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An Inhibitory Monoclonal Antibody to Rabbit Brain Acetylcholinesterase

Studies on Interaction with the Enzyme

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SUMMARY

A recently isolated monoclonal antibody was found to be a potent and powerful inhibitor of the catalytic activity of rabbit brain acetylcholinesterase (AChE; acetylcholine acetylhydrolase, EC 3.1.1.7), with an IC_{50} of about 1 nM and a maximal inhibition of at least 90%. The antibody increased the optimal concentration of acetylthiocholine as much as 50-fold, but analysis of the substrate kinetics did not indicate a simple competitive interaction. The antibody markedly reduced the labeling of purified rabbit brain AChE by tritiated diisopropyl fluorophosphate (DFP) and also impeded the binding of propidium iodide, a fluorescent probe thought to be directed toward the peripheral anionic site. The antibody's affinity for enzyme with active sites that were phosphorylated with DFP or occupied by reversible ligands was measurably less than for native enzyme. It is possible that the mechanism of inhibition involves antibody-induced conformational changes that are unfavorable for catalysis.

INTRODUCTION

Immunochemical studies of AChE[1] (EC 3.1.1.7) have added significantly to our understanding of the structure, function, and localization of this important synaptic enzyme (1–5). For obvious reasons, antibodies capable of inhibiting catalytic activity deserve special attention. Inhibition of non-neuronal AChE by polyclonal antisera was first described by Williams (6), and has been observed several times since (7, 8). Antisera that inhibit neuronal AChE have also been obtained (4, 9), yet, given their heterogeneous nature, they have enabled few inferences about the mechanism of inhibition to be drawn.

Inhibitory monoclonal antibodies to AChE would be considerably more useful than conventional antisera for probing the relationship between enzyme structure and function. Abe *et al.* (10) reported a monoclonal antibody that was able to inhibit 75–80% of the catalytic activity of AChE from the electric ray, *Narke japonica*. The authors suggested that their antibody was directed toward the active site of the enzyme, or to a nearby site, but additional evidence for this view was not given.

We recently obtained several monoclonal antibodies against the AChE of rabbit brain (11). One of them is a potent anticholinesterase which inhibits AChE activity by at least 90%. We now describe experiments aimed at elucidating the site at which this antibody binds to the enzyme.

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[1] The abbreviations used are AChE, acetylcholinesterase (acetylcholine acetylhydrolase); DFP, diisopropylfluorophosphate.

MATERIALS AND METHODS

Antibodies and enzyme preparations. An inhibitory (F3-43) and a noninhibitory (F4-40) monoclonal antibody were selected from our panel of antibodies against rabbit brain AChE (11). The individual immunoglobulins (both IgG) were purified by chromatography on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) as described by Bruck *et al.* (12). For some experiments, Fab fragments were prepared from the purified IgG by digestion with papain (Boehringer-Mannheim, Indianapolis, IN) for 6 hr at 37° (13). Analysis of the digested material by sucrose density gradient ultracentrifugation showed that native 6.6 S IgG was quantitatively removed. The remaining immunoreactivity was associated with a 3.5 S peak, as expected.

Three types of AChE preparations were employed. For general purpose screening, crude extracts were prepared by homogenizing whole brain tissue in 10 volumes of detergent-containing buffer: 50 mM Tris-HCl, pH 7.4, 1% Triton X-100. Unless stated otherwise, the remaining experiments used immunopurified AChE (specific activity 600 units/mg), enriched about 2000-fold from crude extracts by affinity chromatography on an anti-AChE monoclonal antibody coupled to Sepharose 4B (14). When required, electrophoretically homogeneous AChE (specific activity 3000 units/mg) was made from the immunopurified enzyme by means of ligand affinity chromatography on phenyltrimethylammonium Sepharose (14).

Protein determinations. Protein determinations were performed by the dye-binding method of Bradford (15) with bovine serum albumin as a reference standard. The results were used to calculate the molar concentrations of purified antibody and enzyme, assuming molecular weights of 160,000 for IgG and 80,000 for the catalytic subunit of AChE.

Enzyme assay and analysis of substrate kinetics. AChE activity was measured at room temperature by the spectrophotometric method of

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Ellman *et al.* (16) with acetylthiocholine iodide (Sigma Chemical Co., St. Louis, MO) as the substrate. For termination of the assay reaction and for preparation of blank samples, we used the selective AChE inhibitor, BW284C51, in a final concentration of 10 μM (Burroughs-Wellcome, Research Triangle Park, NC). Surface AChE activity was measured in washed erythrocytes by adding substrate directly to the cell suspension, which was centrifuged before spectrophotometry. The selective inhibitor, ethopropazine (0.1 mM), was used in assays of crude brain extracts and whole cells in order to inactivate any butyrylcholinesterase that might have been present. The inhibitor was omitted in assays of immunopurified enzyme, known to be free of other cholinesterases. For routine assays, the concentration of substrate was 1 mM. For kinetics experiments, the substrate concentration was varied from 0.1 to 50 mM. The data obtained from the lower portion of this range were computer analyzed by an iterative, unweighted least squares approximation to the hyperbolic Michaelis-Menten equation (17).

