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20. Abstract

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Differences in Conformational Stability between Native and Phosphorylated Acetylcholinesterase As Evidenced by a Monoclonal Antibody†

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ABSTRACT: Monoclonal antibody 25B1 generated against diisopropyl phosphorofluoridate inhibited fetal bovine serum acetylcholinesterase has been extensively characterized with respect to its anticholinesterase properties. This antibody demonstrated considerably different properties from previously reported inhibitory antibodies raised against acetylcholinesterase in terms of the degree of inhibition (>98%), the high degree of specificity, and the stability of the antigen–antibody complex. Monoclonal antibody 25B1 appears to be directed against a conformational epitope located in close proximity to the catalytic center of the enzyme and was found to be most suitable for studying the stabilization of the active site of acetylcholinesterase against denaturation by heat or guanidine following phosphorylation by organophosphorus anticholinesterase compounds. This approach allowed the determination of stability rank order of various phosphorylated acetylcholinesterases. Among all the organophosphates tested, the combination of a methyl group and a negatively charged oxygen attached to the P atom, $CH_3P(O)(O^-)$–AChE, conferred the greatest protection to the active site of aged or nonaged organophosphoryl conjugates of acetylcholinesterase.

Protein domains which constitute the active site region(s) of both AChE[1] (EC 3.1.1.7) and BChE (EC 3.1.1.8) have been demonstrated to be conformationally less stable than other segments of the protein (Ahmad, 1981; Masson & Goasdoue, 1986; Wu et al., 1987). Ahmad (1981) and Wu et al. (1987) demonstrated that environmental perturbants (urea, Gdn, pH, temperature) caused complete loss of enzyme activity of various forms of AChE (G_2, G_4, A_{12}) from either eel or *Torpedo californica* faster than changes observed in the secondary structure of the enzymes. Studies by Masson and Goasdoue (1986) revealed that although enzyme activity of both normal and atypical human plasma BChE was completely lost at 2.9 and 1.5 M urea, respectively, only 15–35% of protein unfolding occurred. High sensitivity of both AChE and BChE enzymic activities to various environmental changes implies that ligands directed at sites involved in the catalytic machinery of the enzyme affect the denaturation process. Indeed, reversible inhibitors of AChE and BChE (e.g., edrophonium, decamethonium, procainamide) conferred significant protection against thermal inactivation of both enzymes, with rank order parallel to that observed for inhibition of enzyme activity by the same inhibitors (Payne et al., 1989).

Previous studies have used CD and UV absorption spectroscopies to characterize changes in the secondary structure of AChE following its exposure to environmental perturbants (Ahmad, 1981; Wu et al., 1987). Use of fluorescent OP ligands in conjunction with CD spectroscopy has also found successful application in studying differences in conformational stability between aged (nonreactivatable) and nonaged OP–Cht conjugates (Steinberg et al., 1988). A second method of utilizing changes in protein mobility, transverse urea gradient electrophoresis, has been reported by Masson and Goasdoue (1986) to detect enhanced resistance to protein unfolding in aged OP–BChE conjugates.

The present study was undertaken to explore the use of a specific mAb for studying active site domain(s) of FBS-AChE and to elucidate by means of antigen–antibody binding measurements the extent to which various OP conjugates can increase resistance of the enzyme to denaturation. In this report we present results obtained by using a monoclonal antibody raised against DFP-inhibited FBS-AChE. This mAb appears to be directed against a conformational epitope near the active site of native and OP-inhibited enzyme. Phosphorylation of AChE by six OP ligands slightly but definitely lowered affinity of the mAb for the OP–AChE conjugate compared to native enzyme. Thermal and chemical perturbation of enzyme activity decreased binding of mAb 25B1, which could be correlated with decrease in activity of the perturbed enzyme. This mAb was found to be most suitable for studying regional changes near the active site of native and phosphorylated AChEs following exposure to external perturbants. Evidence is provided by antibody–antigen binding experiments that the aged conjugate E–$OP(O)(O^-)CH_3$ constitutes the most stable form among either native or several OP-inhibited AChEs toward various denaturing factors.

Materials and Methods

Methylphosphonic dichloride [$CH_3P(O)Cl_2$], diethyl chlorophosphate [$(C_2H_5O)_2P(O)Cl$], and ethyl dichlorophosphate [$(C_2H_5O)P(O)Cl_2$] were obtained from Aldrich. Diethyl *p*-nitrophenyl phosphate [$(C_2H_5O)_2P(O)(O\text{-}p\text{-}NO_2C_6H_4)$, paraoxon], DFP [$(iPrO)_2P(O)F$], and ethyl(*m*-hydroxy-

† A preliminary communication of this report was presented at the 1988 FASEB meetings, Las Vegas, NV (Ashani et al., 1988).

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[1] Abbreviations: AChE, acetylcholinesterase; FBS, fetal bovine serum; BChE, butyrylcholinesterase; OP, organophosphate; mAb, monoclonal antibody; DFP, diisopropyl phosphorofluoridate; 2-PAM, 1-methyl-2-[(hydroxyimino)methyl]pyridinium iodide; TMB_4, 1,1′-trimethylenebis-[4-(hydroxyiminomethyl)pyridinium] dibromide; DEP, diethylphosphoryl; MEPQ, 7-[(methylethoxyphosphinyl)oxy]-1-methylquinolinium iodide; Gdn, guanidine; Cht, chymotrypsin; CD, circular dichroism; 7-HQ, 7-hydroxy-1-methylquinolinium iodide.

phenyl)dimethylammonium chloride (edrophonium chloride) were purchased from Sigma. Tritiated DFP, 3.5 Ci/mmol, was supplied by Amersham (U.K.) in propylene glycol. MEPQ was prepared as described by Levy and Ashani (1986). Diluted solutions of pinacolyl methylphosphonofluoridate [$CH_3P(O)(O\text{-Pin})F$, soman] were used at the U.S. Army Institute of Chemical Defense, Edgewood, MD. 2,7-Diamino-9-phenyl-10-[(diethylamino)propryl]phenanthridinium iodide methiodide (propidium iodide) was obtained from Calbiochem. 2-PAM and TMB_4 were prepared according to Ginsburg and Wilson (1957) and Poziomek et al. (1958), respectively.

FBS-AChE (11*S*) was purified by affinity chromatography as described by De La Hoz et al. (1986). Specific activity of FBS-AChE was 400 ± 15 units/nmol of active site, with 1 mg of pure enzyme containing approximately 5600 units. AChE from *T. californica* (G_4) was purified according to Lee et al. (1982). Purified bovine caudate AChE (G_4) was obtained from Dr. J. Marquis (Boston University, Boston, MA). Purified AChE from bovine erythrocytes (G_2) and human erythrocytes (G_2) was a gift from Dr. T. Rosenberry (Western Reserve University, Cleveland, OH).

