Organophosphorylation of Acetylcholinesterase in the Presence of Peripheral Site Ligands

DISTINCT EFFECTS OF PROPIDIUM AND FASCICULIN*

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Structural analysis of acetylcholinesterase (AChE) has revealed two sites of ligand interaction in the active site gorge: an acylation site at the base of the gorge and a peripheral site at its mouth. A goal of our studies is to understand how ligand binding to the peripheral site alters the reactivity of substrates and organophosphates at the acylation site. Kinetic rate constants were determined for the phosphorylation of AChE by two fluorogenic organophosphates, 7-{[(diethoxyphosphoryl)oxy]-1-methylquinolinium iodide (DEPQ) and 7{[methylthio(phosphoryl)oxy]-4-methylcoumarin (EMPC), by monitoring release of the fluorescent leaving group. Rate constants obtained with human erythrocyte AChE were in good agreement with those obtained for recombinant human AChE produced from a high level Drosophila S2 cell expression system. First-order rate constants $k_{OP}$ were $1,600 \pm 300$ min$^{-1}$ for DEPQ and $150 \pm 11$ min$^{-1}$ for EMPC, and second-order rate constants $k_{OP}/K_{OP}$ were $193 \pm 13$ M$^{-1}$ min$^{-1}$ for DEPQ and $0.7-1.0 \pm 0.1$ M$^{-1}$ min$^{-1}$ for EMPC. Binding of the small ligand propidium to the AChE peripheral site decreased $k_{OP}/K_{OP}$ by factors of 2–20 for these organophosphates. Such modest inhibitory effects are consistent with our recently proposed steric blockade model (Szegletes, T., Mallender, W. D., and Rosenberry, T. L. (1998) Biochemistry 37, 4206–4216). Moreover, the binding of propidium resulted in a clear increase in $k_{OP}$ for EMPC, suggesting that molecular or electronic strain caused by the proximity of propidium to EMPC in the ternary complex may promote phosphorylation. In contrast, the binding of the polyepptide neurotoxin fasciculin to the peripheral site of AcChE dramatically decreased phosphorylation rate constants. Values of $k_{OP}/K_{OP}$ were decreased by factors of $10^5$ to $10^6$, and $k_{OP}$ was decreased by factors of 300–4,000. Such pronounced inhibition suggested a conformational change in the acylation site induced by fasciculin binding. As a note of caution to other investigators, measurements of phosphorylation of the fasciculin-AChE complex by AChE inactivation gave misleading rate constants because a small fraction of the AChE was resistant to inhibition by fasciculin.

Acetylcholinesterase (AChE)$^1$ terminates neurotransmission by catalyzing hydrolysis of the neurotransmitter acetylcholine at rates near that of a diffusion-controlled process (1). The x-ray crystal structure of AChE reveals that despite the impressive turnover rate of the enzyme, substrate molecules must penetrate 20 Å into a deep active site gorge to be hydrolyzed (2–4). This gorge contains two sites of ligand interaction: a peripheral site at the surface of the enzyme and an acylation site at the base of the gorge where the substrate acyl group is first transferred to residue Ser$^{200}$ (Torpedo californica AChE sequence numbering) and then hydrolyzed. In the acylation site, a catalytic triad consisting of residues Ser$^{200}$, His$^{440}$, and Glu$^{327}$ promotes the acyl transfers, and Trp$^{54}$ binds the acetylcholine trimethylammonium group, positioning the substrate for hydrolysis. Certain ligands can bind selectively to either the acylation site or the peripheral site, and ternary complexes can be formed in which ligands are bound to both sites simultaneously (5, 6). Ligands specific for the peripheral site include the small aromatic compound propidium and the snake venom neurotoxin fasciculin, both of which are potent inhibitors of the hydrolysis of the chromogenic acetylcholine analog, acetylthiocholine.

The AChE peripheral site is an attractive target for the design of new classes of therapeutic agents, so it is important to understand how ligand binding to the peripheral site affects substrate hydrolysis. We recently provided evidence for a steric blockade model which proposes that small peripheral site ligands like propidium inhibit substrate hydrolysis by decreasing the association and dissociation rate constants for an acylation site ligand without significantly altering their ratio, the ligand equilibrium constant (7, 8). Cationic substrates like acetylthiocholine also were shown to bind to the peripheral site as the first step in their catalytic pathway, and steric blockade arising from this substrate binding accounted for the well known phenomenon of substrate inhibition for AChE at very high concentrations of substrate (8). A key feature of the steric blockade model is that ligand binding to the peripheral site results in significant inhibition only if substrate fails to reach equilibrium binding prior to reaction at the acylation site. Substrates that are thought to form equilibrium complexes at the acylation site can be examined to test this prediction. Among these substrates are the organophosphates (OPs), a class of compounds that inactivate cholinesterases because they are poor substrates (9–11). OPs readily phosphorylate the active site serine of AChE, but very slow hydrolysis of this

