Taylor et al., 1974). The fluorescence of *N*-methylacridinium increased in intensity upon adding increasing concentrations of **A** to an AChE solution containing the fluorescent probe (Figure 2a), indicating that **A** binds specifically at the anionic subsite of the active site. In contrast, no decrease in the fluorescence of propidium bound to AChE was observed upon addition of **A** (Figure 2b), showing that **A** does not bind to the peripheral site.

Carbamylation of AChE and BuChE by CCh. It was clearly demonstrated by Wilson et al. (1960, 1961) that CCh serves as a slow substrate of AChE; rapid carbamylation is followed by much slower decarbamylation (Scheme 1) which can be accelerated by dilution, as is usually the case for carbamate inhibitors of serine hydrolases (Aldridge & Reiner, 1972). Although BuChE is known to be inhibited by CCh (Augustinsson et al., 1959; Fellman & Fujita, 1964), a similar kinetic analysis has not been performed.
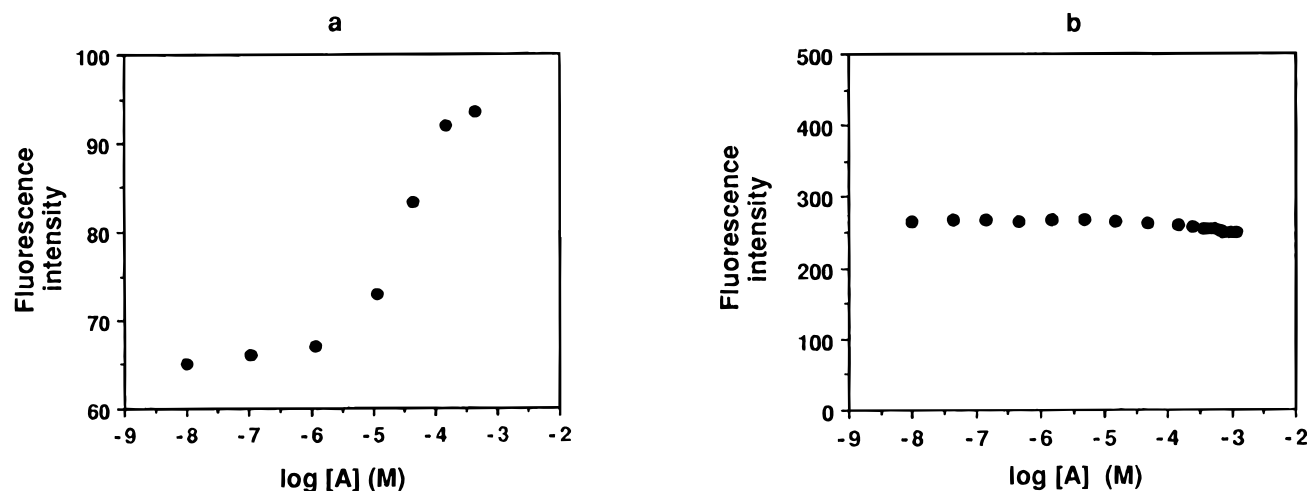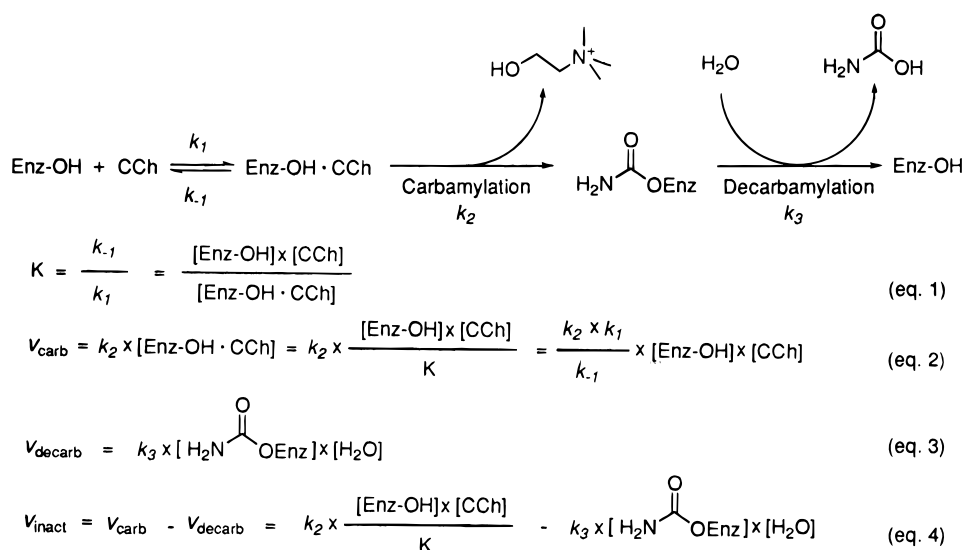
a

b

FIGURE 2: (a) Displacement of *N*-methylacridinium from AChE by **A**. (b) Back-titration of the propidium−AChE complex with **A**.