Monoclonal antibody 25B1 (IgG_1) was prepared by fusion of plasmacytoma cells (P3x63Ag8.653; Kearney et al., 1979) with spleen cells from mice immunized with DFP–FBS-AChE. The details of the fusion technique have been previously published (Gentry et al., 1982). Monoclonal antibody AE-2 was prepared by Fambrough et al. (1982), and the cell line secreting this antibody was obtained from the American Type Culture Collection. Both antibodies were amplified in mouse ascitic fluids and purified by protein A–Sepharose CL-4B by Charles River Biotechnical Services Inc. Fab fragments from purified mAb 25B1 were generated by using a Pierce ImmunoPure Fab kit.

AChE Activity Determinations. FBS-AChE activity and the kinetics of inhibition and reactivation were monitored spectrophotometrically by the method of Ellman et al. (1961). Measurements of enzyme activity of AChE attached to microtiter plates were carried out after extensive washing with phosphate-buffered saline by a modified Ellman procedure (Doctor et al., 1987).

Solid-Phase Immunoadsorbance Assay. Antigens were absorbed to flat-bottom 96-well ELISA plates in phosphate-buffered saline overnight at room temperature. Binding of antibody to antigen was detected with a horseradish peroxidase labeled goat antibody to mouse IgG + IgM (H+L). An automated microplate reader was used to read the absorbance of the colored substrate with a 405-nm filter.

Kinetics of AChE Inhibition by mAb 25B1. Standard stock solutions of mAb 25B1 (0.1–15 μM) were prepared in 50 mM phosphate (pH 8.0) and diluted at time zero into FBS-AChE solution (1–100 nM active site) in 2–50 mM phosphate buffer containing 0.01% BSA (pH 8.0). At selected time intervals 5–50 μL of sample was diluted (either directly or via intermediate dilution) into 3.2 mL of Ellman assay mixture for measurements of residual enzyme activity. Data for the time course of inhibition reactions were analyzed with a computer-assisted Marquardt nonlinear regression analysis using the equation, $\%\ \text{AChE} = A_1e^{-k_1t} + A_2e^{-k_2t}$.

Dissociation of the mAb·AChE Complex. Dissociation of enzyme activity from partial or complete mAb 25B1 complexed AChE was carried out by diluting complexed enzyme into 1.0 M Gdn·HCl containing 0.1 M phosphate buffer (pH 7.4–7.6). Increase in enzyme activity was monitored by diluting 5–50 μL of the Gdn·HCl–enzyme solution directly into 3.2 mL of Ellman assay mixture. SDS–polyacrylamide gel electrophoresis under nonreducing conditions was performed on a minigel (0.75-mm width) as described by Laemmeli (1970).

Titration of AChE. Titration of FBS-AChE was performed by monitoring residual activity of AChE solution (5–100 nM active site) incubated with increasing concentrations of either paraoxon, MEPQ, or mAb 25B1 over a range of 0.1–1.2 times the number of equivalents of enzyme in solution. Inhibition of AChE by paraoxon and mAb 25B1 was carried out for 20–24 h; for MEPQ, incubation time was 30–60 min.

Preparation of OP–AChE Conjugates. The following procedures were employed to obtain OP-AChE conjugates:

(a) $CH_3P(O)(O^-)$–AChE, $(C_2H_5O)P(O)(O^-)$–AChE, and $(C_2H_5O)_2P(O)$–AChE were obtained by dropwise addition (over a period of 10 min) of a fresh concentrated solution (0.01 M in acetonitrile) of either $CH_3P(O)Cl_2$, $(C_2H_5O)P(O)Cl_2$, or $(C_2H_5O)_2P(O)Cl$, respectively, to a solution of FBS-AChE (approximately 1 μM in 50 mM phosphate, pH 8.0) with stirring at room temperature. Decrease in enzymatic activity was monitored until 95–98% of the initial enzyme activity was inhibited. Organic solvent in the final inhibition medium did not exceed 2%.

(b) $CH_3P(O)(O^-)$–AChE, $CH_3P(O)(OC_2H_5)$–AChE, $(C_2H_5O)_2P(O)$–AChE, and $(iPrO)_2P(O)$–AChE were obtained by incubating approximately 1 μM AChE solution with a slightly substoichiometric amount of soman, MEPQ, paraoxon, and DFP, respectively. Decrease in enzyme activity indicated that 94–98% inhibition was achieved. An 87% aged form of $(iPrO)P(O)(O^-)$–AChE was obtained by incubating $(iPrO)_2P(O)$–AChE at pH 5.0 for 24 h at 25 °C.

None of the OP–AChEs contained residual OP inhibitors as evidenced by monitoring the activity of AChE freshly added to the phosphorylated enzyme preparations. Aging was determined by measuring increase in enzymic activity after 16-h incubation with 1 mM TMB_4 (50 mM phosphate, pH 8.0, 25 °C). All OP–AChE conjugates were diluted 20- to 200-fold into 50 mM phosphate buffer for further experiments.

[^3H]DFP Radiolabeling of Free and mAb-Bound AChE. One micromolar AChE and 0.7 μM mAb 25B1 or 2 μM mAb AE-2 were incubated for 16 h at 25 °C followed by addition of 1.5 μM [^{3}H]DFP (3.5 Ci/mmol). After 24 h at 25 °C, the amount of [^{3}H]DFP bound to free or antibody-complexed AChE was determined by gel filtration on a Bio-Rad P-6 column and measurement of radioactivity in the void volume (front peak) relative to the total eluted radioactivity (eluent: 50 mM Tris, pH 9.0). Displacement of radioactivity from protein-bound [^{3}H]DFP at either pH 5.0 (aging-induced reaction) or in the presence of 1 mM TBM_4 at pH 8.0 (reactivation-induced experiments) was also carried out by gel filtration as described above.

Fluorescence Spectroscopy. Fluorescence spectroscopy measurements were performed in 5 mM phosphate (pH 8.0) containing 2–3 μM AChE and 1–2 μM propidium iodide. Dissociation of propidium from AChE was initiated by addition of 1–2 μM mAb 25B1 to the propidium–AChE mixture. Release of the fluorescent chromophore 7-HQ from MEPQ was monitored in 5 mM phosphate, pH 8.0 (Levy & Ashani, 1986).

