Phosphoryl Oxime Inhibition of Acetylcholinesterase during Oxime Reactivation Is Prevented by Edrophonium

Chunyuan Luo,*,‡ Ashima Saxena,‡ Miles Smith,§ Gregory Garcia,‡ Zoran Radić,|| Palmer Taylor,|| and Bhupendra P. Doctor‡

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20307-5100, Drug Discovery Group, Georgetown University Medical Center, Washington, D.C. 20007, and Department of Pharmacology, University of California at San Diego, La Jolla, California 92093-0636

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ABSTRACT: Reactivation of organophosphate (OP)-inhibited acetylcholinesterase (AChE) is a key objective in the treatment of OP poisoning. This study with native, wild-type, and mutant recombinant DNA-expressed AChEs, each inhibited by representative OP compounds, establishes a relationship between edrophonium acceleration of oxime-induced reactivation of OP−AChE conjugates and phosphoryl oxime inhibition of the reactivated enzyme that occurs during reactivation by pyridinium oximes LüH6 and TMB4. No such recurring inhibition could be observed with HI-6 as the reactivator due to the extreme lability of the phosphoryl oximes formed by this oxime. Phosphoryl oximes formed during reactivation of the ethoxy methylphosphonyl−AChE conjugate by LüH6 and TMB4 were isolated for the first time and their structures confirmed by ^{31}P NMR. However, phosphoryl oximes formed during the reactivation of the diethylphosphoryl−AChE conjugate were not sufficiently stable to be detected by ^{31}P NMR. The purified ethoxy methylphosphonyl oximes formed during the reactivation of ethoxy methylphosphonyl−AChE conjugate with LüH6 and TMB4 are 10- to 22-fold more potent than MEPQ as inhibitors of AChE and stable for several hours at pH 7.2 in HEPES buffer. Reactivation of both ethoxy methylphosphonyl− and diethylphosphoryl−AChE by these two oximes was accelerated in the presence of rabbit serum paraoxonase, suggesting that organophosphorus hydrolase can hydrolyze phosphoryl oxime formed during the reactivation. Our results emphasize that certain oximes, such as LüH6 and TMB4, if used in the treatment of OP pesticide poisoning may cause prolonged inhibition of AChE due to formation of phosphoryl oximes.

Acetylcholinesterase (AChE,[1] EC 3.1.1.7) catalyzes the hydrolysis of the neurotransmitter acetylcholine and terminates impulse transmission at cholinergic synapses. Upon inhibition of AChE by organophosphorus (OP) esters, a conjugate between the γ-oxygen of the active-site serine and the OP is formed, and the enzyme recovers activity by itself very slowly. This may result in severe intoxication and death of the exposed individual within a very short period of time. During the past several decades, effective therapeutic measures (antidotes) for OP poisoning have been actively sought. Some nucleophilic oxime compounds, including the monopyridinium compound 2-PAM and the bispyridinium compounds TMB4, LüH6, and HI-6, were shown to reactivate OP-inhibited AChE and greatly attenuate the toxicity of many OP compounds (*1*−*5*) (Chart 1). However, in many cases, little or no reactivation of the OP−enzyme conjugate could be achieved by these oximes, therein limiting their efficacy as antidotes (*6*−*8*).

One factor responsible for retarding the oxime-induced reactivation of OP-inhibited AChE is aging (dealkylation) of the enzyme (*9*). Aging is responsible for the rapid loss of reactivatability of the OP−enzyme conjugate under certain situations. With certain types of OPs, such as nerve agent soman, the $t_{1/2}$ of aging of enzyme varies from a few minutes to half an hour, depending on the source of the enzyme (*10*, *11*). In other cases, poor reactivation by oxime cannot be attributed to aging and may be due to poor orientation or inaccessibility of the attacking group of oxime to the AChE active center, as indicated in recent site-specific mutagenesis studies (*12*, *13*). Another factor which can also affect the reactivation of OP-inhibited AChE is the possibility that a putative phosphoryl oxime (POX, including both phosphorylated and phosphonylated oxime) may be formed during

* Corresponding author: Telephone: (202) 782-7245. Fax: (202) 782-7651. E-mail: dr.chunyuanluo@wrsmtp-ccmail.army.mil.

‡ Walter Reed Army Institute of Research.

§ Georgetown University Medical Center.

|| University of California at San Diego.

[1] Abbreviations: AChE, acetylcholinesterase; FBS AChE, fetal bovine serum AChE; MoAChE, mouse AChE; OPH, organophosphorus hydrolase; PON, paraoxonase; OP, organophosphate; POX, phosphoryl oxime; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; DEPQ, 7-(*O*,*O*-diethylphosphinyloxy)-1-methylquinolinium methyl sulfate; 7-HQ, 7-hydroxy-1-methyl quinolinium cation; ATC, acetylthiocholine iodide; DTNB, 5,5-dithiobis(2-nitrobenzoic acid); LüH6 (toxogonin), 1,1′-(oxybismethylene)bis[4-(hydroxyimino)methyl]-pyridinium dichloride; TMB4, 1,1′-trimethylene bis[4-(hydroxyimino)-methyl]pyridinium dibromide; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxyaminopyridinium) dimethyl ether hydrochloride; EMP−AChE, ethoxyl methylphosphonyl−AChE; DEP−AChE, *O*,*O*-diethylphosphinyloxy−AChE; EMP−LüH6, ethoxyl methylphosphonyl−LüH6; EMP−TMB4, ethoxyl methylphosphonyl−TMB4; BSA, bovine serum albumin.

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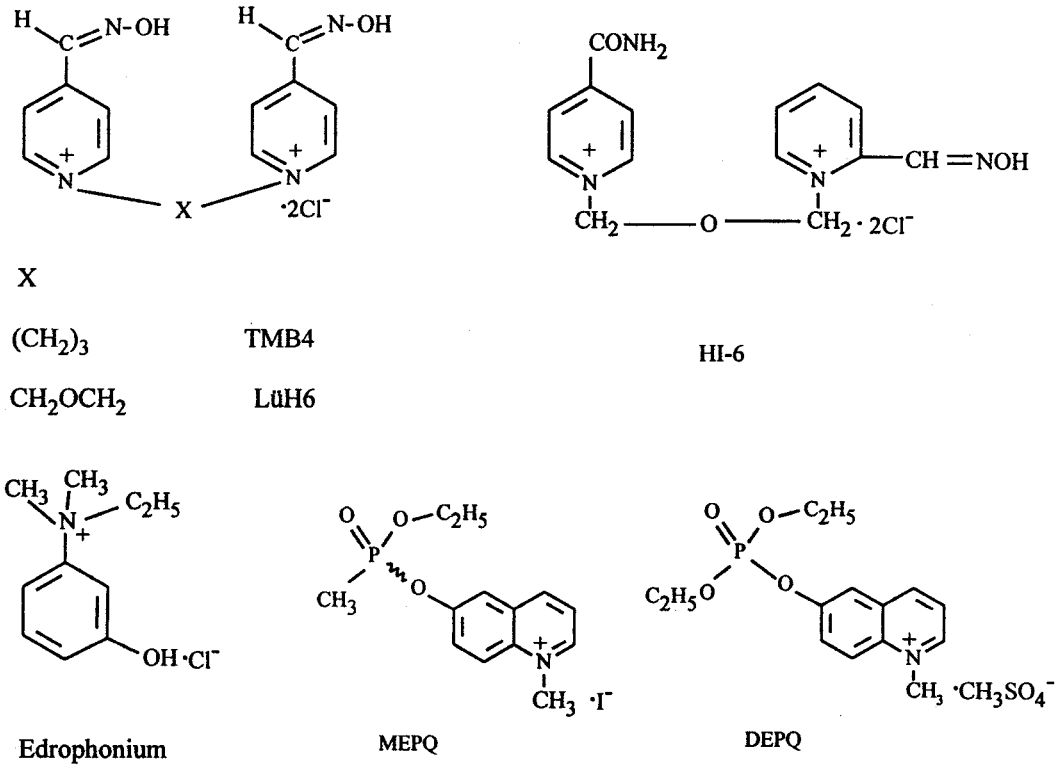
Chart 1



the reactivation and may inhibit the reactivated enzyme (*14*−*20*). POX is believed to be an unstable intermediate, and its presence in the reactivation medium has not yet been confirmed.