Scheme 1: Mechanism of Carbamylcholine Hydrolysis by Cholinesterases[a]

$$\text{Enz-OH} + \text{CCh} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{Enz-OH} \cdot \text{CCh} \xrightarrow[\text{Carbamylation}]{k_2} \text{H}_2\text{N}-\overset{\text{O}}{\text{C}}-\text{OEnz} \xrightarrow[\text{Decarbamylation}]{k_3} \text{Enz-OH}$$

$$K = \frac{k_{-1}}{k_1} = \frac{[\text{Enz-OH}] \times [\text{CCh}]}{[\text{Enz-OH} \cdot \text{CCh}]} \qquad \text{(eq. 1)}$$

$$v_{\text{carb}} = k_2 \times [\text{Enz-OH} \cdot \text{CCh}] = k_2 \times \frac{[\text{Enz-OH}] \times [\text{CCh}]}{K} = \frac{k_2 \times k_1}{k_{-1}} \times [\text{Enz-OH}] \times [\text{CCh}] \qquad \text{(eq. 2)}$$

$$v_{\text{decarb}} = k_3 \times [\text{H}_2\text{N}-\text{C(O)}-\text{OEnz}] \times [\text{H}_2\text{O}] \qquad \text{(eq. 3)}$$

$$v_{\text{inact}} = v_{\text{carb}} - v_{\text{decarb}} = k_2 \times \frac{[\text{Enz-OH}] \times [\text{CCh}]}{K} - k_3 \times [\text{H}_2\text{N}-\text{C(O)}-\text{OEnz}] \times [\text{H}_2\text{O}] \qquad \text{(eq. 4)}$$

[a] Carbamylcholine forms a complex with AChE prior to carbamylation, and decarbamylation proceeds through attack of water. Enz-OH, cholinesterases; CCh, carbamylcholine; K, noncovalent binding constant of CCh to the enzyme; k_1, rate constant for formation of the [Enz-OH·CCh] complex; k_{-1}, rate constant for dissociation of the [Enz-OH·CCh] complex; k_2, carbamylation rate constant; k_3, decarbamylation rate constant; v_{carb}, rate of carbamylation; v_{decarb}, rate of decarbamylation; v_{inact}, rate of inactivation.

CCh is an analogue of ACh and, like ACh, forms a reversible complex with AChE prior to hydrolysis (Scheme 1). The reversible binding constant ($K = k_{-1}/k_1$, eq 1 in Scheme 1) of CCh to AChE, obtained from a Lineweaver-Burk plot (Figure 3a), is 9.23×10^{-5} M, similar to the value for ACh. Unexpectedly, as shown in Figure 3b, CCh has a very weak affinity for BuChE, unlike ACh, which displays an affinity for BuChE similar to the affinity for AChE.

In Figure 4, panels a and b display the time courses of loss of enzymic activity for AChE and BuChE, respectively, upon incubation with CCh. The rate of inhibition of AChE decreases progressively, reaching a plateau at about 90% inhibition, employing 0.1 mM CCh, under the experimental conditions used. In contrast, inhibition of BuChE requires higher concentrations of CCh so as to achieve a comparable initial rate of inactivation and, with 200 mM CCh, follows pseudo-first-order kinetics up to 90% inactivation (Figure 4a,b). This difference may be ascribed both to the different affinities of CCh for the two enzymes and to the different rates of their carbamylation and decarbamylation. As already mentioned, CCh has a very low affinity for BuChE, and

decarbamylation appears to be very much slower than carbamylation ($k_2 \gg k_3$, Scheme 1). Consequently, decarbamylation remains negligible throughout the course of inhibition of BuChE. For AChE, although carbamylation is faster than decarbamylation ($k_2 > k_3$, Scheme 1), the ratio of the rate constants is lower, and since decarbamylation cannot be neglected, this results in a steady-state situation.