Affinity Measurements of mAb 25B1 for OP–AChE. Relative affinity of mAb 25B1 for OP-modified FBS-AChE was determined by measuring the effect of OP–AChE (0.01–0.03 μM) on the inhibition of an equimolar concentration of native AChE by 0.005–0.015 μM mAb 25B1. The control contained native enzyme and half the concentration

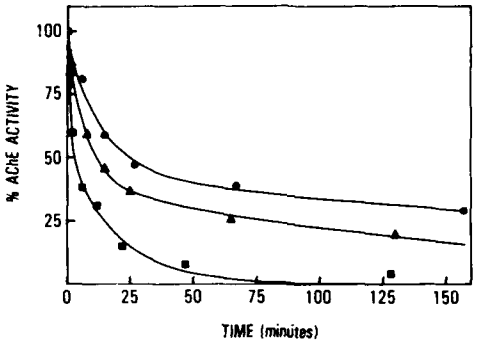

FIGURE 1: Effect of ionic strength on the rate of inhibition of FBS-AChE by mAb 25B1. AChE and 25B1 concentrations were 0.46×10^{-8} and 1×10^{-8} M, respectively (phosphate buffer, pH 8.0, 25 °C). Solid lines show the fit according to the equation % AChE = $A_1e^{-k_1t} + A_2e^{-k_2t}$: (■) 2 mM phosphate ($k_1 = 0.8$, $k_2 = 0.050$ min^{-1}); (▲) 50 mM phosphate ($k_1 = 0.13$, $k_2 = 0.006$ min^{-1}); (●) 2 N NaCl–50 mM phosphate ($k_1 = 0.072$, $k_2 = 0.0026$ min^{-1}).

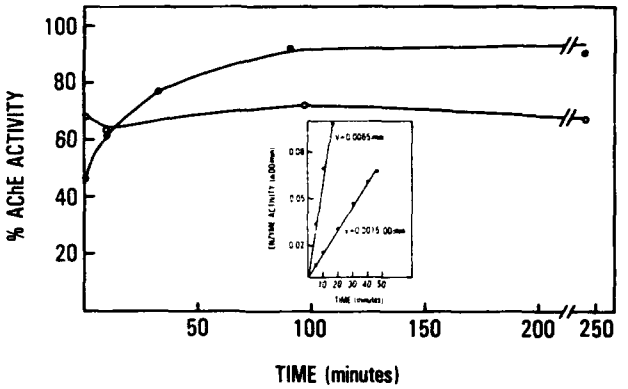

FIGURE 2: Effect of time on restoration of AChE activity following dilution of mAb-AChE complex. MAb-AChE conjugate was prepared by incubation at 25 °C for 20 h and diluted 1000-fold in 50 mM phosphate, pH 8.0, 25 °C. At specified time intervals, 50-μL aliquots were withdrawn for assay: (●) AE-2-AChE, final concentrations of AE-2 and AChE were 3.3×10^{-10} and 7.85×10^{-11} M, respectively; (○) 25B1-AChE, final concentrations of 25B1 and AChE were both 2.0×10^{-11} M. Inset: Effect of time of incubation (at 25 °C) on enzyme activity of 25B1-AChE conjugate in Ellman assay mixture (0.8 mM acetylthiocholine). Final concentrations were 1.0×10^{-12} and 1.48×10^{-12} M for 25B1 and AChE, respectively: (○) AChE; (●) 25B1-AChE.

of mAb 25B1 used for inhibition of the phosphorylated/native enzyme mixture. The extent of inhibition of AChE in the presence and absence of OP–AChE was used to calculate the affinity of mAb 25B1 for OP–AChE relative to native AChE.

Heat- and Gdn·HCl-Induced Denaturation of AChE. Thermal denaturation of AChE was carried out in a shaking water bath at either 50 or 54 ± 1 °C in 50 mM phosphate (pH 8.0). To correlate residual activity of denatured AChE with inhibition induced by mAb 25B1, samples were cooled to room temperature (20–25 °C) at various time intervals, assayed for enzyme activity, and incubated with a fixed amount of mAb 25B1 sufficient to inhibit 20–35% of preexperimental enzyme activity. Samples were allowed to incubate for 20 h at 25 °C, followed by measurement of residual activity.

Loss of binding of heat-treated OP–AChE to mAb 25B1 was determined by measuring unbound mAb 25B1 after 20 h of incubation of thermally treated OP–AChE with a substoichiometric amount of mAb 25B1. Unreacted mAb 25B1 was determined by adding a slight excess of fresh FBS-AChE to the OP–AChE/mAb 25B1 mixture and measuring enzyme activity after 20-h incubation at 25 °C.

The extent of loss of AChE activity upon denaturation with Gdn·HCl was determined as follows: Native or phosphorylated AChE was incubated at 25 °C in Gdn·HCl solutions over a concentration range of 0–2 M in 0.1 M phosphate buffer (pH 7.4–7.6). The samples were diluted 10-fold into 50 mM phosphate buffer (pH 8.0), and enzyme activity was measured as described above, by further diluting 5–50 μL into Ellman assay mixture. Loss of binding of mAb 25B1 to Gdn·HCl-induced denaturation of either native or OP–AChE was carried out in Gdn·HCl/enzyme diluted mixture, as described above for heat-denatured enzyme. All denaturation experiments were carried out concurrently with controls not subjected to denaturation.

Results

Kinetics of Inhibition of AChE by mAb 25B1. More than 98% of the enzymic activity of AChE from FBS (G_4), bovine erythrocytes (G_2), and bovine brain (G_4) was inhibited by mAb 25B1. Pseudo-first-order plots did not produce straight lines despite the presence of a large excess of mAb. A similar profile at a lower rate of inhibition was observed when AChE was incubated with excess purified 25B1 Fab fragments (not shown). To assess changes in the time course of enzyme inhibition by 25B1 under different experimental conditions, kinetic data were fitted to a biexponential decay equation (% AChE = $A_1e^{-k_1t} + A_2e^{-k_2t}$), as illustrated in Figure 1. Corresponding rate constants are referred to as average fast (k_1) and average slow (k_2) inhibition rate constants. These rate constants reflect a continuous decrease in the rate of inhibition of enzyme activity by 25B1 and are not claimed to represent two different populations of FBS-AChE. When k_1 and k_2 were plotted against the concentration of mAb 25B1 (mAb:AChE > 10), straight lines were obtained, and the following bimolecular rate constants for the inhibition of FBS-AChE by 25B1 were calculated from the corresponding slopes (50 mM phosphate, pH 8.0; 25 °C): $k_{fast} = 1.25 \times 10^7$ M^{-1} min^{-1} ($r^2 = 0.931$) and $k_{slow} = 0.07 \times 10^7$ M^{-1} min^{-1} ($r^2 = 0.956$).