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1 The abbreviations used are: AChE, acetylcholinesterase; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); OP, organophosphate; EMPC, 7-{[methylthio(phosphoryl)oxy]-4-methylcoumarin; THTFA, m-(N,N,N-trimethylammonio)trifluoroacetophenone.
phosphoryl enzyme results in essentially irreversible inactivation of the enzyme (12). In this paper we examine the effects of ligand binding to the peripheral site on OP phosphorylation of AChE in the context of the sterice blockade model. Rate constants of phosphorylation are measured in two ways. The classical method involves periodic measurements of AChE activity toward substrates as the enzyme becomes inactivated. The second method involves continuous assay of the phosphorylation reactions either with mixtures of acetic acid ester substrates and OPs (13, 14) or by monitoring loss of a fluorescent OP leaving group (15, 16). This method can be adapted to stopped-flow kinetic techniques to allow determination of both first- and second-order phosphorylation rate constants (13, 14). Here we monitor the reactions of two fluorescent OPs, EMPC and DEPQ (Fig. 1), with erythrocyte and recombinant human AChE. Phosphorylation rate constants obtained directly by fluorescence measurement of their AChE-mediated hydrolysis products 7HMC and 7HMQ, respectively (Fig. 1), are compared with those obtained by enzyme inactivation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human erythrocyte AChE was purified as outlined previously, and active site concentrations were determined by assuming previously, and active site concentrations were determined by assuming 1 unit corresponds to 1 nmol of acetylthiocholine hydrolyzed/min under standard pH-stat assay conditions (3.67 \( \Delta A_{122} \) nm/min in our standard spectrophotometric assay (17)).

Fig. 1. Chemical structures of OPs and their fluorescent hydrolysis products in these studies.

**Experimental Human AChE**—The full-length cDNA for human G4 AChE was obtained from Dr. Avigdor Shafferman in the vector pACHE10 (20). To obtain a secreted dimeric form of human AChE, a 96-base pair truncation sequence including a stop codon was synthesized and inserted just downstream from the exon 4/5 boundary (see Ref. 21). Insertion of the modified exon 4/5 sequence (corresponding to 5′AASEAPSTC-DGDSS-stop, human AChE sequence numbering) resulted in a partial duplication of the 3′-end of the exon 4 region of the gene. To remove this duplicated segment, the NotI-NheI 3′-segment of the gene was cloned into NotI-NheI-digested pCIneo (Promega Corp.). This construct, pCIneo′-AChE, contained an EspI site in both the original and modified sections of exon 4. The unwanted duplicated gene segment was removed by digestion with EspI followed by cloning of the resolved NotI-NheI fragment back into the AChE gene cassette. The final gene construct was confirmed by DNA sequencing carried out at the Mayo Clinic Rochester Molecular Biology Core Facility. The modified human AChE cDNA was moved into the pPac vector for transfection into and expression from Drosophila S2 cells in tissue culture (21). S2 cells were maintained in Schneider’s Drosophila medium (Life Technologies, Inc.) with 10% fetal bovine serum and appropriate antibiotics at 28 °C. S2 cells were cotransfected with pPac carrying the hygromycin phosphotransferase gene for selection of cells with hygromycin B. After selection with 0.2 mg/ml hygromycin B, monoclonal cell lines were isolated from colonies formed using a modified soft agar cloning protocol (21). Briefly, 10\(^6\) to 10\(^7\) selected cells were suspended in complete medium with 0.3% low melting temperature agarose. This mixture was plated onto a base layer of solidified 1.5% low melting temperature agarose (in complete medium with 0.15 M NaCl) in 12-well tissue culture plates. After cell/medium layer solidification, a layer of complete medium was placed on top of the agarose. Colonies (>2 mm) were picked and grown in 24-well plates until confluence. At this point clones were assayed for AChE activity, and lines with high activity were kept for large scale culturing and long term propagation. AChE was purified from culture medium by two cycles of affinity chromatography on acridinium resin (17). Purified recombinant AChE samples analyzed by SDS-polyacrylamide gel electrophoresis (22) showed no contaminants. In the absence of disulfide reducing agents, a prominent band of 140-kDa dimer and a minor band of 70-kDa monomer were apparent, and under these conditions data were fitted by nonlinear regression (23). Ratios of OP to AChE concentrations were adjusted to at least 20 for EMPC and 9 for DEPQ in all cases to prevent significant depletion of OP during the course of the reaction. Formation of 7HMC or 7HMQ did not follow a simple exponential time course.