In Figure 4, panels c and d display the time courses of regeneration of enzymic activity of carbamylated AChE and BuChE, respectively, upon 500-fold dilution. AChE could be regenerated >90% within 20 min upon 500-fold dilution from an initial CCh concentration of 1.0×10^{-4} M to a final concentration of 2.0×10^{-7} M (Figure 4c), whereas only 45% of the activity of BuChE was regenerated at 90 min, after 500-fold dilution from an initial CCh concentration of 10 mM to a final concentration of 0.02 mM (Figure 4d). These different rates of decarbamylation confirm the explanation offered above for our observation that the inactivation kinetics of AChE by CCh approach a steady state, whereas inactivation of BuChE by CCh follows pseudo-first-order kinetics almost to completion (Figure 4a,b).
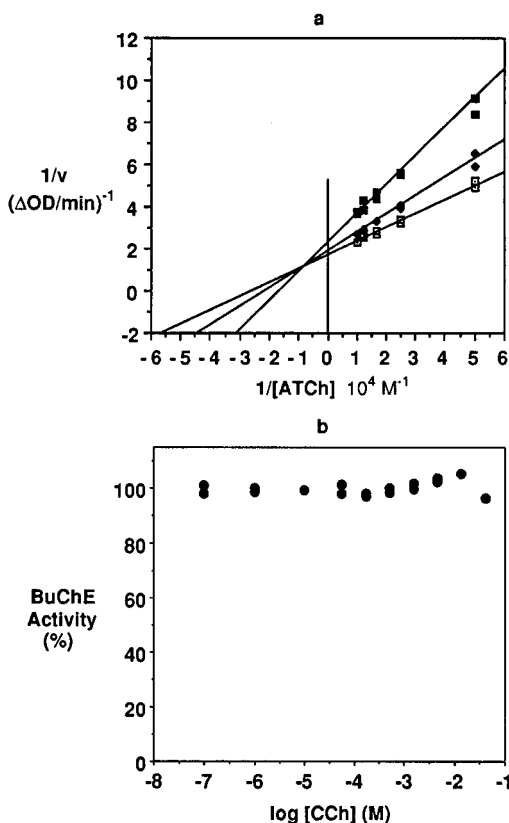
FIGURE 3: (a) Lineweaver−Burk plot of non-covalent inhibition of AChE by CCh. (□) no CCh; (◆) 3.3×10^{-5} M CCh; (■) 1.0×10^{-4} M CCh. (b) Non-covalent inhibition of BuChE by CCh.

Protection by Edrophonium of AChE against Carbamylation by CCh. Inhibition of AChE by CCh (4.0×10^{-5} M and 1.0×10^{-4} M) was prevented by the reversible inhibitor, edrophonium, at 2.0×10^{-6} M (Figure 5).

Regeneration of the Enzymic Activity of AChE and BuChE after Photolysis of Enzyme Solutions Containing Compound A. Laser flash photolysis of **A**, at 351 nm, generates choline very rapidly, with a half-time of 10 μs at 20 °C, pH 6.5 (Peng & Goeldner, 1996). AChE and BuChE were inhibited, with 1.5×10^{-4} M and 5.4×10^{-5} M concentrations of **A**, to 42% and 45% residual activity, respectively (Figure 6). Both enzymes regained most of their control activity after laser flash photolysis, as a consequence of the photolysis of a large fraction of **A**. BuChE could be regenerated up to 90% of the activity of a control sample (Figure 6b), and AChE up to 75% (Figure 6a). Free choline at a concentration of 1.5×10^{-4} M produces 9% inhibition of AChE activity. Thus photochemical restoration of AChE activity may approach 84%.

Carbamylation of AChE by Carbamylcholine Photogenerated from B. Laser flash photolysis of a solution of AChE containing **B** resulted in time-dependent inactivation of AChE (Figure 7a). This strongly suggested that AChE was being carbamylated by CCh generated from its photolabile precursor. The rate of inactivation increased as the number of pulses was increased (Figure 7a), as would be expected to occur due to increased photogeneration of CCh. The activity of AChE could be regenerated upon dilution of the enzyme solution (Figure 7b). This confirmed that inactivation was due to carbamylation which could be reversed by dilution, just as is the case for AChE inhibited directly by free CCh. Experiments of this type were not performed on BuChE

because of the requirement of a high concentration of CCh and due to the slow rate of carbamylation of BuChE by CCh.