The nonhomogeneous inhibition profile (i.e., deviation from linearity for first-order plots) for the inactivation of FBS-AChE by mAb 25B1 was found to be associated with an increase in the size of enzymatically active complex formed between AChE and mAb 25B1. Sucrose density gradient centrifugation profiles (not shown) revealed that when an amount of 25B1 sufficient to produce approximately 50% inhibition of the catalytic activity was added, more than 90% of the residual activity was bound to multiple molecular complexes heavier than unbound AChE. Increasing the ratio mAb:AChE was associated with an increase in the size of the enzymatically active complexes. Antibody 25B1 displayed high specificity and variable affinity toward AChEs from various bovine sources (e.g., brain, erythrocytes) but neither cross-reacted with nor inhibited AChE isolated from eel, *T. californica* (both G_4), human erythrocytes (G_2), or human serum BChE (G_4).

Dissociation of the mAb-AChE Complex. When 25B1-AChE complex was diluted extensively into buffer solutions (5–50 mM phosphate, pH 6–9) containing a wide range of NaCl concentration (0.01–2 N), no regeneration of enzyme activity could be detected for several hours at 25 °C, even at a concentration as low as 10^{-12} M of the diluted complex. Stability was independent of the time of incubation or mAb:AChE ratio. Residual enzyme activity (68%) in the supernatant was not changed after high-speed centrifugation (150000*g*, 2 h) of the mAb-AChE complex, ruling out aggregation as a possible explanation for the apparent stability of the complex. The effect of time on restoration of AChE activity in the absence or presence of acetylthiocholine is shown

Table I: Effect of the Presence of Propidium and Edrophonium on Inhibition Rate Constants of FBS-AChE by mAb 25B1

ligand	buffer[a] molarity (mM)	dissociation constants[b] (K_I, μM)		inhibition rate constants[c] (min^{-1}) by 0.05 μM 25B1	
		propidium	edrophonium	k_1	k_2
controls (AChE only)	5	0.35	0.11	3.30	0.36
controls (AChE only)	50	3.50[d]	0.19[d]	0.75	0.035
mAb-treated AChE[e]	50	3.96[d]	0.16[d]	0.41	0.023
propidium, 0.35 μM	5			1.70	0.11
propidium, 3.5 μM	5			0.80	0.05
propidium, 11.5 μM	5			0.54	0.04
edrophonium, 1.1 μM	5			3.20	0.42
propidium, 3.5 μM, + edrophonium, 1.1 μM	5			3.30	0.34

[a] Phosphate, pH 8.0. All reactions were carried out at 25 °C. [b] Determined from measurements of AChE residual activity in accordance with Hofstee plot. Noncompetitive and competitive inhibition curves were observed for propidium and edrophonium, respectively, in the presence of acetylthiocholine substrate. [c] Calculated from computer fit of experimental data to the equation: % AChE = $A_1e^{-k_1t} + A_2e^{-k_2t}$. See Figure 1. [d] Similar K_m values for acetylthiocholine were obtained for native enzyme (0.07 mM) and mAb-inhibited AChE at 75% level (0.06–0.07 mM). K_m was determined from Hofstee plot. [e] 75% inhibition of FBS-AChE enzyme activity was obtained after 20 h with sufficient amounts of mAb 25B1.

in Figure 2. Acetylthiocholine did not regenerate enzyme activity from mAb 25B1, nor did specific-site cationic ligands such as 2-PAM, TMB_4, propidium, and edrophonium (not shown). In contrast, mAb AE2 (Fambrough et al., 1982) raised against human erythrocyte AChE, which inhibited FBS-AChE, appeared to dissociate from the enzyme–antibody complex, as suggested by the restoration of enzyme activity (Figure 2).

In view of the stability of the 25B1·AChE complex, the possibility that a covalent bond might be involved in the formation of the conjugate was tested. Nonreducing SDS–PAGE patterns showed a dissociation of the mAb–enzyme complex, as evidenced by bands at molecular weights corresponding to mAb (150000) and FBS-AChE (dimer, 140000). In addition, an 80% restoration of enzyme activity could be demonstrated by transferring a completely inhibited mAb·AChE from 50 mM phosphate (pH 8.0) to 1.0 M Gdn·HCl solution (pH 7.5). Regeneration of enzyme activity was independent of the time of incubation of mAb 25B1 with FBS-AChE prior to dilution into Gdn·HCl. Sucrose density gradient centrifugation in 1.0 M Gdn·HCl for 17 h at 20 °C (24000 rpm) clearly demonstrated that regeneration of enzyme activity was accompanied with dissociation of mAb·AChE, while no such dissociation was observed at 1 M NaCl (not shown).

Stoichiometry of Inhibition of FBS-AChE by mAb 25B1. The stoichiometry of inhibition of FBS-AChE by mAb 25B1 was determined by measuring the amount of mAb 25B1 required to inhibit nearly 100% of enzyme activity. When enzyme was treated with increasing concentrations of either MEPQ or paraoxon and the residual activity determined after completion of reaction, similar titration curves were obtained for both inhibitors. However, the molar concentration of mAb 25B1 required for approximately 100% inhibition of enzyme activity was half the concentration required for complete inhibition by the OPs MEPQ or paraoxon, suggesting that 1 molecule of mAb inactivated approximately 2 active sites of FBS-AChE. By use of four preparations of mAb 25B1 it was calculated that the >98%-inhibited mAb·AChE complex contained an average of 2.2 ± 0.2 active site molecules of bound AChE per molecule of mAb.[2]

Effect of Cationic Ligands on the Kinetics of AChE Inhibition by mAb 25B1. Since AChE has a high affinity for certain positively charged ligands, the rate of inhibition of FBS-AChE activity by mAb 25B1 was determined in the presence of edrophonium and propidium, previously demonstrated to bind at the active site (Wilson & Quan, 1958; Taylor & Lappi, 1975) and at the peripheral anionic site (Taylor & Lappi, 1975) of AChE, respectively. Analysis of enzyme velocity data using a Hofstee plot (data not shown) revealed a competitive-type inhibition for edrophonium and a noncompetitive inhibition for propidium. The dissociation constants for edrophonium and propidium with respect to 75%-inhibited mAb·AChE conjugate were similar to those for the free enzyme (Table I), indicating that antibody-bound residual activity exhibited the same properties as unbound FBS-AChE, as far as binding of cationic ligands was concerned. This was consistent with the observation that K_m for acetylthiocholine was independent of the degree of enzyme inhibition by mAb 25B1 (Table I).