In our previous study with oxime-induced reactivation of EMP−AChE from fetal bovine serum (FBS AChE), protection of the reactivated enzyme from POX reinhibition by some quaternary ligands, such as edrophonium, was proposed (*21*). In the study presented here, we have attempted to establish a relationship between edrophonium-induced acceleration of reactivation and POX inhibition of the reactivated enzyme using FBS AChE, wild-type mouse AChE (MoAChE), and D74N MoAChE expressed from recombinant DNA, all inhibited with MEPQ and DEPQ. Our results with three oximes, TMB4, LüH6, and HI-6, confirm that acceleration of oxime-induced reactivation of AChE by edrophonium is due to the prevention of POX inhibition of the reactivated enzyme. The two POXs formed during the reactivation of the EMP−AChE conjugate by LüH6 and TMB4 were isolated and characterized using ^{31}P NMR. The POXs were purified using HPLC, and their stability and their potency as inhibitors of AChE were studied. The results highlight the importance of POX inhibition of the reactivated enzyme during oxime reactivation both in vitro and in vivo.

MATERIALS AND METHODS

Materials. MEPQ (racemic) and DEPQ were provided by Y. Ashani and H. Leader (Israel Institute for Biological Research, Ness Ziona, Israel). The oximes LüH6, TMB4, and HI-6 were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. HEPES [*N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid] and MOPS [3-(*N*-morpholino)propanesulfonic acid] were from Sigma Chemical Co. (St. Louis, MO). Recombinant wild-type and D74N MoAChEs were prepared as described previously (*22*, *23*). Large quantities of native FBS AChE were purified as described previously (*24*). Procainamide Sepharose 4B affinity gel with a specific activity of 27 μM procainamide/mL was obtained from the Israel Institute for Biological Research.

All solvents that were used were HPLC grade. Other chemicals were analytical grade and purchased from commercial sources. Rabbit serum paraoxonase (PON) was purified by modification of the method used by Gan et al. (*25*) with Cibacron Blue 3GA-Agarose chromatography (Sigma) followed by DEAE-Sephacel chromatography (Pharmacia, Uppsala, Sweden) in the presence of a nonionic detergent, Tergitol NP-10. SDS−PAGE showed that the purified enzyme migrated as two bands with molecular weights of about 41 000 and 44 000, in agreement with a previous report (*26*). The activity of rabbit serum PON was assayed using 1 mM paraoxon as a substrate in 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$.

AChE Assay. AChE activity was determined spectrophotometrically by the method of Ellman et al. (*27*). The assay mixture contained 1 mM ATC (or 5 mM for D74N MoAChE) as the substrate and 0.5 mM DTNB in 50 mM $NaPO_4$ buffer (pH 8.0). All measurements were taken at 25 °C.

Titration of AChE Active Sites with DEPQ. Increasing amounts of DEPQ (1−20 μL) were added to 0.5−2 units/mL AChE [60−100 μL of 50 mM $NaPO_4$ buffer (pH 8.0) containing with 0.1% BSA]. The mixture was incubated at 25 °C for 1 h. Titration curves were constructed by plotting residual enzyme activity against the amount of DEPQ added to the mixture. The enzyme active-site concentration was calculated by extrapolation of the curve to zero activity.

Effect of Edrophonium on the Reactivation of EMP− and DEP−FBS AChE and MoAChE. Stoichiometric amounts of MEPQ or DEPQ were added to 2 units of FBS AChE or 1.15 units of MoAChE (0.005 nmol, each) in 50 mM $NaPO_4$ buffer (pH 8.0) containing 0.1% BSA, in a total volume of 25 μL, and incubated at room temperature for 60 min to inhibit enzyme activity. Oxime (0.1 mM) in the absence or presence of different concentrations of edrophonium was added to the mixture in a total volume of 50 μL. After a fixed time, the mixture was diluted 200-fold with 50 mM $NaPO_4$ buffer (pH 8.0) containing 0.1% BSA. A 100 μL aliquot of the reactivation mixture was assayed for AChE activity using 3.2 mL of assay buffer. The percent reactivation was calculated as described previously (*21*).

Influence of Edrophonium on the Reactivation Kinetics of EMP− and DEP−Wild-Type and D74N MoAChE. The time courses of the reactivation were monitored in the absence and presence of 10 μM edrophonium using procedures reported previously (*12*, *21*). To analyze POX inhibition under comparable conditions, the enzyme conjugate concentration was fixed at 1.25 nM in all kinetic studies. Due to the nonequivalent reactivation rate of the two diastereomers of EMP−AChE conjugates, an equation for the two-component first-order approach to equilibrium was used to fit the data, assuming an equal distribution of the two species under the experimental conditions (*12*). Since only one species was created when AChE was inhibited with DEPQ, a one-component equation was used to fit the reactivation data.

^{31}P NMR of POXs Formed by Reaction of MEPQ with Oximes. ^{31}P NMR spectroscopy of POXs formed by reaction of MEPQ with LüH6, TMB4, and HI-6 was performed using a method similar to that reported for the measurement of POX formed by mixing sarin with LüH6 (*28*). A mixture of 1 mg of MEPQ and 0.5 mL of 50 mM oximes in 300 mM MOPS buffer (pH 7.7) in D_2O (equal to pH 7.4 in H_2O) was incubated for 3 min at room temperature. The pH was then lowered to 3.5 using 0.2 mL of 2 M citric acid/sodium citrate buffer (pH 3.0) in D_2O. The ^{31}P NMR spectra were recorded at 121.4 MHz in 5 mm tubes on a Varian INOVA-300 spectrometer at ambient probe temperature. All spectra were recorded H-decoupled and referenced to (external) 85% phosphoric acid (0 ppm). The spectra were measured under the following conditions: spectral width, 8500 Hz; acquisition time, 1.6 s; and pulse delay, 0. The total acquisition time was 30 min.

HPLC Purification of POXs Formed by the Chemical Reactions. Since POXs prepared by mixing oximes with MEPQ contained excess oxime, and hydrolytic products of MEPQ and POXs, they were purified by ion-pair reverse phase high-performance liquid chromatography (RP-HPLC). Aliquots of the mixture were injected into a Vydac narrow bore C_{18} column (2.1 mm × 25 cm, 5 μm, 300 D) attached to a Beckman model 126 pump and a model 168 diode array detector controlled by Beckman Gold Nouveau version 6.1 software. POX separations were accomplished by isocratic elution using a solvent system of 0.005 M 1-heptanesulfonic acid (PIC-7; Waters), 20% acetonitirile, and 0.5% acetic acid in water (pH 3.8) at a flow rate of 0.3 mL min^{-1} at ambient temperature. The samples were maintained at 4 °C prior to injection, and peak fractions were collected on ice. The eluate was monitored at 284 and 354 nm for the maximum sensitivity for oximes and MEPQ, respectively. Oximes and MEPQ were identified by comparison of retention times and absorbance spectra with those of standard solutions examined from identical RP-HPLC runs. The POX peak in the sample of the MEPQ and LüH6 mixture was initially identified by the hypsochromic shift (286 to 276 nm) of the LüH6 spectra, as described by Waser et al. (*28*). A hypsochromic shift from 284 to 274 nm was also found for the POX peak in the mixture of MEPQ and TMB4. Large-scale isolation of POXs was accomplished as described above except that a Waters μBondapak analytical C_{18} column (3.9 mm × 30 cm) was used. POX peaks from eight runs were pooled, concentrated by lyophilization, and equilibrated in D_2O for ^{31}P NMR measurements.