DISCUSSION

As outlined in the introduction, the unusual structure of AChE, in which the active site is located near the bottom of a long and narrow gorge, raises cogent questions concerning traffic of substrate and products to and from the active site, especially since AChE is an unusually rapid enzyme (Sussman et al., 1991; Gilson et al., 1994). Time-resolved macromolecular crystallography (Cruickshank et al., 1992; Bolduc et al., 1995; Farber, 1995) offers a direct experimental approach which may permit visualization of the route of clearance of choline, the cationic product of enzymic hydrolysis, from the active-site gorge, and putative conformational changes in the AChE molecule which may occur concomitantly. Such an experimental approach demands, however, rapid and efficient initiation of the dynamic process within the AChE crystal. This can, in principle, be achieved by photochemical triggering, using photolabile precursors which undergo a fast photolytic reaction with a high quantum yield (Corrie et al., 1992).

In the present study we have studied photosensitive precursors both of choline (the cationic product of ACh hydrolysis) and of CCh (a slow substrate of both AChE and BuChE) and have demonstrated their potential as photochemical triggers in time-resolved crystallographic studies on the two enzymes.

All three probes, one precursor of choline (**A**) and two precursors of CCh (**B** and **C**), were shown to be reversible inhibitors of both *Torpedo* AChE and human plasma BuChE, but only **A** and **B** display high enough affinity, *viz.*, possess inhibition constants in the micromolar range (Table 1), to render them suitable candidates for time-resolved crystallography studies. The inhibition constants for inhibition of both AChE and BuChE by **C**, the α-carboxy-2-nitrobenzyl derivative of CCh, were much higher than those for **A** and **B** (Table 1). This is presumably due to a negative influence of the α-carboxylic function on recognition of the positively charged quaternary ammonium ion by both cholinesterases. Advantage was already taken of this reduced affinity to utilize **C** as an ideal "caged" compound for time-resolved studies on the nicotinic acetylcholine receptor, in which no interaction between **C** and the receptor was required before photoactivation (Milburn et al., 1989; Hess, 1993). In our case, the interaction of the probes with the target protein is required before light activation. Thus, the reduced affinity rendered **C** unsuitable for time-resolved crystallographic studies on the cholinesterases.

Both **A** and **B** display rapid kinetics of product release upon pulsed illumination. Their photochemical half-times, 10 μs for **A** and 24 μs for **B** (Table 1), are in the range required for studying even an enzyme as rapid as AChE, since turnover rates in the range of 70−300 μs, depending on the species, have been reported for its action on its natural substrate, ACh (Vigny et al., 1978). The quantum yields of both **A** and **B** ensure satisfactory conversion to choline and CCh, respectively. Furthermore, the byproduct of photochemical fragmentation, 2-nitrosoacetophenone, has no effect on the enzymic activity of either AChE or BuChE, even at concentrations as high as 1 mM (not shown).