In the presence of propidium, however, at a concentration equal to 10-fold its dissociation constant, inhibition of FBS-AChE by mAb 25B1 occurred at rates 4- to 7-fold slower than in the absence of propidium (Table I), and the extent to which propidium retarded the inhibition of AChE by 25B1 could be correlated with the concentration of this peripheral site cationic ligand. Edrophonium neither affected the time course of inhibition of AChE by mAb 25B1 nor interfered with propidium-induced retardation of enzyme inhibition by 25B1. To further test the possibility that propidium binds at or near a region which constitutes the epitope for mAb 25B1, the displacement of propidium from its conjugate with FBS-AChE was monitored in the presence of mAb 25B1. On the basis of changes observed in the excitation and emission spectra of AChE-bound propidium, a nearly complete dissociation of propidium could be demonstrated following the addition of stoichiometrically sufficient 25B1 (Figure 3). Brimijoin et al. (1985) reported similar results for a monoclonal antibody raised against rabbit brain AChE.

Edrophonium and propidium did not change the stoichiometry observed for the inhibition of AChE by mAb 25B1. The affinity of both propidium (K_I) and mAb 25B1 (k_1 and k_2) for FBS-AChE increased in low ionic strength, while inhibition of AChE by edrophonium (K_I) was considerably less sensitive to changes in ionic strength over the range studied (Table I).

Effect of mAb 25B1 on the Phosphorylation of AChE and on Reactivation and Aging of the Corresponding OP–AChE Conjugate. To characterize the nature of inhibition of AChE by mAb 25B1, [^{3}H]DFP binding to mAb·AChE complex was determined. mAb 25B1·AChE failed to show any binding of [^{3}H]DFP when more than 98% of enzyme activity was preinhibited by mAb 25B1. The extent of radiolabeling of 50%

[2] In the abstract submitted to FASEB, it was erroneously reported as 4:1 stoichiometry.

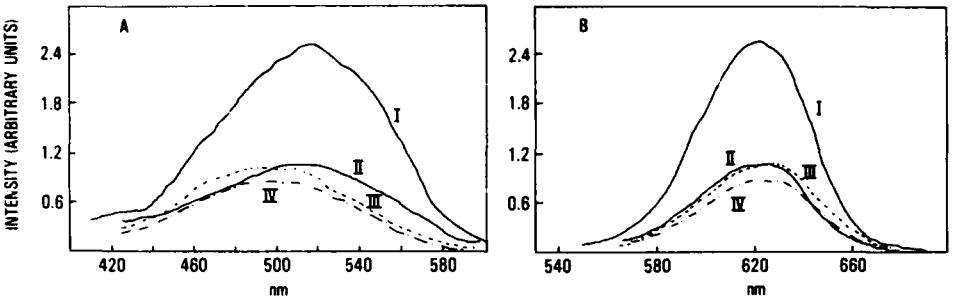

FIGURE 3: Displacement of propidium from AChE by mAb 25B1 as shown by changes in the fluorescence properties of AChE-bound propidium (I). mAb 25B1 (1.6 μM) was added to a mixture of 2.3 μM AChE and 1.4 μM propidium (I). The spectra recorded 30 min later (II) show that the fluorescence intensity was reduced to the level observed for propidium + mAb 25B1 (III) or propidium alone (IV). All measurements were carried out in 5 mM phosphate (pH 8.0, 25 °C). Panel A: Excitation spectrum (λ_{em} = 625 nm). Panel B: Emission spectrum (λ_{ex} = 516 nm).

mAb-inhibited AChE was approximately half that obtained for either free enzyme or AE-2·AChE. These results are similar to previous reports by Brimijoin et al. (1985) showing that mAb raised against rabbit brain AChE reduced the labeling of the enzyme by [^{3}H]DFP, whereas Sorensen et al. (1987) demonstrated that mAb AE-2 could not prevent the labeling of human erythrocyte AChE by [^{3}H]DFP.

To further determine the decrease in the nucleophilicity of AChE following complexation with mAb 25B1, the release of fluorescence leaving group 7-HQ from MEPQ was monitored in the presence or absence of either AChE or mAb·AChE. 7-HQ was quantitatively released within 10 s following the addition of 0.16 μM MEPQ to 0.1 μM AChE. The same amount of AChE preincubated 20 h with 0.19 μM mAb 25B1 released the same amount of 7-HQ with a half-time of approximately 120 min. Fab–AChE complex displayed a similar time course as observed for the release of MEPQ by mAb-25B1.

To determine the ability of oximes to reactivate mAb·OP–AChE conjugate, it was necessary to dissociate reactivated enzyme from its complex with mAb 25B1, since the latter is enzymatically inactive. OP–AChE conjugates (0.08–0.7 μM) obtained with paraxon, DFP, or MEPQ were prepared with a slight stoichiometric excess of mAb 25B1 prior to the addition of 1 mM of either 2-PAM or TMB_4. At various time intervals, the reactivation mixture was diluted 100-fold into 1.0 M Gdn·HCl solution to dissociate the mAb·AChE complex and restore the enzyme activity. In a control experiment the presence of 0.01 mM oxime reactivator (either 2-PAM or TMB_4) in 1.0 M Gdn·HCl did not significantly reactivate the OP–AChE conjugates tested. Results indicate that mAb 25B1 decreased the rate of reactivation of various OP-inhibited AChEs by either 2-PAM or TMB_4 but did not prevent approach of the oxime nucleophile to the phosphorus atom at the active site, since the oxime-induced reactivation of mAb·OP–AChE was significantly higher than the reactivation without the oximes and only 2–10 times less susceptible to reactivation than OP–AChE alone. No significant changes were observed with respect to the spontaneous reactivation in the presence or absence of mAb 25B1.

The effect of mAb on the rate of aging and oxime-induced reactivation was correlated with chemical changes occurring in the covalent conjugate OP–AChE by measuring release of radioactivity from enzyme-bound [^{3}H]DFP over a period of 24 h in the absence or presence of 1 mM of TMB_4. Results are shown in Figure 4. The rate of displacement of enzyme-bound radioactivity from [^{3}H]DFP–AChE in the presence of 1 mM TMB_4 is significantly lower for mAb-treated DFP–AChE than in absence of mAb 25B1 (panel A). It appeared that mAb-treated DFP–AChE was slowly converted