**Peripheral Site Inhibition of AChE Organophosphorylation**

Here we monitor the reactions of two fluorogenic OPs, EMPC or DEPQ, with AChE (or AChE with inhibitor) and OP solutions rapidly, and fluorescence was recorded at fixed intervals as short as 20 ms. Formation of 7HMC or 7HMQ did not follow a simple exponential time course. Nonenzymatic hydrolysis of EMPC under all conditions and of DEPQ in the presence of propidium or fasciculin as inhibitors was significant, and under these conditions data were fitted by nonlinear regression analysis (Fig. P version 6.0, BioSoft, Inc.) to Equation 1.

\[
f = f_{\text{initial}} + \Delta f(1 - e^{-kt}) + Ct \quad (\text{Eq. 1})
\]

In Equation 1, \( f_{\text{initial}} \) is the fluorescence at time zero, \( \Delta f \) is the fluorescence change corresponding to an amount of fluorescent product equal to the AChE concentration (23), \( C \) is the nonenzymatic hydrolysis rate, and \( k \) is the rate constant for the approach to the steady-state level of phosphorylation. With DEPQ in the absence of inhibitors the release of phosphorylated enzyme occurred in two phases (see "Results") for both erythrocyte and recombinant AChE. These data were fitted to Equation 2, where \( \Delta f' \) and \( h \) were the respective amplitude and the rate constant for the slower phase, and the other parameters were as defined in Equation 1.

\[
f = f_{\text{initial}} + \Delta f'(1 - e^{-kh}) + \Delta f'(1 - e^{-kh}) \quad (\text{Eq. 2})
\]

The rate constants \( k \) were analyzed according to the catalytic pathway in Scheme 1. In this Scheme, OPX is the intact OP with leaving group X; EOPX is the initial complex of the OP with AChE, characterized by the equilibrium dissociation constant \( K_{d} \); and EOP is the phosphorylated enzyme. The inhibitor I can bind to the peripheral site in each of the enzyme species (as denoted by the subscript P). This scheme is identical to a general pathway for substrate hydrolysis by AChE considered elsewhere (7). Kinetic analysis of this scheme was simplified here in two ways. First, dephosphorylation rate constants (\( k_{3} \) in Scheme 1), which appeared consistent with a value of 2–4 \times 10^{-3}.
Peripheral Site Inhibition of AChE Organophosphorylation

$$\text{OPX + E} \xrightarrow{k_{1}} \text{EOPX} \xrightarrow{k_{2}} \text{X} + \text{EOP} \xrightarrow{k_{3}} \text{E} + \text{OPOH}$$

Scheme I

$$\text{OPX + I} \xrightarrow{k_{2}} \text{EOPXI} \xrightarrow{d_{k}} \text{X} + \text{EOPI} \xrightarrow{k_{3}} \text{I} + \text{OPOH}$$

These rate constants are related to the intrinsic rate constants in Scheme 1 as shown Equations 4 and 5.

$$k = \frac{k_{\text{OP}}[\text{OP}]}{K_{\text{OP}} + [\text{OP}]} \quad \text{(Eq. 3)}$$

$$k_{\text{OP}} = k_{2} \left[\frac{1 + a[I]}{[I]_{\text{Ki}}} \right] \quad \text{(Eq. 4)}$$

$$K_{\text{OP}} = K_{a} \left[\frac{1 + a[I]}{[I]_{\text{Ki}}} \right] \quad \text{(Eq. 5)}$$

In the absence of I, $k_{\text{OP}} = k_{2}$ and $K_{\text{OP}} = K_{a}$. In the presence of I, $K_{\text{OP}}$ is given by Equation 6.

$$K_{\text{OP}} = \frac{k_{a} + [I]}{[I]_{\text{Ki}}} \quad \text{(Eq. 6)}$$

Assays with the peripheral site inhibitors propidium (30 μM) or fasciculin (0.5–10 μM) were conducted at inhibitor concentrations at least 30 times their respective $K_{a}$ values to ensure that most of the AChE was complexed with inhibitor. Values of $K_{a}$ were taken as 1.0 ± 0.1 μM for propidium (7), 11 ± 2 pm for fasciculin (6), and 100 pm for fasciculin in the presence of DTNB and acetylthiocholine in the standard assay (6, 8). Measurements that included fasciculin were modified in several ways. First, the enzyme was incubated with fasciculin for 5–10 min to generate equilibrated complex before the addition of OP. Second, the reaction buffer with EMPC was adjusted to pH 7.0 to reduce nonenzymatic hydrolysis of the OP. Finally, a four-cell cuvette changer was employed (except where noted) for reaction times as long as 4 h. This device minimized fluorophore photobleaching because the sample was cycled in and out of the light path. Each reaction was measured in parallel with cuvettes corresponding to an air blank and a nonenzymatic hydrolysis control devoid of AChE.