Determination of Hydrolysis Rate Constants (k_h) of POXs. The concentrations of purified POXs in solution were determined by titration with FBS AChE at a defined concentration. For the determination of k_h, 500 μL of 0.1 μM POX in different buffers was incubated at 25 °C. At specified time intervals, 5 μL was transferred to tubes containing 45 μL of 10 nM FBS AChE and the mixture incubated for 30 min and assayed for enzyme activity. The k_h values were calculated by fitting the inhibition data to the following one-phase exponential decay equation:

$$(E_i)_t = (E_i)_0 e^{-kht} \quad (1)$$

where $(E_i)_t$ and $(E_i)_0$ are the concentration of FBS AChE at time t and prior to reaction, respectively.

Determination of Bimolecular Rate Constants (k_i) of POXs for the Inhibition of AChEs. An initial trial of sample removal for determination of the k_i of POXs indicated that the inhibition rate was too fast to be measured by this method. Thus, a continuous monitoring method was used to determine the k_i values (*29*). In this method, 5 μL of approximately 5−30 nM enzyme was added to a cuvette with 1 mL of assay mixture, containing 0.15 mM DTNB, 0.075 mM ATC, 0.1% BSA, and different concentrations of POX in 20 mM HEPES buffer (pH 7.2). Changes in absorbance at 412 nm were monitored at 6−9 s intervals. Pseudo-first-order inhibition constants (k_{obs}) were obtained by fitting data to the following equation:

$$A = A_0 + (v_0 - v_{ss})/(1 - e^{-k_{obs}t})k_{obs} + v_{ss}t \quad (2)$$

where A and A_0 are absorbances at times t and 0, respectively, v_{ss} is the slope of the steady-state phase, v_0 is the initial velocity of the time course for inhibition, and k_{obs} is the observed pseudo-first-order rate constant. Replots of k_{obs} versus POX concentration yielded second-order inhibition constants in the presence of substrate, which were then converted to the second-order constants (k_i) in the absence of substrate by multiplying by a factor of $(1 + [A]/K_m)$. When the plot was linear, k_i was estimated from the slope of the line, assuming that the concentration of POX is far below the dissociation constant of the POX−AChE complex. To ensure the accuracy of this determination, measurements were taken in the linear range of substrate hydrolysis over the entire reaction course.

Reactivation Kinetics of POX-Inhibited Wild-Type MoAChE. Wild-type MoAChE was inhibited with a stoichiometric

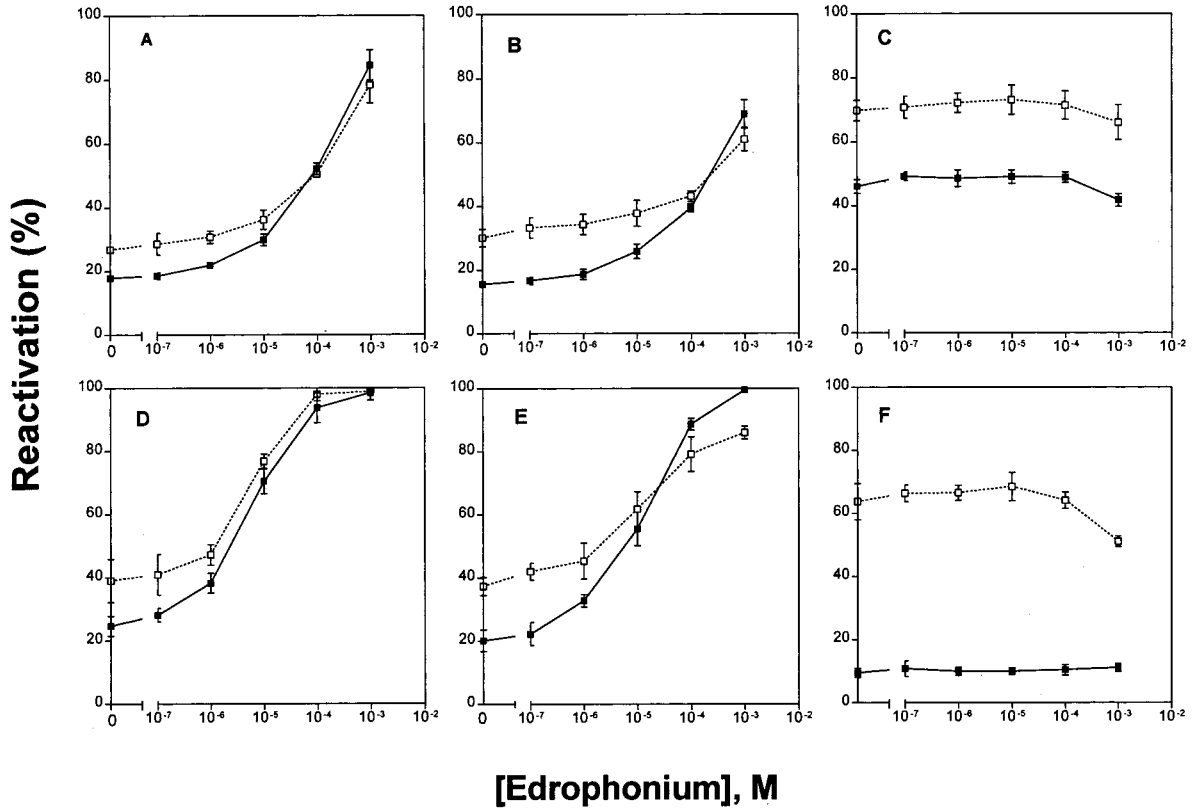


FIGURE 1: Concentration dependence of edrophonium on oxime-induced reactivation of EMP− and DEP−AChE. AChE (0.2 μM) was inhibited using stoichiometric amounts of MEPQ and DEPQ. Reactivation was carried out in 50 mM $NaPO_4$ buffer at pH 8.0 and 25 °C by 0.1 mM oximes in 0.1 μM conjugated AChE. FBS AChE (■) and MoAChE (□). (Upper panels) EMP−AChE conjugate and (lower panels) DEP−AChE conjugate. (A and D) Reactivation by LüH6 for 30 min, (B and E) reactivation by TMB4 for 30 min, and (C and F) reactivation by HI-6 for 15 min. Data are the mean ± SD of three experiments.

amount of racemic, HPLC-purified POX (>93% pure), and reactivation with HI-6 was performed exactly as described above for the reactivation of the MEPQ-inhibited enzyme.

^{31}P NMR Spectroscopy of Isolated POXs Formed during the Reactivation of the EMP−FBS AChE Conjugate. Isolation of POX formed during the reactivation of the EMP−FBS AChE conjugate was performed at 4 °C. Approximately 80 000 units of FBS AChE was adsorbed to a column containing approximately 3 mL of procainamide−Sepharose 4B affinity gel (0.6 cm × 3 cm). MEPQ (1 mM) in 4 mL of 6 mM MOPS buffer (pH 7.4) was used to inhibit AChE on the column for 30 min. Excess MEPQ was removed by washing the column with 6 mM MOPS buffer. Reactivation of the enzyme conjugate in the column was carried out using 4 mL of 6 mM MOPS buffer (pH 7.4) containing 1 mM oxime and 0.5 mM edrophonium. At 10 min intervals, fresh reactivation buffer was added to the column and the eluate was collected. The pH of the eluate was immediately lowered to 3.5 by adding 0.4 mL of 40 mM citric acid/sodium citrate buffer (pH 2.9) to 1 mL of eluate. Reactivation was nearly complete after 40 min. The procedure was repeated five times so a sufficient sample for each ^{31}P NMR measurement could be collected. The eluate (about 110 mL) was lyophilized to less than 1 mL, and the H_2O in the buffer was replaced with D_2O. ^{31}P NMR spectra of the POX isolated from the enzyme conjugate were measured using the same procedure as described for POX formed from the direct mixing of MEPQ and oxime, but overnight signal accumulation was required to obtain a good signal-to-noise ratio.