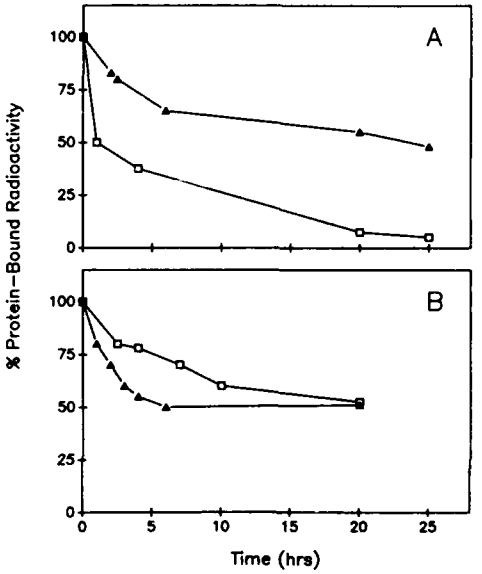

FIGURE 4: Time course for displacement of radioactivity from either free (□) or mAb-bound [^{3}H]DFP–AChE (▲). Approximately 1 μM [^{3}H]DFP–AChE was incubated 20 h with a stoichiometric amount of mAb 25B1 and than subjected to oxime-induced reactivation and aging. Panel A: Oxime-induced reactivation in the presence of 1 mM TMB_4 (50 mM phosphate, pH 8.0, 25 °C). Panel B: Aging reaction in 50 mM phosphate adjusted to pH 5.0 with HCl.

in the presence of TMB_4 at pH 8.0 to a form resistant to further release of radioactivity. When DFP–AChE was transferred to pH 4.9–5.1, aging apparently occurred, as evidenced by progressive loss of protein-bound radioactivity, with a half-time of approximately 3 h at 25 °C. Pretreatment of DFP–AChE with a stoichiometric amount of mAb 25B1 enhanced 4-fold the rate of the assumed aging (Figure 4, panel B).

Binding of mAb 25B1 to Thermally Denatured or Gdn-Denatured FBS-AChE. To determine whether conformational changes in regions of FBS-AChE that are involved in the catalytic mechanism alter the binding of mAb 25B1, the extent of binding of mAb to either partially or completely inactivated enzyme was correlated with the residual activity of perturbed AChE (Figure 5). The same amount of antibody 25B1 was incubated with FBS–AChE that was first inactivated to different levels by increasing time of heating or increasing the concentration of Gdn·HCl. Since loss of AChE activity in Gdn·HCl (pH 7.4–7.6) was time dependent, the extent of inactivation could be controlled by dilution of AChE/Gdn·HCl solution to stop the assumed protein unfolding by Gdn·HCl. No further changes in enzyme activity were observed in the diluted solution over a period of 20 h. After completion of

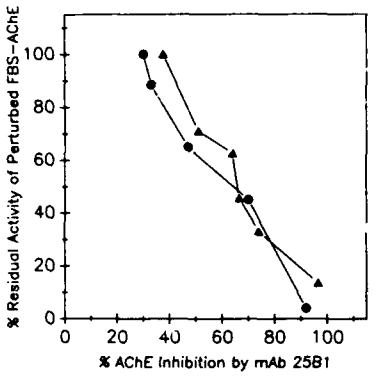

FIGURE 5: Correlation between denaturation of FBS-AChE and loss of binding of mAb 25B1 to denatured enzyme. Thermal denaturation (▲): 21.6 nM AChE (50 mM phosphate, pH 8.0) was heated at 50–51 °C for 0, 10, 22, 45, 70, and 135 min. Each sample was incubated 20 h (25 °C) with 3.7 nM mAb 25B1. Gdn·HCl denaturation (●): 71.4 nM AChE was incubated for 60 min in Gdn·HCl solution, pH 7.4–7.6, at the following concentrations: 0, 0.9, 1.1, 1.35, and 1.60 M. Each sample was diluted 10-fold in 12 nM mAb 25B1 and incubated for 20 h at 25 °C. The loss of binding of mAb 25B1 to denatured enzyme is expressed as increased inhibition of residual AChE by fixed concentration of mAb 25B1.

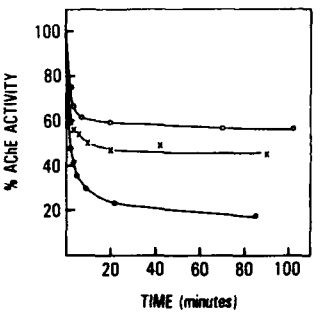

FIGURE 6: Effect of presence of phosphorylated AChE on rate of inhibition of native AChE by mAb 25B1: (○) 0.023 μM AChE + 0.006 μM 25B1 (42% inhibition); (●) 0.023 μM AChE + 0.012 μM 25B1 (83% inhibition); (×) 0.023 μM AChE + 0.023 μM $(C_2H_5O)_2P(O)$–AChE + 0.012 μM 25B1. The extent of inhibition of AChE by mAb 25B1 is proportional to the concentration of antibody added to reaction mixture (○; ●). In the presence of equal amounts of native and phosphorylated AChE, the extent of inhibition was not proportional to the concentration of mAb 25B1 added (×), indicating that the affinity of native enzyme for mAb 25B1 is higher than that of phosphorylated enzyme (affinity ratio = 1.3).

the reaction between Gd-treated AChE and mAb 25B1, residual activity of the perturbed enzyme was measured. The results demonstrated that the percent inhibition of residual activity of AChE by mAb 25B1 could be correlated with the residual activity of the perturbed enzyme before the addition of mAb 25B1. It would appear that the affinity of mAb 25B1 for either thermally perturbed or Gdn·HCl-perturbed AChE decreased significantly and that mAb 25B1 preferentially bound to the native form of the enzyme. When enzyme activity (0.04 μM AChE) was protected against heat denaturation by 35 μM of either propidium or edrophonium (70–89% loss of enzyme activity in unprotected AChE), no significant changes were observed in the stoichiometry of the binding of mAb 25B1 to the protected enzyme compared to unprotected enzyme. AChE, when complexed to mAb 25B1, could be protected (>55%) against thermal denaturation under conditions that caused complete loss of free AChE activity (5 min at 56 °C).

Relative Affinity of mAb 25B1 for Native and Phosphorylated FBS-AChE. Since mAb 25B1 appears to significantly affect the catalytic machinery of FBS-AChE, it was important to determine whether modifying the active site of the enzyme by irreversible OP inhibitors would cause any detectable changes in its binding to mAb 25B1. Solid-phase immunoassay technique (ELISA) could not be used for quantitation of these experiments, since partial and variable inactivation (denaturation) of enzyme was observed as a result of its coating to the microtiter plate wells. Therefore, the effect of the presence of OP–AChE (>95% inhibition of enzyme activity) on the inhibition of native FBS-AChE by mAb 25B1 was measured in homogeneous solution to determine the relative affinity of various OP–AChEs for mAb 25B1. For example, if 0.023 μM AChE was 42% inhibited by 0.006 μM mAb 25B1, one would expect that the addition of 0.023 μM OP–AChE (assuming that mAb 25B1 displayed the same affinity for OP–AChE as for native AChE) and an increase in the concentration of mAb 25B1 to 0.012 μM would lead to the same degree of inhibition (i.e., 42%). However, as illustrated in Figure 6 for $(C_2H_5O)_2P(O)$–AChE and summarized in Table II for six different OP–AChE conjugates, the affinity of mAb 25B1 for phosphorylated AChE was slightly but definitely lower than the affinity observed for the native enzyme. Despite the small differences among the various OP conjugates, $CH_3P(O)(O^-)$–AChE appeared to have the highest affinity for mAb 25B1, irrespective of the ligand used for the preparation of the phosphonylated enzyme (i.e., CH_3POCl_2 or soman).