AChE Phosphorylation Determined by OP Inactivation of AChE-Catalyzed Substrate Hydrolysis—AChE activity was monitored by a modified acetylthiocholine assay (25). Standard assays were conducted in 3.0 ml of 20 mM sodium phosphate, 0.02% Triton X-100, 0.33 mM DTNB, and 0.5 mM acetylthiocholine (pH 7.0) at 25°C. Enzyme hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm ($\lambda_{\text{exc}} = 14.15 \text{~mm}^{-1} \text{~cm}^{-1}$ (26)) for 1–5 min on a Varian Cary 3A spectrophotometer.4 The inactivation of AChE by an OP was initiated by mixing AChE and OP at 23°C in 20 mM phosphate buffer and 0.02% Triton X-100 (pH = 7.0). At various times a 1.0-ml aliquot was removed to a cuvette, 40 μl of acetylthiocholine and DTNB were added to final concentrations of 0.5 mM and 0.33 mM, respectively, and a continuous assay trace was recorded immediately at 412 nm. Background hydrolysis rates in the absence of AChE were subtracted. To assess the effects of peripheral site inhibitors on OP inactivation rates, propidium (30 μM) or fasciculin (50–250 nM) was incubated with AChE for at least 10–30 min prior to the addition of the OP. In some cases, DEPQ was also added (10–80 μM) for 60–120 min after incubation of fasciculin with AChE to eliminate a minor population of AChE which was refractory to normal fasciculin inhibition (see “Results”). Titrations of AChE activity with substoichiometric amounts of DEPQ were conducted by procedures similar to those in other inactivation measurements except that initial incubation mixtures contained higher concentrations of AChE (28–260 nM) and fasciculin (0–2 μM) and that after 90–120 min small aliquots of the mixtures (15–20 μl) were diluted into the standard acetylthiocholine assay solution.

OP inactivation reactions were measured under pseudo first-order conditions in which the ratio of OP to AChE concentrations was adjusted to at least 5. Assay rates $v$ during inactivation were divided by the control assay rate in the absence of OP to give a normalized value $v_{\text{N}/v_{\text{N}0}}$, and these values were fitted by nonlinear regression analysis (Fig. 2) to Equation 7, where $v_{\text{N}0}$ is $v$ at time zero and in the final steady state, respectively.

$$v_{\text{N}(t)} = v_{\text{N}0} \left(1 + \frac{v_{\text{N}0} - v_{\text{N}0}}{v_{\text{N}0} - v_{\text{N}0}}\right) e^{kt} \quad \text{(Eq. 7)}$$

OP concentrations also were sufficiently low that the observed inactivation rate constant $k$ was proportional to [OP]. The second-order rate constant for inactivation $k_{\text{OP}}/K_{\text{OP}}$ was fitted by weighted linear regression analysis of the relationship $k = (k_{\text{OP}}/K_{\text{OP}})[\text{OP}]$ (see Equation 3), assuming a constant percent error in $k$.

RESULTS

Direct Fluorometric Measurement of AChE Phosphorylation by OPs in the Presence and Absence of Peripheral Site Ligands—The rapid reactions of EMPC and DEPQ with AChE require the use of stopped-flow methods if both first- and second-order phosphorylation rate constants are to be measured. Fig. 2A illustrates the measurement of an individual k value for the reaction of DEPQ with AChE. The release of the fluorescent product 4HMQ occurred largely with a single rapid exponential time course, but a slower phase corresponding to about 10% of the overall reaction also was apparent. The amplitude, or amount of product released, in the predominant faster reaction phase equaled the AChE concentration (Equations 1 and 2) and thus corresponded to a fluorescence titration of the enzyme normality which reacted rapidly with DEPQ (15, 23). Fitted $k$ values for the rapid phase were analyzed according to Equation 3 (Fig. 2B) to obtain the first-order phosphorylation rate constant $k_{\text{OP}}$ and the second-order rate constant $k_{\text{OP}}/K_{\text{OP}}$. A second, slower phase was not apparent in reactions of EMPC with AChE or in reactions of either OP in the presence of the pe-

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3 To ensure that EMPC and DEPQ were close enough to equilibrium with AChE in the presence of 30 μM propidium or 0.5–10 μM fasciculin to justify application of Equations 4–6, we applied the ScOP simulation program (7) to Scheme 1 with the following rate constant assignments: $k_{a} = 1 \times 10^{7} \text{~M}^{-1} \text{~s}^{-1}$ for EMPC and $2 \times 10^{7} \text{~M}^{-1} \text{~s}^{-1}$ for DEPQ, values similar to those assigned previously for neutral and cationic acetic acid ester substrates (7); $k_{1} = 2 \times 10^{5} \text{~M}^{-1} \text{~s}^{-1}$ for propidium (7); $k_{1} = 3 \times 10^{5} \text{~M}^{-1} \text{~s}^{-1}$ for fasciculin with free AChE, and $k_{1} = 1 \times 10^{5} \text{~M}^{-1} \text{~s}^{-1}$ for fasciculin with AChE in the presence of DTNB and acetylthiocholine (6); $k_{a} = 5 \times 10^{5} \text{~M}^{-1} \text{~s}^{-1}$; $k_{a} = k_{a}$; and $a$ from Table I and “Experimental Procedures” (with $k_{a} = K_{a}k_{a}$; $k_{a}$ from Equation 6; and $k_{a} = k_{a}K_{a}K_{a}K_{a}$), $k_{a}$ and $K_{a}$ were then compared for complete equilibrium ($k_{a}/k_{a} = 1$) and pronounced steric blockade ($k_{a}/k_{a} = 0.00001$) and found to differ by less than 10%, justifying the equilibrium assumption.