RESULTS

Concentration-Dependent Enhancement of Oxime-Induced Reactivation of EMP− and DEP−FBS AChE and Wild-Type MoAChE by Edrophonium. In our previous study, we examined ligand-induced acceleration of reactivation of the FBS AChE−MEPQ conjugate with edrophonium, decamethonium, and propidium, and showed that edrophonium significantly enhanced reactivation when LüH6 and TMB4, but not HI-6, were used as reactivators (*21*). In the study presented here, we examined the concentration dependencies of edrophonium-induced acceleration of reactivation using both FBS AChE and MoAChE inhibited with MEPQ and DEPQ. The effect of oxime and edrophonium on AChE activity measurements in the assay was minimized by diluting the reactivation mixture 200-fold with 50 mM $NaPO_4$ buffer (pH 8.0) containing 0.1% BSA. As shown in Figure 1, the concentration dependencies of edrophonium-induced acceleration of reactivation of EMP− and DEP−AChE were similar for both enzymes. With EMP−AChE, significant increases in the extent of reactivation were observed in the presence of 100 μM and 1 mM edrophonium when LüH6 and TMB4 were used as reactivators (Figure 1A,B). By contrast, edrophonium did not accelerate HI-6-induced reactivation. In fact, the level of reactivation was reduced considerably in the presence of 1 mM edrophonium. A significantly higher level of reactivation was observed with EMP−MoAChE than with EMP−FBS AChE with HI-6 as the reactivator (Figure 1C). Similar results were observed with DEP−AChE (Figure 1D−F). However, the acceleration

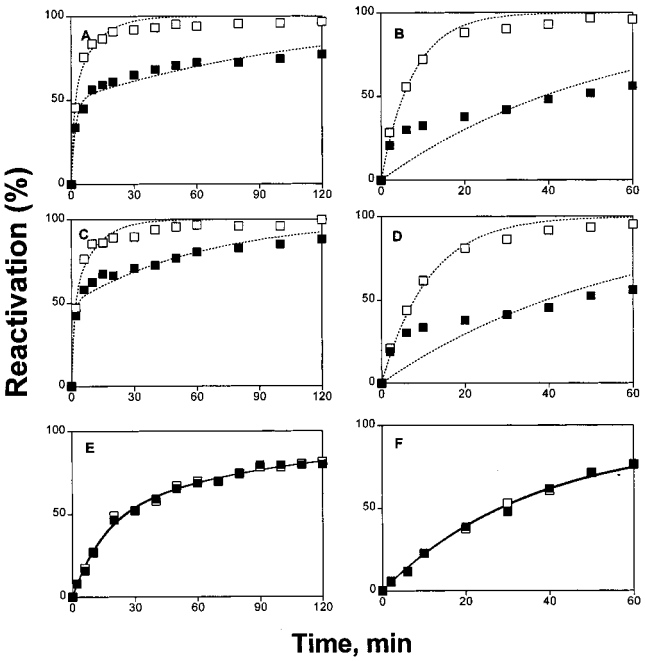


FIGURE 2: Oxime-induced reactivation of EMP− and DEP−MoAChE in the absence (■) and the presence (□) of 10 μM edrophonium. Reactivation was carried out in 50 mM $NaPO_4$ buffer at pH 8.0 and 25 °C using the enzyme conjugate at a concentration of 1.25 nM. (Left panels) EMP−MoAChE and (right panels) DEP−MoAChE. (A and B) Reactivation by 30 and 5 μM LüH6, respectively, (C and D) by 30 and 5 μM TMB4, respectively, and (E and F) by 50 μM HI-6. Data were fit (solid lines) or not fit (dotted lines) to exponential association equations and are representative of three or four experiments.

was evident at lower edrophonium concentrations with DEP−AChE than with EMP-AChE (Figure 1D,E). These results demonstrate that ligand-induced acceleration of reactivation is dependent primarily on the reactivating oximes and inhibiting OPs.

Effect of Edrophonium on the Reactivation Kinetics of EMP− and DEP−Wild-Type and D74N MoAChE. Reactivation kinetic curves of EMP− and DEP−wild-type MoAChE in the absence and presence of edrophonium are shown in Figure 2. In the presence of edrophonium, an increased level of reactivation was always observed for both EMP− and DEP−AChE when LüH6 and TMB4 were used as reactivators, but the curves for the time courses of reactivation were biphasic instead of the theoretical monophasic curves (Figure 2A−D). Biphasic kinetics observed with DEP−AChE were obvious both in the absence and in the presence of edrophonium but were not so apparent with EMP−AChE due to complication of the two-component kinetic model in the analysis. In the presence of 10 μM edrophonium, significant increases in reactivation were observed. This suggests formation of a putative POX in the reactivation medium, since edrophonium as a reversible inhibitor of the enzyme could protect the reactivated enzyme from inhibition by accumulating POX. With HI-6, the data for the reactivation time courses of both EMP− and DEP−AChE fit monophasic reactivation well, and the presence of edrophonium did not affect the reactivation kinetics, indicating that POX, if it accumulated, did not influence the reactivation kinetics (Figure 2E,F).

Since putative POXs are cationic OPs and it has been shown that D74 is a primary determinant for the specificity

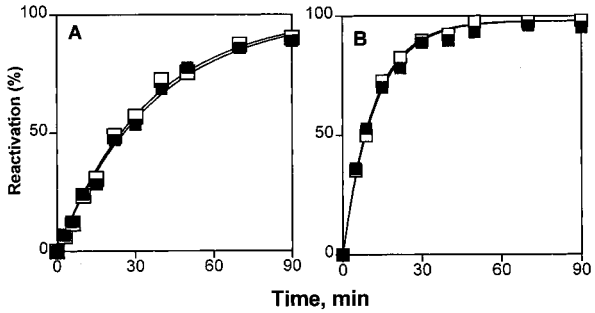


FIGURE 3: Oxime-induced reactivation of DEP-D74N MoAChE in the absence (■) and presence (□) of 10 μM edrophonium. D74N MoAChE was inhibited by incubating the mutant enzyme with excess DEPQ at pH 8.0 for 60 min at 25 °C. Excess DEPQ was removed by dialysis overnight at 4 °C against 50 mM $NaPO_4$ buffer at pH 8.0. Reactivation was carried out in 50 mM $NaPO_4$ buffer at pH 8.0 and 25 °C using the enzyme conjugate at a concentration of 1.25 nM: (A) reactivation by 0.5 mM LüH6 and (B) reactivation by 0.5 mM TMB4. Data were fit to the one-phase exponential association equation and are representatives of three experiments.

of the enzyme for positively charged OPs (*31*), we conducted reactivation studies with D74N MoAChE inhibited by DEPQ to show that this mutant was less sensitive to inhibition by POX than the wild-type enzyme. The time courses for reactivation of D74N MoAChE shown in Figure 3 support this hypothesis since the reactivation data fit a monophasic kinetic model well and no acceleration of LüH6- or TMB4-induced reactivation was observed in the presence of edrophonium. These results further support POX accumulation and inhibition of reactivated enzyme during reactivation with LüH6 and TMB4, and edrophonium accelerates reactivation by preventing POX inhibition of the reactivated enzyme.