The experiments in which we studied the recovery of enzymic activity after photochemical fragmentation of solu-
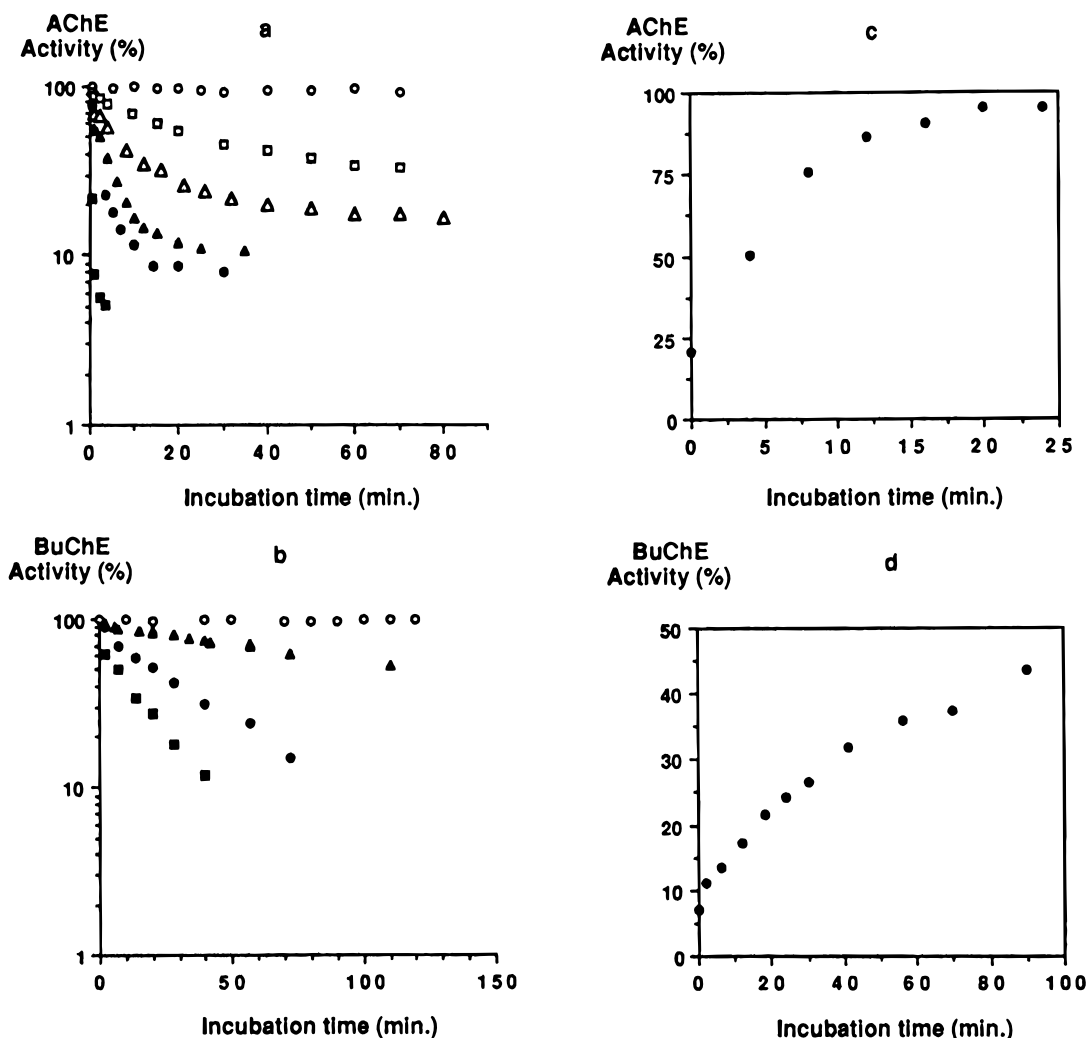
FIGURE 4: Inactivation of (a) AChE and (b) BuChE by CCh and regeneration of enzymic activity of (c) AChE and (d) BuChE after subsequent dilution. (a) (○) no CCh; (□) 2.0×10^{-5} M CCh; (△) 4.0×10^{-5} M CCh; (▲) 1.0×10^{-4} M CCh; (●) 2.0×10^{-4} M CCh; (■) 2.0×10^{-3} M CCh. (b) (○) no CCh; (▲) 2.0×10^{-3} M CCh; (●) 2.0×10^{-2} M CCh; (■) 2.0×10^{-1} M CCh.
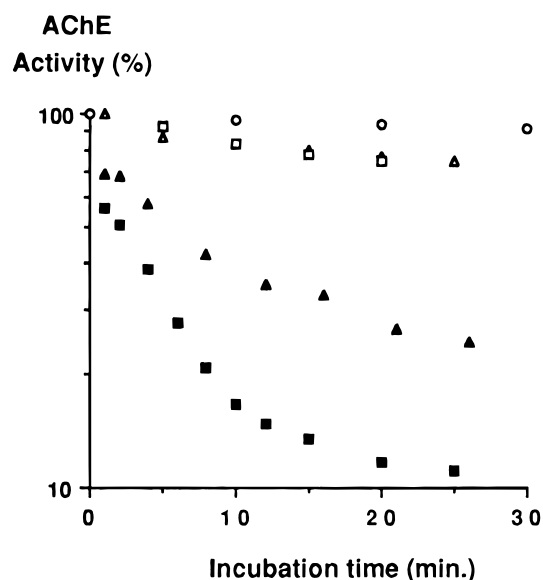

FIGURE 5: Protection by edrophonium of AChE against carbamylation by CCh. (○) 2.0×10^{-6} M edrophonium; (▲) 4.0×10^{-5} M CCh; (■) 1.0×10^{-4} M CCh; (△) 4.0×10^{-5} M CCh and 2.0×10^{-6} M edrophonium; (□) 1.0×10^{-4} M CCh and 2.0×10^{-6} M edrophonium.

tions of AChE and BuChE containing a large excess of compound **A** showed that fragmentation of the photolabile

compound in the bulk phase did not result in substantial loss of activity of either enzyme. Not only did 2-nitrosoacetophenone have no inhibitory effect on the two enzymes, as suggested by the control experiment mentioned above, but both the byproduct and choline, which would be generated concomitantly within the active sites of the enzymes under such experimental conditions, were cleared from their active sites. Thus, the experimental system can indeed serve as a paradigm for studying the clearance of choline from the active site of AChE and of BuChE under the conditions of time-resolved crystallography.