4 Enzyme activities were standardized to 0.1 ΔA_{125} u/min by applying the observed relationship $v_{\text{std}} = 0.1(0.1)^{2}$, where $v$ was the measured activity, $v_{\text{std}}$ was the standardized activity, and $r = 0.95$. $R$ was the slope of a plot of log measured activity versus log enzyme concentration over a 200-fold range of enzyme dilution.
ribosomal site inhibitors propidium or fasciculin. These reaction time courses, however, were superimposed upon significant nonenzymatic OP hydrolysis rates that were incorporated into the curve fitting of the k values. Estimates of \( k_{OP} \) and \( k_{OP/K_{OP}} \) were obtained from these k values by analysis similar to that in Fig. 2B (Table I). Purified recombinant human AChE expressed in Drosophila S2 cells gave rate constants for both OPs and relative amplitudes for DEPQ which were in good agreement with those for purified human erythrocyte AChE. Furthermore, our \( k_{OP/K_{OP}} \) value for DEPQ (1.9–2.1 × 10^8 M⁻¹ min⁻¹) agreed with previous estimates of this second-order phosphorylation rate constant determined by inactivation of eel AChE (15, 27; see below and Table I). No previous estimates of \( k_{OP} \) for either DEPQ or EMPC or of \( k_{OP/K_{OP}} \) for EMPC have been reported. We observe that \( k_{OP/K_{OP}} \) is about 200–300 times larger for DEPQ than for EMPC and that \( k_{OP} \) is about 10 times larger for DEPQ than for EMPC. These differences are consistent with previous expectations that the cationic nature of DEPQ and the lower pKₐ of its leaving group relative to neutral EMPC should result in higher rates of AChE phosphorylation (14, 27, 28).

The effects of the small peripheral ligand propidium on phosphorylation of AChE by OPs have not been widely studied.
precise estimates were not possible.

Unlike propidium, the binding of fasciculin to the AChE peripheral site had a drastic effect on the phosphorylation of AChE by OPs (Fig. 3B). At saturating fasciculin concentrations (10^6 to 10^8 times greater than its K_i), k_OP/K.OP was decreased about 700-fold for EMPC and by about 10^5 for DEPQ. Bound fasciculin also decreased k.OP for both OPs by factors of 30–4,000 (Table I). The amplitudes of the OP reactions with the fasciculin-AChE complex again were consistent with the AChE normality, indicating that most of the enzyme was involved in the slowly reacting complex. A previous report of the effects of fasciculin-AChE complex could react with DEPQ at the previous rate, only about 17% of the residual activity should have been inactivated before DEPQ was completely depleted; in fact we continued to observe 80% inactivation with about the same value (data not shown).

TABLE I
Rate constants for the phosphorylation of AChE by OPs

Rate constants were calculated from the dependence of [OP] as outlined under “Experimental Procedures.”

| Enzyme, OP, and inhibitor | k_OP | k_OP/K_OP | k_OP/K_OP | Enzyme inactivation |
|---------------------------|------|-----------|-----------|---------------------|
| Erythrocyte | 149 ± 9 | 0.95 ± 0.04 | 0.82 ± 0.03 | 630 |
| EMPC | 900 ± 500° | 0.37 ± 0.02 | 2.6 | 25 |
| None | 0.06 ± 0.09 | 0.0001 ± 0.0001 | 600 |
| Fasiculin | 0.04 ± 0.09° | 0.0003 | 200,000 |
| 1,600 ± 300 | 193 ± 13 | 151 ± 8 |
| Propidium | 1,100 ± 500 | 11 ± 1 | 25 |
| None | 0.45 ± 0.09° | 0.0003 | 63 ± 10 | 2.4 |
| Fasiculin | 0.23 ± 0.08 | 0.002 | 0.0019 ± 0.0002 | 440 |
| Recombinant | 150 ± 11 | 0.67 ± 0.03 | 0.83 ± 0.02 |
| EMPC | 570 ± 140° | 0.45 ± 0.02 | 1.5 |
| None | 0.02 ± 0.08 | 0.0011 ± 0.0002 | 600 |
| Propidium | 0.12 ± 0.10° | 0.0003 | 0.02 ± 0.02 | 3.5 |
| Fasiculin | 0.001 ± 0.001 | 0.0001 ± 0.0001 | 200,000 |
| None | 205 ± 11 | 99 ± 3 |
| Propidium | 1,200 ± 400 | 15 ± 1 | 19 |
| Fasiculin | 0.06 ± 0.10° | 0.0003 | 32 ± 17 | 3.0 |

- The maximum [OP] employed did not exceed 80% of the estimated k.OP, and therefore estimates of k.OP are approximate.
- AChE was first preincubated with fasciculin then with DEPQ to eliminate the fasciculin-resistant AChE population (see “Results”).