^{31}P NMR Spectroscopy of POXs Formed by the Chemical Reaction of MEPQ with Oximes and Purification of POXs by HPLC. A POX generated by mixing LüH6 with sarin with a $t_{1/2}$ of 13.3 min was readily detected by ^{31}P NMR spectroscopy (*28*). Therefore, we measured the ^{31}P NMR spectra of mixtures of MEPQ with oximes. When MEPQ and LüH6 were mixed at a ratio of 1:10, the MEPQ ^{31}P resonance signal completely disappeared within 3 min, and a new resonance signal appeared downfield at about 41.6 ppm (POX) in addition to the organophosphoric acid signal (27.6 ppm) (Figure 4C). The ^{31}P NMR spectrum of the mixture of MEPQ and TMB4 also exhibited POX resonance at about the same position (41.4 ppm), but the organophosphoric acid signal was much smaller, suggesting that the decomposition of this POX is slower than that of the POX formed between MEPQ and LüH6 (Figure 4D). When MEPQ was mixed with HI-6, no signal with a frequency close to the POX position was observed; only the organophosphoric acid signal appeared on the ^{31}P NMR spectrum (Figure 4E). At neutral pH, the new signals at 41.6 ppm disappeared within 1 h at room temperature. However, when the pH of the mixtures was lowered to 3.5, resonances with amplitudes of at least half of the original value were evident even after 26 h at room temperature (not shown). This result further supports the fact that the new compounds that are stable at low pH are indeed POXs formed by reaction of MEPQ with LüH6 and TMB4 (named EMP−LüH6 and EMP−TMB4, respectively).

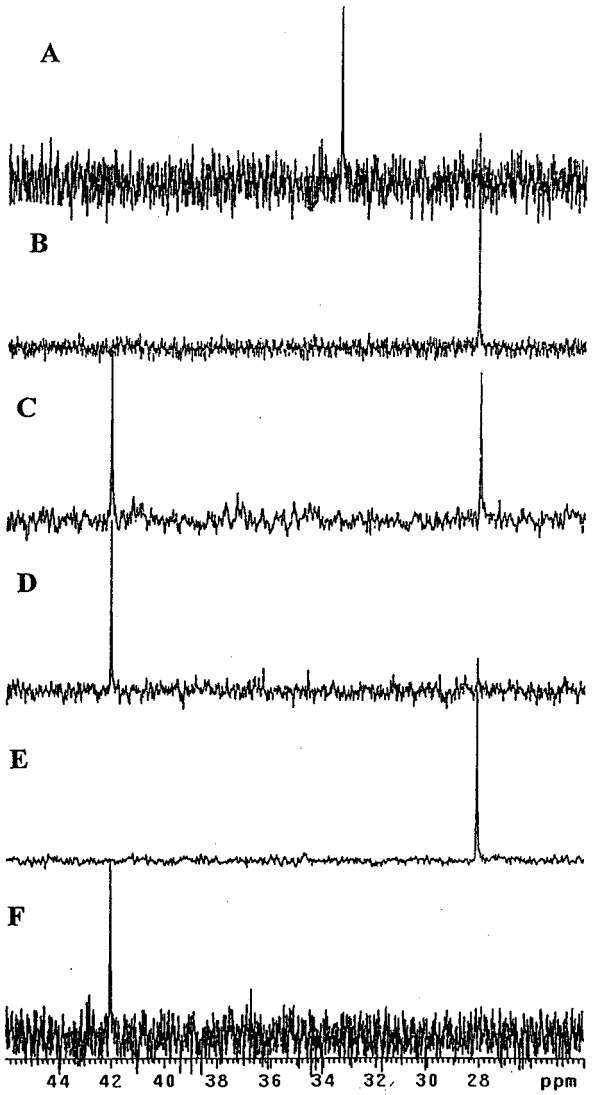


FIGURE 4: ^{31}P NMR spectra of mixtures of MEPQ with different oximes. (A) A strong signal at 33.1 ppm for MEPQ and a small signal at 27.6 ppm for hydrolyzed MEPQ (organophosphoric acid) were present in the sample. (B) MEPQ was completely hydrolyzed by 10 mM NaOH before measurement. (C) A new signal appeared at 41.6 ppm showing the formation of EMP−LüH6. (D) A new signal appeared at 41.4 ppm showing the formation of EMP−TMB4. (E) A signal appeared for MEPQ with HI-6, showing that all MEPQ was converted to the organophosphoric acid. (F) A signal appeared for HPLC-purified EMP−LüH6, showing the disappearance of the organophosphoric acid signal (27.6 ppm).

HPLC analysis of the mixture of MEPQ and LüH6 showed the appearance of a peak with a retention time of 13 min behind the major oxime peak (retention time of 9 min) at 280 nm (Figure 5). The new peak at 13 min showed a slight shift in the absorption spectra compared to LüH6 (Figure 5, inset), corresponding to the spectrum change reported previously for POX formed between sarin and LüH6 (*28*). The ^{31}P NMR spectrum of this peak from HPLC exhibited only a single resonance signal at 41.6 ppm (Figure 4F), demonstrating that HPLC separated the POX from the reactants and hydrolysis products. Similar results were observed with HPLC analysis of the MEPQ and TMB4 mixture, indicating a common POX structure (not shown). ^{31}P NMR spectra of the mixture of these two oximes and DEPQ or paraoxon (an analogue of DEPQ also yielding a diethyl phosphoryl enzyme or phosphoryl oxime) did not reveal resonances characteristic of diethyl phosphoryl LüH6 or diethyl phosphoryl TMB4, suggesting that these POXs, if formed, were not stable. Therefore, no attempt was made to isolate POXs formed in the mixtures of DEPQ with oximes.

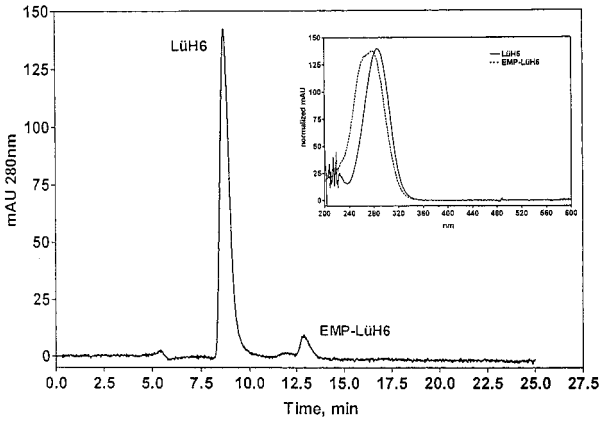


FIGURE 5: HPLC analysis of the mixture of MEPQ with LüH6. POX formed by direct chemical reaction was examined by RP-HPLC as described in Materials and Methods. The major peak at a retention time of 9 min was identified as the original LüH6 by comparing it with a standard sample. The new peak at a retention time of 13 min was identified as EMP−LüH6 by examining the spectrum of the peak. MEPQ and 7-HQ did not exhibit any appreciable absorbance at 280 nm. The inset shows a comparison of the spectra of LüH6 and EMP−LüH6 showing the hypsochromic shift.

Table 1: Hydrolysis Rate Constants (k_h) of MEPQ and POXs in Different Buffers[a]

	k_h ($\times 10^{-3}$ min^{-1})		
buffer	MEPQ	EMP−LüH6	EMP−TMB4
20 mM HEPES (pH 7.2)	0.21 ± 0.05 (3300)	4.1 ± 0.5 (169)	2.2 ± 0.1 (315)
50 mM HEPES (pH 8.0)	0.81 ± 0.03 (855)	53.9 ± 5.5 (13)	36.1 ± 4.4 (19)
20 mM phosphate (pH 7.2)	2.4 ± 0.1 (288)	9.7 ± 1.7 (71)	5.2 ± 0.3 (133)
50 mM phosphate (pH 8.0)	7.8 ± 1.3 (89)	26.7 ± 4.2 (26)	17.4 ± 0.5 (39)

[a] Data are the mean ± standard errors of three typical determinations, and values in parentheses are the half-life times of hydrolysis in minutes.