Use of photolabile precursors of CCh may better mimic the situation within the active site of the enzyme during conditions of catalysis, since clearance of choline in this latter case would be expected to occur concomitantly with carbamylation of the active-site serine by the CCh generated by photofragmentation. This process should closely mimic acylation of the serine by the natural substrate, ACh (see Scheme 1).

To study the effect of probe **B** on AChE, we used a high concentration of the probe, thus producing high occupancy of the active site, so as to ensure efficient generation of CCh within the active site. Indeed, even a single pulse, under the experimental conditions employed, caused progressive loss of enzymic activity to 60% of the control value, with
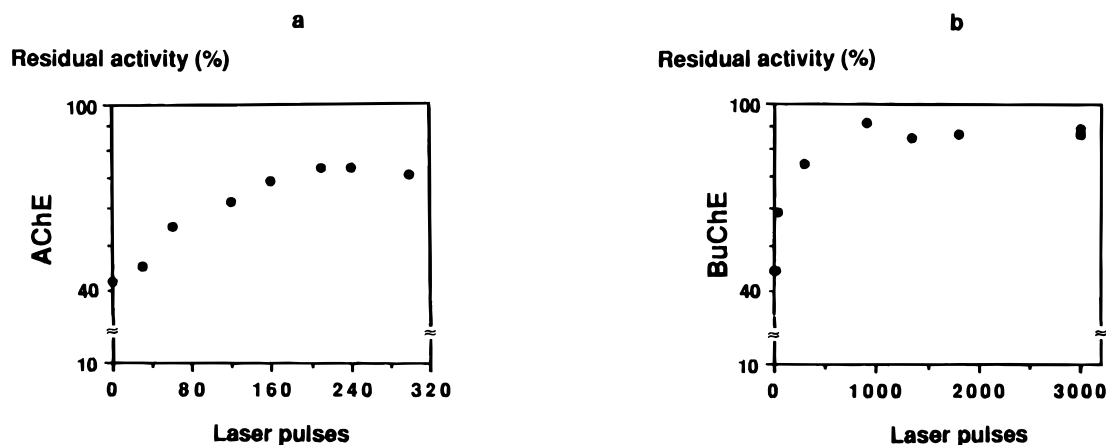
FIGURE 6: Regeneration of enzymic activity after laser flash photolysis of (a) AChE in the presence of **A** and (b) BuChE in the presence of **A**. The wavelength of the employed laser pulses was 351 nm and experimental conditions are given under Methods.
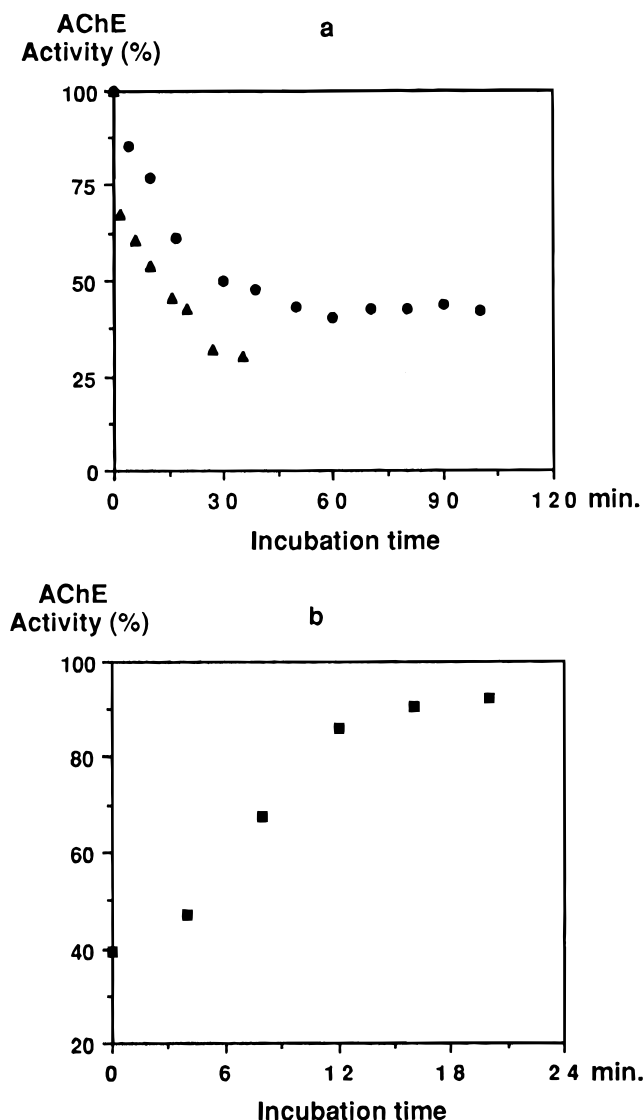