Table II: Affinity Ratios of Native AChE/Phosphorylated AChE for mAb 25B1[a]

OP inhibitor	OP–AChE structure	affinity ratio[e] (native/phosphorylated)
$CH_3P(O)Cl_2$[b]	$CH_3(O)(O^-)$–AChE (aged)[d]	1.15
$CH_3P(O)$(O-Pin)F[c] (soman)	$CH_3P(O)(O^-)$–AChE (aged)	1.10
$(iPrO)_2P(O)F$[c] (DFP)	$(iPrO)P(O)(O^-)$–AChE (aged)	1.40
$(C_2H_5O)P(O)Cl_2$[b]	$(C_2H_5O)P(O)(O^-)$–AChE (aged)	1.25
$(C_2H_5O)_2P(O)Cl$[b]	$(C_2H_5O)_2P(O)$–AChE (nonaged)[d]	1.40
$CH_3P(O)(OC_2H_5)$(7-HQ)[c] (MEPQ)	$CH_3P(O)(OC_2H_5)$–AChE (nonaged)	1.25
$(iPrO)_2P(O)F$[c]	$(iPrO)_2P(O)$–AChE (nonaged)	1.35

[a] Measurements were based on inhibition of FBS-AChE enzymic activity (23 nM) by mAb 25B1 (12 nM) in the presence of equimolar concentration of OP–AChE conjugate (23 nM) in 50 mM phosphate buffer, pH 8.0 at 25 °C. [b] Inhibition (95–97%) was obtained by stepwise addition of small volumes of concentrated stock solution of OP in organic solvent to FBS-AChE in 50 mM phosphate buffer (pH 8.0). [c] OP–AChE was obtained by using stoichiometric amounts of AChE and OP; 95–98% inhibition of AChE was obtained. [d] Determined by monitoring regeneration of enzyme activity in presence of 1 mM TMB_4 (16 h, 25 °C). [e] Determined by dividing percent inhibition observed for the mixture AChE/OP–AChE/25B1 (23:23:12) by inhibition observed by incubating 23 nM AChE/6 nM mAb 25B1. See Figure 6; n = 2–3, estimated error <15%.

Stability of OP–AChE to Heat and Gdn·HCl Perturbation. Among all complexes studied, $CH_3P(O)(O^-)$–AChE [obtained by using either $CH_3P(O)Cl_2$ or soman] displayed the highest stability to heat and Gdn·HCl perturbation in terms of maintaining its ability to bind to mAb 25B1 (Table III). The stability of OP–AChEs obtained by using MEPQ and $(C_2H_5O)P(O)Cl_2$ [$CH_3P(O)(OC_2H_5)$–AChE and $(C_2H_5O)P(O)(O^-)$–AChE] appeared to be similar to each other, although consistently less stable than $CH_3P(O)(O^-)$–AChE. The DEP-AChE [$(C_2H_5O)_2P(O)$–AChE] and the aged and

Table III: Stability of OP-AChE Conjugates to Conformational Perturbation Expressed as Loss of Binding to mAb 25B1

OP–enzyme[b]	loss of binding to mAb 25B1[a] for perturbant: heat I[c]	heat II[d]	1.6 M Gdn·HCl, 25 °C I[e]	1.6 M Gdn·HCl, 25 °C II[f]
native FBS-AChE	87	>98	92	86
$CH_3P(O)(O^-)$–AChE (aged)[g]	30	27	21	<2
$CH_3P(O)(O^-)$–AChE (aged)[h]	NM[i]	25	NM	4
$(C_2H_5O)P(O)(O^-)$–AChE (aged)	55	45	54	28
$(iPrO)P(O)(O^-)$–AChE (aged)	NM	97	NM	72
$CH_3P(O)(OC_2H_5)$–AChE (nonaged)	58	67	63	16
$(C_2H_5O)_2P(O)$–AChE (nonaged)	90	94	87	66
$(iPrO)_2P(O)$–AChE (nonaged)	NM	95	NM	69

[a] A stoichiometric amount of mAb 25B1 was added to both perturbed and nonperturbed enzyme and incubated overnight at 25 °C to complete mAb–enzyme complex formation. Unbound mAb 25B1 was measured by adding a slight stoichiometric excess of fresh FBS-AChE. Inhibition by residual mAb 25B1 was determined and used to calculate the amount of unbound mAb 25B1. Figures are percent (%) loss of binding of mAb 25B1 relative to nonperturbed enzyme. Estimated error, <15% of the reported values. [b] For OP ligands used for the preparation of OP-enzyme see Table II. [c] 0.092 μM of either native or OP–AChE conjugates heated at 50 °C in 50 mM phosphate buffer for 120 min. Native AChE was 90% inactivated under these conditions. [d] 0.06 μM OP–AChE containing 0.01% BSA heated 360 min at 50 °C and for additional 2 h at 54 °C to decrease enzyme activity to 4% of control activity. [e] 0.16 μM of either native or OP–AChE conjugate; 98% loss of enzyme activity was observed after 120 min. [f] 0.06 μM OP–AChE containing 0.01% BSA; 97% loss of enzyme activity after 6 h. [g] Obtained by using $CH_3P(O)Cl_2$. [h] Obtained by using soman. [i] NM = not measured.

nonaged forms of DFP-inhibited AChE [$(iPrO)P(O)(O^-)$–AChE and $(iPrO)_2P(O)$–AChE] were the least stable forms of OP–AChE. The stability rank order shown in Table III suggests that negative charge on the phosphoryl residue in aged OP–AChE conjugates is only partially responsible for the increased protection conferred to the active site region against external perturbants. The relative stability of the various OP–AChE conjugates toward heat and Gdn·HCl denaturation can be correlated with their affinity for mAb 25B1 (Table II). The phosphonyl group in $CH_3P(O)(O^-)$–AChE, which only slightly changed the affinity of AChE for mAb 25B1, significantly stabilized the conformation around the active site of AChE. In contrast, DFP-induced inhibition of AChE (both aged and nonaged forms) and the conjugate $(C_2H_5O)_2P(O)$–AChE, which displayed the greatest decrease in affinity of AChE for mAb 25B1 (Table II), were also found to provide the least stabilization among OP–AChEs in this study (Table III).