Stability of POXs and the Bimolecular Rate Constants for the Inhibition of AChEs by EMP−LüH6 and EMP−TMB4. HPLC-purified EMP−LüH6 and EMP−TMB4 were used to determine their hydrolysis rate constants (k_h) in different buffers and the second-order inhibition constants (k_i) for the AChEs. The hydrolysis rate constant, k_h, of MEPQ in 50 mM $NaPO_4$ buffer (pH 8.0) was 0.0078 min^{-1} (Table 1), which is close to the value (0.01 min^{-1}) reported previously at this pH (*31*). Lowering the pH of $NaPO_4$ buffer to 7.2 significantly stabilized MEPQ where the $t_{1/2}$ increased by 3.2-fold. Some differences were noticed in HEPES buffer. In 20 mM HEPES buffer (pH 7.2), EMP−LüH6 and EMP−TMB4 were very stable with $t_{1/2}$ values of 169 and 315 min, respectively. But in 50 mM HEPES buffer (pH 8.0), they are even less stable than in 50 mM $NaPO_4$ buffer at the same pH. The k_i values of these two POXs for FBS AChE and wild-type and D74N MoAChE were about 10−22-fold higher than that of the parent compound MEPQ. Mutation of D74 to N in MoAChE yielded k_i values 44- and 60-fold lower than that of the wild-type enzyme (Table 2).

Table 2: Bimolecular Rate Constants (k_i) for the Inhibition of AChEs by MEPQ and POXs[a]

	k_i ($\times 10^8$ M^{-1} min^{-1})		
enzyme	MEPQ	EMP−LüH6	EMP−TMB4
MoAChE	5.91 ± 0.47 (1)	130.0 ± 19.0 (22.0)	91.2 ± 3.2 (15.4)
FBS AChE	4.89 ± 0.18 (1)	75.9 ± 8.9 (15.5)	45.7 ± 2.2 (9.3)
D74N MoAChE	0.11 ± 0.01 (1)	2.17 ± 0.16 (19.7)	2.08 ± 0.11 (18.9)

[a] All k_i values measured in 20 mM HEPES buffer (pH 7.2) containing 0.1% BSA; data are the mean ± standard errors of three typical determinations, and values in parentheses are relative potencies for the inhibition of different enzymes with MEPQ having a value of 1.

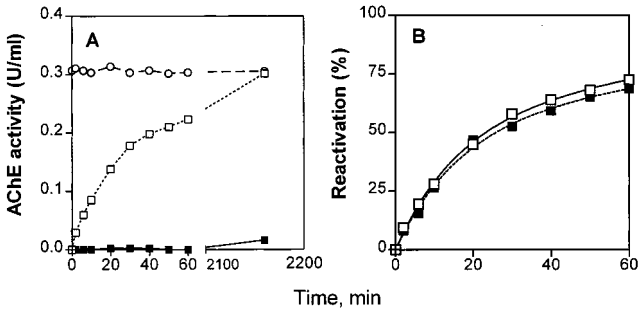


FIGURE 6: Reactivation kinetics of EMP−TMB4-inhibited wild-type MoAChE with HI-6. Reactivation was carried out under the same conditions as described in the legend of Figure 2. (A) Time courses of reactivation of EMP−TMB4-inhibited AChE (1.25 nM) with 50 μM HI-6: (○) uninhibited AChE, (■) AChE inhibited by EMP−TMB4 (prepared by mixing MEPQ with excess TMB4 and purified by HPLC) in the absence of HI-6, and (□) AChE inhibited by EMP−TMB4 in the presence of HI-6. (B) Comparison of HI-6-induced reactivation kinetics of AChE inhibited by EMP−TMB4 (□) with those of AChE inhibited by MEPQ (■), with the data fit to the two-component exponential association equation.

Reactivation Kinetics of EMP−TMB4-Inhibited Wild-Type MoAChE. As shown in Figure 6, the reactivation kinetics of EMP−TMB4-inhibited wild-type MoAChE with 50 μM HI-6 were identical to those of the MEPQ-inhibited enzyme. In the absence of HI-6, the EMP−TMB4-inhibited enzyme did not recover significant activity over 36 h, suggesting that the binding of POX to AChE is not reversible.

Formation of POXs during the Oxime-Induced Reactivation of EMP−FBS AChE. Since EMP−LüH6 and EMP−TMB4 are stable even at neutral pH, we attempted to isolate POXs from the reactivation of the FBS AChE−MEPQ conjugate by oximes and confirm their structures using ^{31}P NMR spectroscopy. In the reactivation medium, free POX interacts with the reactivated enzyme to form inhibited enzyme. Edrophonium, a reversible inhibitor, was added to the reactivation medium to promote the accumulation of free POX. The procainamide affinity gel column-adsorbed FBS AChE enabled the separation of reactivation medium from the column in less than 2 min. After overnight scanning of the concentrated sample from the reactivation of the EMP−AChE conjugate by LüH6 and TMB4, the ^{31}P NMR spectra exhibited signals of EMP−LüH6 and EMP−TMB4 at about 41.6 ppm and a smaller signal at 27.6 ppm (organophosphoric acid) (Figure 7B,D). An additional 24 h after the first overnight scan, the EMP−LüH6 signal diminished and the organophosphoric acid signal increased, confirming the gradual decomposition of POX even at low pH (Figure 7C). When the same procedure was used for the ^{31}P NMR measurement of POX from the reactivation medium of enzyme conjugate with HI-6 as the reactivator, there was no POX signal and only the organophosphoric acid signal was observed (Figure 7E).

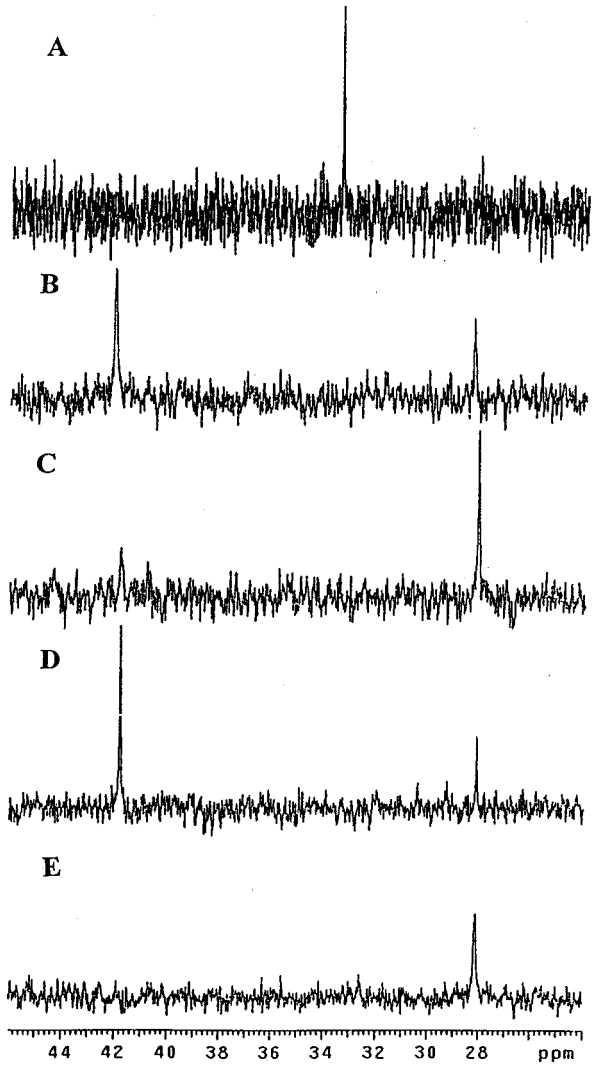


FIGURE 7: ^{31}P NMR signals of samples from reactivation eluates of the EMP−FBS AChE conjugate. (A) MEPQ. (B) Reactivation by LüH6, with a major peak at 41.6 ppm showing EMP−LüH6 still in the reactivation medium. (C) Second overnight scanning 24 h after the first overnight scanning of sample B, showing the decomposition of EMP−LüH6 at low pH. (D) Reactivation by TMB4, showing that EMP−TMB4 is the major peak in the reactivation medium. (E) Reactivation by HI-6, with only one signal at 27.6 ppm showing that all the OP conjugate was converted to organophosphoric acid.