To quantify this point, we titrated several AChE stocks with DEPQ in the presence and absence of fasciculin by measuring inactivation. Examples of these titrations are shown in Fig. 5. As expected in the absence of fasciculin, the stoichiometric amount of DEPQ required for complete inactivation was within about 15% of the AChE active site concentration calculated from the initial activity (Fig. 5A). In the presence of saturating fasciculin, however, less than 100% of the residual activity was rapidly inactivated (Fig. 5, B and C). We fitted these titration data to a model with two enzyme populations, one that was rapidly inactivated and the other that reacted with DEPQ at the very low rate constants measured by the fluorescence assays in Table I. The rapidly inactivated population corresponded to 5% of the total AChE concentration in Fig. 5B and 40% in Fig. 5C. These percentages varied among AChE stocks, with erythrocyte AChE typically giving about 5%
and two preparations of recombinant AChE exhibiting 2 and 40%, respectively. These data thus are consistent with the assignment of a small but variable fraction of the AChE as a population that is largely resistant to fasciculin inhibition.

We next confirmed that the residual activity remaining after the rapid inactivation by DEPQ in Fig. 5, B and C, in fact did correspond to the fasciculin-AChE complex. This involved demonstrating that this residual activity was slowly inactivated by OPs at the same low rate constants determined with the fluorescence assays in Table 1. AChE was incubated with fasciculin, and activity from the fasciculin-resistant population was removed by rapid inactivation with 10–60 nM DEPQ (see “Experimental Procedures”). The activity remaining after this treatment (e.g. the activity remaining after 60 min in the lower trace of Fig. 4) was then progressively inactivated by further incubation with EMPC, and \( \frac{k_{op}}{k_{OP}} \) was determined as above. These values of \( \frac{k_{op}}{k_{OP}} \) from inactivation were now in good agreement with the values of \( \frac{k_{op}}{k_{OP}} \) obtained for the reaction of EMPC with the fasciculin-AChE complexes by fluorescence assay (e.g. \( 1.3 \times 10^3 \text{ m}^{-1} \text{ min}^{-1} \) for erythrocyte AChE in Table 1).

Detection of More Than One Population of AChE in the Presence of Fasciculin by Fluorometry—As our last demonstration of the consistency between the fluorescence- and inactivation-based assays, we reexamined the release of fluorescent 7HMQ from the reaction of DEPQ with AChE when fasciculin was present. Because this method does not depend on residual enzyme activity, the fasciculin-resistant population can be monitored separately from the fasciculin-AChE complex simply by altering the time of measurement and the concentration of DEPQ (Fig. 6). In Fig. 6A, DEPQ was 7–14-fold in excess of the expected fasciculin-resistant population of AChE. A burst of 7HMQ was released in the initial minute of reaction, and the amplitude of this burst indicated that approximately 2–3% of the recombinant AChE concentration had reacted. This percentage agreed with the percentage of rapidly inactivated AChE obtained by an inactivation titration like those in Fig. 5 for this recombinant AChE sample (data not shown). When the DEPQ concentration was increased by a factor of 25 (Fig. 6B), the initial burst in Fig. 6A became too fast to measure, but the slower reaction of DEPQ with the fasciculin-AChE complex became apparent. As expected, the amplitude of this reaction corresponded to the total AChE concentration, and the \( k \) value

![Fig. 4. Inactivation of the residual activity of the fasciculin-AChE complex by DEPQ at two ratios of the DEPQ to AChE concentrations. Erythrocyte AChE at 0.16 nM (C) and 50 nM (E) was incubated for 10 min with fasciculin at 50 nM (C) and 500 nM (E), and inactivation was initiated by the addition of DEPQ to a final concentration of 1.4 nM. Aliquots were assayed at the indicated times as outlined under “Experimental Procedures.” Assay points \( v \) were normalized to corresponding control residual activities with fasciculin but without DEPQ \( v_{DEPQ=0} \) and fitted to Equation 7 (lines) to obtain a value of \( k \) for each curve.](image)

![Fig. 5. Titration of AChE with DEPQ in the presence and absence of fasciculin. Erythrocyte (eryth) or recombinant (rec) AChE was incubated with or without fasciculin for 10–30 min and mixed with an equal volume of DEPQ for 90–120 min as outlined under “Experimental Procedures.” Each point represents one mixture with the indicated final concentrations of DEPQ, AChE, and fasciculin. Aliquots (15–20 µl) were then incubated 50-fold (panels B and C) or 200-fold (panel A) into the standard acetylthiocholine solution for assay. Observed \( v \) were normalized to \( v_{DEPQ=0} \) obtained in the absence of DEPQ, and titration lines fitting the stoichiometric amount of DEPQ required to give complete rapid inactivation were calculated with the SCoP program. The calculated concentrations of rapidly inactivated AChE were 32 nM (panel A), 13 nM (panel B), and 43 nM (panel C) and correspond closely to the intersections of the lines in the plots. (The SCoP simulation program (7) was applied to two populations of AChE which reacted with DEPQ according to Scheme 1 to fit the data in Fig. 5, B and C. Rate constant assignments for the fasciculin-inhibited population were taken from Footnote 3; \( k_{op}/k_{OP} \) for DEPQ with the fasciculin-resistant population was assigned as \( 1 \times 10^{-4} \text{ m}^{-1} \text{ min}^{-1} \), and the measured nonenzymatic DEPQ hydrolysis rate was \( 1.4 \times 10^{-4} \text{ min}^{-1} \) (data not shown). The fitted variables were the ratio of the concentrations of the two populations and the ratio of their acetylthiocholine hydrolysis rates.)](image)
Peripheral Site Inhibition of AChE Organophosphorylation