FIGURE 7: (a) Time-dependent inactivation of AChE after laser flash photolysis of AChE in the presence of **B**. (●) One pulse; (▲) 20 pulses. (b) Regeneration of AChE activity after subsequent dilution.

20 pulses decreasing activity by a further 15%. That the progressive inactivation observed was indeed due primarily to carbamylation by CCh generated by photofragmentation of **B** was clearly demonstrated by the fact that >90% of control activity could be regenerated in a time-dependent fashion upon dilution of the sample of inactivated enzyme, just as is observed for AChE inactivated directly by CCh.

Carbamylation could be achieved both by CCh generated directly within the active site and by entry into the active site of CCh generated in the bulk phase. Nevertheless, carbamylation by CCh generated directly within the active site has both kinetic and thermodynamic advantages over that by CCh generated in the bulk phase. Kinetically, carbamylation occurs at the active site, involving the active-site serine of AChE. CCh generated in the bulk solution must diffuse into the active site prior to carbamylation, while CCh generated in situ, within the active site, from **B**, can carbamylate AChE directly. CCh forms a reversible complex with AChE prior to carbamylation (Scheme 1). Formation of a complex between AChE and CCh generated in the bulk solution is a bimolecular process, while carbamylation by CCh generated in the active site from **B**, which is already complexed with AChE (Scheme 1), may be considered as an intramolecular process. The binding constant of CCh on AChE (9.23×10^{-5} M) is similar to that of its precursor, probe **B** (4.40×10^{-5} M). Thus, in the first instance, it must be primarily CCh, generated by photofragmentation of **B** bound within the active site should be involved in carbamylation of the enzyme and only subsequently will CCh generated in the bulk participate.

The data which we have obtained in solution show that **A** and **B** possess photochemical and specificity characteristics which render them suitable to serve as probes for time-resolved crystallography studies. Since the two probes generate choline in two different ways, either by a direct photocleavage reaction (**A**) or by enzymatic hydrolysis of a substrate generated by photocleavage (**B**), they constitute complementary tools for the time-resolved crystallographic studies envisaged. Work is presently in progress to establish the biochemical and photochemical properties of **A** and **B** soaked into crystals of *Torpedo* AChE.

ACKNOWLEDGMENT

REFERENCES

Aldridge, W. N., & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, North Holland, Amsterdam.

Antosiewicz, J., McCammon, J. A., Wlodek, T. S., & Gilson, M. K. (1995a) *Biochemistry 34*, 4211−4219.

Antosiewicz, J., Gilson, M. K., Lee, I. H., & McCammon, J. A. (1995b) *Biophys. J. 68*, 62−68.

Augustinsson, K.-B., Fredriksson, T., Sundwall, A., & Jonsson, G. (1959) *Biochem. Pharmacol. 3*, 68−76.

Bazelyansky, M.; Robey, E., & Kirsch, J. F. (1986) *Biochemistry 25*, 125−130.

Bolduc, J. M., Dyer, D. H., Scott, W. G., Singer, P., Sweet, R. M., Koshland, D. E., Jr., & Stoddard, B. L. (1995) *Science 268*, 1312−1318.

Bourne, Y., Taylor, P., & Marchot, P. (1995) *Cell 83*, 503−512.

Chatonnet, A., & Lockridge, O. (1989) *Biochem. J. 260*, 625−634.

Corrie, J. E. T., & Trentham, D. R. (1993) Caged nucleotides and neurotransmitters, in *Bioorganic Photochemistry Volume 2: Biological applications of photochemical switches* (Morrison, H., Ed.) pp 243−305, John Wiley & Sons, New York.

Corrie, J. E. T., Katayama, Y., Reid, G. P., Anson, M., & Trentham, D. R. (1992) *Philos. Trans. R. Soc. London, A 340*, 233−244.

Cruickshank, D. W. J., Helliwell, J. R., & Johnson, L. N. (1992) *Time-Resolved Macromolecular Crystallography*, Oxford University Press, Oxford, England.