Immunoadsorbance Assays. Solid-phase immunoadsorbance assays consistently showed that FBS-AChE phosphorylated by either DFP (aged and nonaged forms), paraoxon, MEPQ, or $(C_2H_5O)P(O)Cl_2$ displayed reduced affinity for mAb 25B1. In contrast, a small but definite increase in affinity of $CH_3P(O)(O^-)$–AChE for 25B1 was observed, irrespective of the ligand used for its preparation. Brimijoin et al. (1985) also reported that DFP-inhibited AChE from rabbit brain reduced the affinity of AChE for a monoclonal antibody raised against native AChE. Since native FBS-AChE partially lost enzymic activity during the process of coating of microtiter wells, data (not shown) represent the combined effects of denaturation of antigens and the inherent affinity of immobilized AChE and OP–AChE for mAb 25B1. Despite the uncertainty in the interpretation of results from the binding experiments it is important to emphasize that $CH_3P(O)(O^-)$–AChE displayed the highest affinity for 25B1 among all OP–AChEs tested, including native enzyme. These results are in agreement with the stability rank order for OP–AChEs in homogeneous solutions (Table III).

Discussion

In this study we have placed particular emphasis on the analysis and characterization of a unique mAb-AChE complex which permitted its use for the determination of stability rank order of various OP–AChE conjugates against denaturation.

Characterization of mAb 25B1·AChE Complex. Sensitivity of mAb 25B1 to conformational changes around the catalytic domains of FBS-AChE induced by external perturbants suggests that the antigenic determinants constitute a conformational epitope located, at least in part, within protein segments that control the catalytic activity of AChE. Stability of the mAb 25B1·AChE complex ($K_d < 10^{-12}$ M) is >1000-fold higher than values reported for an inhibitory mAb raised against rabbit brain AChE (Brimijoin et al., 1985). High specificity and the stability of the complex formed between mAb 25B1 and FBS-AChE indicate the existence of a remarkable complementarity between interacting surfaces.

In contrast to the loss of reactivity (i.e., nucleophilicity) of mAb-bound AChE toward both substrates and inhibitors (>10^4-fold decrease in the reactivity toward MEPQ), mAb-OP–AChE was only 2–10 times less susceptible to reactivation in the presence of external nucleophiles than OP–AChE. Accessibility of both the anionic site and the esteratic site serine to small ligands was not severely compromised despite a decrease in the nucleophilicity of mAb-bound FBS-AChE. These results suggest that it is possible that inactivation of FBS-AChE by mAb 25B1 is largely due to binding to an antigenic determinant(s) located outside the amino acid sequence region containing the esteratic serine. Indeed, propidium, a peripheral site ligand, clearly interfered with the rate of inhibition of AChE by mAb 25B1 and was displaced from AChE upon addition of mAb 25B1. In contrast, edrophonium, a specific active site ligand, which presumably binds at the anionic site, neither affected the kinetics of inhibition of AChE nor altered the effect of propidium on the inhibition of AChE by mAb 25B1. Also binding of both propidium and mAb 25B1 to FBS-AChE was significantly enhanced in low ionic strength solutions, whereas edrophonium responded quite modestly to such changes (Table I).

Monoclonal antibody 25B1 demonstrated considerably different properties than inhibitory antibodies raised against AChE (Abe et al., 1982; Brimijoin et al., 1985; Sorensen et al., 1987), in degree of inhibition of enzyme activity, specificity, and stability of the complex formed between the two proteins. Loss of AChE activity upon heating or Gdn·HCl-induced denaturation could be correlated with loss of binding of mAb 25B1 to the perturbed enzyme. This is consistent with the empirical concept of utilizing mAb 25B1 to monitor changes in the three-dimensional structure of the catalytic domains of native and phosphorylated FBS-AChE following their exposure to denaturing agents.

Resistance to Denaturation of OP–AChE. Phosphorylation of FBS-AChE decreased slightly its affinity for mAb 25B1. This suggests that various phosphoryl residues are well accommodated within the active site pocket and cause only slight changes in the spatial arrangement of the segments which constitute the conformational epitope. The modest changes observed could be correlated with neither the structure of the alkyl groups nor the assumed presence of negatively charged oxygen attached to the phosphorus atom of aged OP–AChE.

As evidenced by the use of mAb 25B1, phosphorylation significantly increased the conformational stability of AChE

to heat and Gdn·HCl denaturation. Similar protection was also achieved by reversible inhibitors such as propidium, edrophonium, and mAb 25B1, provided that sufficient ligand was present when the AChE was exposed to environmental perturbants. Since the enhancement of conformational stability was achieved by ligands that inhibit AChE by different mechanisms, it is suggested that the common denominator underlying the enhanced conformational stability is restriction of movement of catalytic domain(s) through internal cross-linking of protein segments by protecting ligands. The OP residue attached to the esteratic serine may offer four simultaneous binding sites for formation of internal cross-bridges between protein segments constituting the active site.

Perhaps the most important finding of this work is the conformational stability displayed by $CH_3P(O)(O^-)$–AChE. This conjugate consistently demonstrated the highest resistance to conformational changes in AChE induced by either temperature or Gdn·HCl denaturation. The present report provides the first experimental evidence that among the OP ligands tested in this study the combination of a methyl group and a negatively charged oxygen attached to a P atom, e.g., soman-inhibited AChE, constitutes the most efficient stabilization of the active site region toward denaturing effects. The $CH_3P(O)(O^-)$–AChE complex closely resembles the assumed shape of the transition-state geometry for the hydrolysis of acetylcholine and thus substantiates the hypothesis that the tetrahedral configuration centered around the P atom of the soman-inhibited AChE presents the closest transition-state analogue for the hydrolysis of acetylcholine (Ashani & Green, 1981).

Conclusions. Monoclonal antibody 25B1 defines a conformational epitope on FBS-AChE in close proximity to the catalytic center of FBS-AChE. Its unique properties permitted its use as a molecular probe for studying the relationship of the structure of organophosphoryl residues attached to the active site serine of the inhibited enzyme and the enhanced resistance of the catalytic domains of AChE to denaturing agents. The results suggest that the increased resistance of $CH_3P(O)(O^-)$–AChE to denaturation relative to either the native enzyme or other OP–AChE conjugates may be employed to describe in detail the relative spatial orientation of several binding sites within the catalytic center of FBS-AChE.

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