A, recombinant AChE (200 nM) was preincubated with fasciculin (1.0 μM) before mixing an equal volume of 380 nM DEPQ in the stopped-flow accessory, and generation of 7HMQ was monitored by fluorometry as in Fig. 2. Panel B, recombinant AChE (200 nM) was preincubated with fasciculin (1.0 μM) before conventional mixing with an equal volume of 10 μM DEPQ, and generation of 7HMQ was monitored as in panel A. A value of k = 0.01 min⁻¹ was estimated by fitting the data in panel B with Equation 1. Dashed lines indicate blank DEPQ hydrolysis rates measured in the absence of AChE.

was consistent with the kD/OP determined by fluorometry for the reaction of DEPQ with AChE in the presence of fasciculin (Table I).

**Discussion**

In this paper, we report kinetic parameters for the phosphorylation of AChE by two fluorogenic OPs, EMPC and DEPQ (Fig. 1). Both human erythrocyte AChE and recombinant human AChE produced from a high level *Drosophila* S2 cell expression system were examined. Because this expression system yields more than 20 mg of purified AChE from 2 liters of medium after 10 days of continuous culture, it is attractive for the preparation of wild type and site-specific mutants of AChE for comparative kinetic analyses and x-ray crystallography. The agreement of the phosphorylation kinetic parameters in Table I for the two AChEs provides important confirmation that the recombinant enzyme retains the catalytic properties of endogenous AChEs. EMPC and DEPQ were particularly useful organophosphorylation reagents because their reactions with AChE were observed both directly by fluorometry and indirectly by enzyme inactivation, their high phosphorylation rate constants approximated those for OPs used in chemical warfare applications, and their charges differed, allowing comparison of neutral EMPC with cationic DEPQ. We focused specifically on the effects of bound peripheral site ligands on AChE phosphorylation by OPs. Characterization of these effects is of great interest because it may be possible to design a peripheral site ligand that will block OP inactivation of AChE specifically while allowing sufficient acetylcholine hydrolysis activity to maintain synaptic transmission.

To pursue this goal we first compared the effects on AChE phosphorylation of two ligands that bind specifically to the peripheral site, the small phenanthridinium derivative propidium and the 61-residue polypeptide fasciculin. Propidium is a potent inhibitor of substrate hydrolysis by AChE, decreasing the second-order rate constant k₆/HK₉ for acetylthiocholine and phenyl acetate by factors of 15–50 and the first-order rate constant kₛ by factors of 2–10 (7). To account for this inhibition, we proposed a steric blockade model in which the primary effect of a small peripheral site ligand like propidium is to slow the association and dissociation rate constants for ligand binding to the acylation site without significantly altering their ratio, the equilibrium constant (7, 8). One objective in proposing this model was to demonstrate that inhibition by peripheral site ligands could be explained without invoking a conformational change in the acylation site induced by the binding of ligand to the peripheral site. Our steric blockade model was supported by direct measurements with the acylation site ligands huperzine A and TMTFA: bound propidium decreased the association rate constants 49- and 380-fold and the dissociation rate constants 10- and 60-fold, respectively, relative to the rate constants for these acylation site ligands with free AChE (7). The model also was supported by computer simulations of substrate hydrolysis based on Scheme 1. When the binding of substrate to the acylation site failed to reach equilibrium, the observed level of propidium inhibition could be reproduced (7). On the other hand, the model predicts that propidium should have little effect on the reaction of a substrate that essentially equilibrates with the acylation site. Few reports in the literature include data that allow this prediction to be examined, but it is supported by a recent investigation of aryl acylamidase activity in AChE (31). The peripheral site ligands propidium and gallamine failed to inhibit AChE-catalyzed hydrolysis of aryl acylamides, which are hydrolyzed slowly by AChE and thus should equilibrate with the acylation site (32), but gave typical inhibition of acetylthiocholine hydrolysis.