Effect of PON on the Oxime-Induced Reactivation Kinetics of EMP− and DEP−MoAChE. Organophosphate hydrolases (OPH) have been shown to be able to hydrolyze charged OP DEPQ (*32*). If OPH can catalyze POX hydrolysis, then reactivation by oxime may be accelerated in its presence due to the continuous elimination of POX. Therefore, the effects of rabbit serum PON (one of the enzymes that hydrolyze OPs) on oxime-induced reactivation of EMP− and DEP−MoAChE were examined. The reactivation kinetics showed that reactivation rates of LüH6 and TMB4 for reactivation of EMP−MoAChE were increased in the presence of 0.01 unit/mL rabbit serum PON (Figure 8A,C). Increases in the extent of reactivation were more pronounced for DEP−AChE when the same concentration of PON was present (Figure 8B,D). This may be due to the possibility that PON hydrolyzes only one of the two diastereomers of the POX

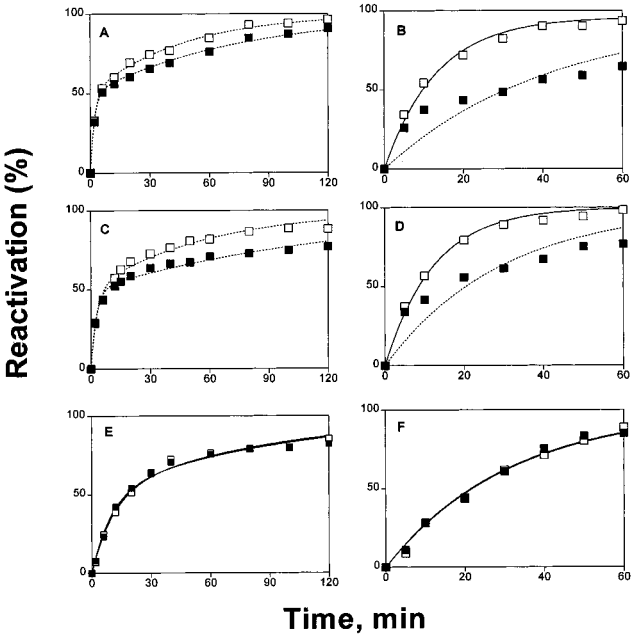


FIGURE 8: Acceleration of oxime-induced reactivation of EMP− and DEP−MoAChE in the absence (■) and presence (□) of 0.01 unit/mL rabbit serum PON. Reactivation was carried out at 25 °C in 50 mM HEPES buffer (pH 8.0) containing 1 mM $CaCl_2$ and 0.1% BSA. The initial enzyme conjugate concentration of 1.25 nM was used. (Left panels) EMP−MoAChE and (right panels) DEP−MoAChE. (A and B) Reactivation by 30 and 5 μM LüH6, respectively, (C and D) reactivation by 30 and 5 μM TMB4, respectively, and (E and F) reactivation by 50 μM HI-6. Data were fit (solid lines) or not fit (dotted lines) to exponential association equations and are representative of three experiments.

formed by EMP−AChE. In fact, acceleration of reactivation by PON was evident only when more than 50% of the EMP−MoAChE was reactivated, suggesting that only the POX formed by the slow reactivation component of the enzyme conjugate could be hydrolyzed by rabbit serum PON. When HI-6 was used as the reactivator, no change in reactivation rate was observed in the presence of PON for reactivation of both EMP− and DEP−MoAChE (Figure 8E,F). These results further support the conclusion from acceleration of reactivation by edrophonium that POX inhibition occurs only during reactivation with LüH6 and TMB4, but not with HI-6.

DISCUSSION

Oximes have been used in the treatment of OP poisoning for several decades to reactivate OP-inhibited AChE. Although in vitro reactivation studies indicated that oximes, such as LüH6, TMB4, 2-PAM, and MMB4, might form unstable POXs with OP−AChE conjugates and cause inhibition of the newly reactivated enzyme, the presence of POX in the reactivation medium had not been confirmed (*14*−*20*). In our previous study with EMP−AChE, ligand-induced accelerations of reactivation were shown to be due to the protection of the reactivated enzyme from POX inhibition. Because of the complication arising from the formation of two reactivatable species of EMP−AChE from the chiral (*R*,*S*)-MEPQ, the biphasic nature of the reactivation kinetics with oximes was not identified (*21*). However, greater increases in the second-order reactivation rate constants were observed in the presence of these ligands with the slow reactivation component than with the fast-reactivation component. In this study using both EMP− and DEP−wild-type and D74N MoAChE, we have established the relationship between ligand-induced acceleration of reactivation and POX inhibition of the reactivated enzyme. We also provide here the first characterization of POX formed during the reactivation of EMP−FBS AChE by LüH6 and TMB4.

The concentration dependence of edrophonium on reactivation of EMP− and DEP−FBS AChE and MoAChE showed that edrophonium accelerated LüH6 and TMB4 reactivation. However, when HI-6 was used as the reactivator, edrophonium did not accelerate reactivation. The reduced reactivation at an edrophonium concentration of 1 mM reflects competition between edrophonium and HI-6 for the active-site gorge of the enzyme conjugate, in agreement with the 1000-fold higher K_i value of edrophonium for phosphonylated AChE compared to that for the free enzyme (*33*). The significantly lower extent of reactivation of EMP− and DEP−FBS AChE compared with those of the corresponding MoAChE conjugates by HI-6 may reflect species differences for HI-6 binding (Figure 1E,F). A study by Grosfeld et al. (*34*) showed that the binding affinity of HI-6 is 50-fold lower than that of TMB4 for DEP−wild-type human AChE, which indicated that HI-6 has a low affinity for certain mammalian AChE−OP conjugates.

There are three possible explanations for the lack of POX inhibition observed when HI-6 was used as the reactivator: (1) no POX was formed; (2) POX was formed, but it was not sufficiently stable to accumulate and inhibit the enzyme during reactivation; and (3) stable POX was formed, but it is a poor inhibitor of AChE. To delineate these possibilities, we compared the ^{31}P NMR spectra of mixtures of MEPQ with the three oximes. MEPQ mixed with LüH6 or TMB4 showed that the main ^{31}P resonance signals were due to POXs, but the mixture of MEPQ and HI-6 exhibited no POX signal, indicating that this POX was unstable, if it formed. Due to the structural similarity and the capacity for reactivation of HI-6 with LüH6 and TMB4, they should undergo a similar reaction with the same OP. Therefore, a possible answer is that the POX formed by this oxime is too labile to allow it to accumulate and inhibit the reactivated enzyme. Determination of the inhibition constants of EMP−LüH6 and EMP−TMB4 showed that they are very powerful inhibitors of MoAChE with k_i values of approximately 10^{10} M^{-1} min^{-1}. This indicates that, at the conjugate concentration of 1.25 nM used in the reactivation study, if 20% of the conjugate generated POX, half of the reactivated enzyme would be inhibited by POX within 10−20 s. If it is assumed that the POX formed during reactivation by HI-6 was as potent as the two POXs described above in inhibiting AChE, it is estimated that this POX decomposed in less than 1 s, since no POX inhibition was observed.

A study by Hosea et al. (*30*) showed that D74N MoAChE was 2−3 orders of magnitude less sensitive to inhibition by charged OPs than the wild-type enzyme. Since POXs are also positively charged OPs with a bulky cationic moiety, they are expected to exhibit similar behavior. Reactivation studies of DEP−D74N MoAChE with LüH6 and TMB4 showed that the reactivation curves were monophasic in all cases, and edrophonium did not affect the reactivation kinetics, further substantiating the involvement of POX inhibition in edrophonium acceleration of reactivation ob-

served with the wild-type enzyme. The lack of edrophonium acceleration and POX inhibition during the reactivation of DEP−D74N MoAChE is not due to the absence of POX but rather to the decreased sensitivity of the mutant enzyme to inhibition by POX.