Ehret-Sabatier, L., Schalk, I., Goeldner, M., & Hirth, C. (1992) *Eur. J. Biochem. 203*, 475−481.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, M. R. (1961) *Biochem. Pharmacol. 7*, 88−95.

Farber, G. K. (1995) *Curr. Biol. 5*, 1088−1090.

Fellman, J. H., & Fujita, T. S. (1964) *Biochim. Biophys. Acta 89*, 360−362.

Gilson, M. K., Straatsma, T. P., McCammon, J. A., Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., & Sussman, J. L. (1994) *Science 263*, 1276−1278.

Hajdu, J., & Andersson, I. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 467−498.

Harel, M., Sussman, J. L., Krejci, E., Bon, S., Chanal, P., Massoulié, J., & Silman, I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10827−10831.

Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P. H., Silman, I., & Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9031−9035.

Harel, M., Kleywegt, G. J., Ravelli, R. B. G., Silman, I., & Sussmann, J. L. (1995) *Structure 3*, 1355−1366.

Harel, M., Quinn, D. M., Nair, H. K., Silman, I., & Sussmann, J. L. (1996) *J. Am. Chem. Soc. 118*, 2340−2346.

Hess, G. P. (1993) *Biochemistry 32*, 989−1000.

Kalow, W., & Lindsay, H. A. (1953) *Can. J. Biochem. Physiol. 33*, 568−574.

Kronman, C., Ordentlich, A., Barak, D., Velan, B., & Schafferman, A. (1994) *J. Biol. Chem. 269*, 27819−27822.

Lockridge, O., & La Du, B. N. (1978) *J. Biol. Chem. 253*, 361−366.

McCray, J. A., & Trentham, D. R. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 239−270.

Milburn, T., Matsubara, N., Billington, A. P., Udgaonkar, J. B., Walker, J. W., Carpenter, B. K., Webb, W. W., Marque, J., Denk, W., McCray, J. A., & Hess, G. P. (1989) *Biochemistry 28*, 49−55.

Mooser, G., Schulman, H., & Sigman, D. S. (1972) *Biochemistry 11*, 1595−1602.

Peng, L., & Goeldner, M. (1996) *J. Org. Chem. 61*, 185−191.

Pillai, V. N. R. (1980) *Synthesis 1*, 1−26.

Porter, N. A., Bruhnke, J. D., & Koenigs, P. (1993) Photoregulation of enzymes, in *Bioorganic Photochemistry Volume 2: Biological applications of photochemical switches* (Morrison, H., Ed.) pp 197−241, John Wiley & Sons, New York.

Quinn, D. M. (1987) *Chem. Rev. 87*, 955−979.

Ripoll, D. R., Faerman, C. H., Axelsen, P. H., Silman, I., & Sussman, J. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5128−5132.

Ripoll, D. R., Faerman, C. H., Gillilan, R., Silman, I., & Sussman, J. L. (1995) in *Enzymes of the cholinesterase family* (Balasubramanian, A. S., Doctor, B. P., Taylor, P., & Quinn, D. M., Eds.) pp 67−70, Plenum Press, New York.

Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. S. (1990) *Nature 345*, 309−315.

Shafferman, A., Ordentlich, A., Barak, D., Kronman, C., Ber, R., Bino, T., Ariel, N., Osman, R., & Velan, B. (1994) *EMBO J. 13*, 3448−3455.

Singer, P. T., Smalås, A., Carty, R. P., Mangel, W. F., & Sweet, R. M. (1993) *Science 259*, 669−673.

Stoddard, B. L., Koenigs, P., Porter, N., Petratos, K., Petsko, G. A., & Ringe, D. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5503−5507.

Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253*, 872−879.

Taylor, P., Lwebuga-Mukasa, J., Lappi, S., & Rademacher, J. (1974) *Mol. Pharmacol. 10*, 703−708.

Tripathi, R. K., Telford, J. N., & O'Brien, R. D. (1978) *Biochim. Biophys. Acta 525*, 103−111.

Vigny, M., Bon, S., Massoulié, J., & Leterrier, F. (1978) *Eur. J. Biochem. 85*, 317−323.

Walker, J. W., McCray, J. A., & Hess, G. P. (1986) *Biochemistry 25*, 1799−1805.

Wilson, I. B., Hatch, M. A., & Ginsburg, S. (1960) *J. Biol. Chem. 235*, 2312−2315.

Wilson, I. B., Harrison, M. A., & Ginsburg, S. (1961) *J. Biol. Chem. 236*, 1498−1500.