The reaction of OPs, including EMPC and DEPQ, with AChE appears to involve equilibration of the OP with the acylation site (see Footnote 3). Table I indicates that propidium did have modest effects on kD/OP and kD/K7 for both EMPC and DEPQ. Do these observations invalidate our steric blockade model and require that the binding of propidium induce a conformational change in the acylation site? We argue that they do not, if the model is extended to allow an unfavorable electrostatic interaction or a steric overlap between propidium at the peripheral site and an OP at the acylation site in the AChE ternary complex. The need for such an extension in fact has been recognized in our previous studies because small decreases in the affinity of ligands in ternary AChE complexes relative to the corresponding binary complex are observed consistently (7). For example, from the rate constants noted above one can calculate that the affinities of huperzine A and TMTFA for the acylation site decreased by factors of 5–6 when propidium was bound to the peripheral site. Computer modeling revealed no steric overlap between the ligands in these ternary complexes (7), so the decreased affinity must result from unfavorable electrostatic interaction between these cationic ligands. Extending these observations to the OPs, a decrease in affinity for EMPC and DEPQ also was apparent when propidium was bound to the peripheral site. Insertion of data from Table I into Equation 6 indicated that this decrease (given by K₉/K₁ = K₅/K₉) was about an order of magnitude for both OPs. There was also a clear increase in the kD/OP for EMPC (a > 1 in Table I) and a possible increase in kD/OP for DEPQ when propidium was bound, consistent with an acceleration of first-order phosphorylation rate constants by bound peripheral site ligands reported recently by Radic (33, 34). It has long been known that kD/OP for the reaction of neutral OPs with AChE varies smoothly and monotonically with the pKa of the leaving group (27). This suggests that cleavage of the leaving group ester bond of the OP is prominent in the rate-limiting step for phosphorylation of AChE. Computer modeling revealed a clear unfavorable steric overlap between propidium in the peripheral site and the leaving group of either EMPC or DEPQ in the acylation site (data not shown; 35). This steric overlap could contribute to the decrease in affinity for both neutral EMPC and cationic DEPQ, and it could induce molecular or electronic strain caused by the proximity of propidium to the OP in the ternary complex to increase kD/OP. This increase would not require an induced conformational change in the acylation site.
Peripheral Site Inhibition of AChE Organophosphorylation

The consequences of fasciculin binding to the peripheral site of AChE on phosphorylation kinetic parameters for EMPC and DEPQ were qualitatively different from those of propidium. Values of $k_{OP}/K_{OP}$ were decreased by factors of $10^3$ to $10^5$, and $k_{OP}$ was decreased by factors of $300$ to $4000$ (Table I). Fasciculin has been shown to present a substantial steric blockade to the entrance and exit of ligands that bind to the acylation site: association and dissociation rate constants for the binding of $N$-methylacridinium were decreased $8000$- and $2000$-fold, respectively, when fasciculin was bound (36). However, steric blockade of OP association and dissociation rate constants cannot account for the effects of fasciculin on the phosphorylation rate constants. Because OPs essentially equilibrate with the acylation site, a steric blockade of OPs by fasciculin that resulted in even a $100,000$-fold decrease in association rate constant ($k_{OP}/k_{a}$) would result in less than a $10\%$ decrease in $k_{OP}/K_{OP}$ and no change in $k_{OP}$ (see Footnote 3). The pronounced fasciculin inhibition of AChE phosphorylation requires an additional interaction between fasciculin and the acylation site. One possibility might be an unfavorable steric overlap between fasciculin at the peripheral site and an OP at the acylation site in the AChE ternary complex, but the three-dimensional structure of the fasciculin-AChE complex shows no penetration of the acylation site by fasciculin which would lead to such an interaction. Therefore, the additional interaction must involve a conformational change in the acylation site induced by bound fasciculin. Crystal structure analyses of fasciculin-AChE complexes (3, 4) show that fasciculin 2 interacts not only with Trp$^{279}$ in the peripheral site but also with residues on the outer surface of an $\alpha$-loop within $4\AA$ of Trp$^{84}$ in the acylation site, well beyond the region of the peripheral site occupied by propidium (7, 37). These more extensive surface interactions provide a structural basis for an inhibitory conformational effect on the acylation site when fasciculin but not when propidium is bound to the peripheral site.

Second-order phosphorylation rate constants $k_{OP}/K_{OP}$ obtained for EMPC or DEPQ alone or in the presence of propidium were in good agreement when measured either by release of the fluorescent leaving group or by enzyme inactivation (Table I). In the presence of fasciculin, however, $k_{OP}/K_{OP}$ values determined by enzyme inactivation were $100$-fold greater for EMPC and $10^2$-fold greater for DEPQ than the corresponding values measured fluorometrically. Through a series of titrations like those in Fig. 5, this discrepancy was shown to arise from misleading inactivation measurements caused by a small fraction of the total AChE (less than $5\%$, except for one preparation) which remained largely resistant to inhibition by fasciculin. This fraction thus accounted for most of the enzyme resistant population than in the predominant conventional AChE. Fasciculin does appear to interact weakly with this resistant population, resulting in $3$- to $5$-fold decreases in $k_{OP}/K_{OP}$ for EMPC and DEPQ (Table I). A fasciculin-resistant population also may dominate the activity of recombinant mouse AChE in the presence of fasciculin: the addition of fasciculin induced biphasic phosphorylation rates and only modest decreases in phosphorylation rate constants and TMTFA association and dissociation rate constants (less than $20\%$; 30). It is possible that the population of AChE resistant to fasciculin can be distinguished even in the absence of fasciculin as the fraction of AChE that underwent a slower reaction with DEPQ in Fig. 2. The amount of this fraction (about $10\%$ of the total AChE) and its phosphorylation rate constant (about $10\%$ of the $k$ for the faster phase) are roughly consistent with the data for the fasciculin-resistant population.

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