Inhibition constants determined with purified EMP−LüH6 and EMP−TMB4 showed that the two POXs are 10−22 times more potent than the parent OP (MEPQ) in inhibiting MoAChE or FBS AChE. The results support the contention from reactivation kinetic studies that POXs are 10−100 times more potent inhibitors of AChE than parent OPs (*16*−*19*). The 44−60-fold decrease in k_i values of EMP−LüH6 and EMP−TMB4 for D74N MoAChE compared to that of the wild-type enzyme is smaller than the 100−1000-fold decreases which were observed by Hosea et al. (*30*) using OPs with a thiocholine leaving group. The bis-pyridinium rings of EMP−LüH6 and EMP−TMB4 exhibit enhanced charge delocalization and accordingly would be less sensitive to removal of a single negative charge. Similarly, the potency of MEPQ, a cationic OP with a quinolinium leaving group, was decreased only 54-fold after the D74N mutation.

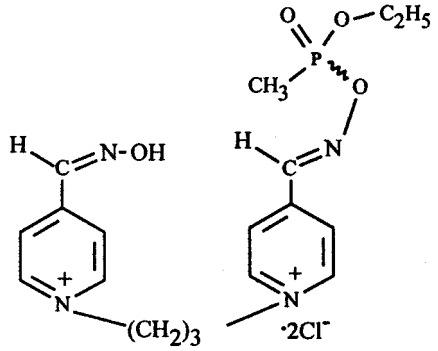
If the inhibition of AChE by POX results in the formation of covalent complex like with other OPs, then the conjugate formed by interaction of POX with AChE should have the same reactivation kinetics as the MEPQ-inhibited enzyme. Examination of the reactivation kinetics of EMP−TMB4-inhibited MoAChE using HI-6 as a reactivator that does not form a stable POX showed that the reactivation kinetics of this conjugate were identical to those of the MEPQ-inhibited enzyme, suggesting that POX inhibits the enzyme by covalently bonding to the active-site serine. Therefore, the following mechanism describes the role of POX in controlling oxime-induced reactivation of the AChE conjugate:

$$\text{E}-\text{OP} + \text{oxime} \rightleftharpoons [\text{E}-\text{OP}\cdot\text{oxime}] \rightleftharpoons \text{E} + \text{POX} \rightarrow \text{decomposed products of POX}$$

According to the scheme, as reactivation proceeds, it results in the accumulation of POX in the reactivation medium such that a steady state is established between the reactants (enzyme conjugate, E−OP, and oxime) and the products (free enzyme, E, and POX). Once this steady state is established, reactivation of the enzyme conjugate will not be evident until appreciable POX decomposes and shifts the steady state to drive the reactivation reaction. Therefore, the formation of stable POX results in the deceleration of the reactivation process due to the inhibition of the reactivated enzyme by POX.

Measurements of the k_h values of EMP−LüH6 and EMP−TMB4 showed that their decomposition rates differ with the buffer. In HEPES buffer (pH 7.2), they have $t_{1/2}$ values of 3 and 5 h; this stability was surprising since POXs were believed to be unstable intermediates formed during reactivation. At higher pH or in $NaPO_4$ buffer, the stability of EMP−LüH6 and EMP−TMB4 decreased significantly (Table 1). Although the inhibition potency of EMP−LüH6 and EMP−TMB4 for D74N MoAChE was decreased considerably compared with that of the wild-type enzyme, the k_i values for this mutant are still very high ($>2.0 \times 10^8$ M^{-1} min^{-1}). At the conjugate concentration of 1.25 nM, inhibition of the reactivated D74N MoAChE is still possible, since the half-life of POXs is greater than 20 min. On the other hand, POXs formed during the reactivation of DEP−AChE by LüH6 or TMB4 are much less stable than EMP−LüH6 and EMP−TMB4. Therefore, even if these POXs are as potent as EMP−LüH6 and EMP−TMB4 in inhibiting D74N MoAChE, their short lifetime precludes them from inhibiting the reactivated enzyme. MEPQ is a phosphonate, and the POXs it forms with LüH6 and TMB4 (EMP−LüH6 and EMP−TMB4) are stable. However, the POXs formed by the same oximes with DEPQ (a phosphorate) are less stable. These results suggest that nerve agents such as sarin, soman, and VX, which are all phosphonates, are likely to form stable POXs with LüH6 and TMB4 and inhibit the reactivated enzyme. This provide an explanation for why reversible ligands, decamethonium and SAD 128, accelerate reactivation of sarin-inhibited AChE when present in the reactivation medium (*35*).

The results with wild-type and D74N MoAChE suggest that edrophonium acceleration is due to the prevention of POX inhibition of the reactivated enzyme when LüH6 and TMB4 were used as reactivators. When HI-6 was used as the reactivator, no evidence of POX inhibition during the reactivation was observed either from the kinetic curves or from edrophonium acceleration. ^{31}P NMR measurement of POX signals in the mixture of MEPQ with these three oximes showed that only LüH6 and TMB4 formed stable POXs, a finding that supports results from the enzyme studies. Most importantly, POXs formed during the reactivation of EMP−AChE by LüH6 and TMB4 were isolated from the concentrated reactivation medium, and shown to be identical to the POX signals in the MEPQ−oxime mixtures by ^{31}P NMR spectroscopy. Attempts were made to determine the structure of POXs by electron-spray mass spectrometry analysis, and it was found that they decomposed during evaporation even when the lowest temperature was used. However, the structure of EMP−TMB4 formed from the MEPQ−TMB4 reaction mixture was determined by ^{1}H NMR spectrometry (manuscript in preparation) and is shown below:



EMP-TMB4

This is the structure expected for monophosphonylated TMB4. On the basis of the initial ^{1}H NMR observations and the ^{31}P NMR data, the structure of EMP−LüH6 from the MEPQ−LüH6 mixture and enzyme-generated POXs should also correspond to monophosphonylated oximes. This provides the first direct identification of POX in the reactivation medium of AChE, though its existence was predicted more than 40 years ago by Wilson et al. (*14*) and Hackley et al. (*15*) using different OPs and 2-PAM. The resistance of POX formation with HI-6 might arise from the oxime being ortho to the pyridinium nitrogen. A near neighbor attack through

formation of a six-membered ring may contribute to the instability. One, however, would also predict instability of POX formed by 2-PAM.

The role of POX accumulation and inhibition in vivo is not clear yet. However, results of this study showed that POXs formed by LüH6 and TMB4 should be extremely toxic if they are not removed and allowed to accumulate. The finding that rabbit serum PON catalyzes the hydrolysis of POX suggests that other factors may affect POX disposition in vivo. PON activity is high in serum or liver in most mammals, including humans (*36*, *37*). Although highly toxic POXs may be produced in vivo when certain oximes, such as LüH6 and TMB4, are administered to the body, hydrolysis catalyzed by PON will eliminate these POXs. In addition, poor penetration of oxime into the brain will also reduce the toxic effect of POX, since POX should only be formed outside the central nervous system and will not cross the blood−brain barrier. Therefore, if POX was to inhibit AChE, it would be formed at local sites not accessible to PON in the plasma. Hence, it is extremely unlikely that POXs would survive after entry into the circulation.

When large amounts of OP are present in vivo (such as in a megadose pesticide suicide), the POX formed by direct reaction of OP with oxime may accumulate fast enough to saturate PON activity and manifest its toxic effect. This may be the reason for the unexplained peripheral paralysis of respiratory muscle, the primary cause of death in the Intermediate Syndrome (IMS) observed during the treatment of poisoning by some OP pesticides and often correlated with a sharp drop of cholinesterase activity in the blood when it occurred (*38*−*41*). Therefore, oximes, such as LüH6 and TMB4, that may form relatively stable POXs and produce inhibition of reactivated enzyme during reactivation in vitro as confirmed by this study, or 2-PAM and MMB4, as indicated by several former studies (*15*−*18*, *20*, *21*), should be used with caution during the treatment of OP pesticide poisoning. On the other hand, oximes that do not form stable POXs, such as HI-6, may be good candidates in these situations. The formation of POX during reactivation by some oximes poses a very important consideration both in vitro for comparing the reactivation potency of different oximes and in vivo in choosing a proper oxime for the treatment of OP pesticide poisoning.

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