Rates of onset and offset of enzyme inhibition. The rate of onset of enzyme inhibition was determined in the following manner. Immunopurified enzyme was mixed in a cuvette at room temperature with 0.95 ml of buffer (50 mM sodium phosphate, pH 7.4, 0.05% Triton X-100, 0.2% bovine serum albumin). The final concentration of AChE was 0.025 units/ml (estimated concentration of catalytic subunits, approximately 0.1 nM). For antibody exposures of 1 min or longer, 10 μl of antibody were added to the curvette in a final concentration of 500 nM or 5 nM and 50 μl of substrate were added at the end of the exposure period. For shorter exposures, antibody and substrate solutions were premixed before addition to the enzyme. Reaction velocity was then determined spectrophotometrically over successive 15-sec intervals.

The rate of offset of enzyme inhibition was determined by a rapid dilution method. Antibody in a concentration of 500 nM was mixed with enzyme in an estimated concentration of 100 nM (total volume, 20 μl) and incubated for 30 min at room temperature. After this exposure the incubation mixture was quickly subjected to two successive 100-fold dilutions, and 1 ml was transferred to a cuvette. Substrate solution (50 μl) was added at once or after a definite interval ranging from 1 to 160 min. The reaction velocity was then followed spectrophotometrically for 1 min. Control measurements were made with uninhibited enzyme (which generated an absorbance change of 0.02 optical density units/min after final dilution), and with enzyme and antibody diluted 10,000-fold before mixing.

Fluorescent ligands. Propidium iodide was purchased from Sigma Chemical Co. *N*-Methylacridinium iodide was synthesized from acridine and iodomethane (Aldritch Chemical Co., Milwaukee, WI) as described by Mooser *et al.* (18). Needle-like red crystals were collected in 30% yield by recrystallization from hot methanol-anhydrous ether. The ultraviolet absorption spectrum contained a sharp peak at 357 nm, with a shoulder at 341 nm. Mass spectrometry by fast atom bombardment indicated a mass of 194.073 daltons for the cation (theoretical value, 194.097). As reported (18), excitation at 360 nm led to fluorescence emission with peak intensity at 490 nm.

Fluorescence spectroscopy. Fluorescence measurements were performed in sodium phosphate buffer (10 mM, pH 7.4, with 0.05% Triton X-100) in a 0.3-cm^2 quartz cuvette (0.2 ml total volume). An SLM 4800 fluorometer (SLM Instruments, Urbana, IL) was used with EMI 9813QA photomultiplier tubes. The sample chamber was thermostatically controlled at 25°. To eliminate errors owing to variations in light output from the lamp, fluorescence was read as a ratio of intensities from the sample and from a reference cuvette containing a solution of dimethyl-1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene) in ethanol. For all measurements the excitation light was selected by use of a monochromator (2 nm bandwidth), and appropriate filters were placed in the excitation path. The excitation beam was unpolarized but the emission was observed through a vertical polarizer. Technical fluorescence emission spectra were adjusted for wavelength variation in instrument response by means of correction factors generated for this instrument with an NBS standard lamp.

DFP labeling of AChE. Immunopurified rabbit brain AChE (0.5–5 μg) was mixed with [^{3}H]DFP (1 μCi; specific radioactivity, 5.2 Ci/mmol; Amersham Corp., Arlington Heights, IL) in a total volume of 50–150 μl. The preparations were allowed to react for a defined period. When maximal labeling was desired, the reaction interval was 1 hr at 37°, followed by 16 hr at 4°. Unbound DFP was removed on a Centricon-30 microconcentrator (Amicon Corp., Danvers, MA) by four washes with 1 ml of 50 mM sodium phosphate, pH 7.4, and reconcentration to 0.16 ml. Total labeling was calculated to be about 15,000 dpm/μg; enzyme activity was inhibited by 99.9%. When the competition of antibody with DFP was to be tested, labeling was for 10 or 60 min at 37° and was terminated by addition of BW284C51 in a final concentration of 6 μM, followed by rinsing with phosphate buffer as just described.

Antibody binding assay. A solid phase immunoadsorbance assay was used to measure the binding of AChE by monoclonal antibody. As described in detail elsewhere (19) the assay employed rabbit antimouse immunoglobulin adsorbed to protein A (Pansorbin, Calbiochem-Behring, La Jolla, CA) in order to ensure quantitative precipitation of immune complexes. Unless stated otherwise, monoclonal antibodies to AChE were preadsorbed to the solid phase by incubation for 1 hr at 37°, and subsequent incubation with antigen was also for 1 hr at 37°. Binding was assessed after low speed centrifugation. In the case of DFP-labeled enzyme, binding was determined by scintillation counting of the rinsed pellets in the presence of 1% sodium dodecyl sulfate. In other cases, binding was determined by assays of AChE activity in the supernatants (when low molecular weight inhibitors were present, the supernatants were first dialyzed for 48 hr with three changes of buffer).

RESULTS

Antibody properties and enzyme inhibition. Of the 22 monoclonal antibodies which we have raised to AChE, only two show any tendency to inhibit enzyme activity, and only one (designated F3-43) causes near-total inhibition. Some general properties of F3-43, described in a recent report (11), may be briefly summarized: 1) the antibody is an IgG1 with κ light chain; 2) affinity for rabbit brain AChE is in the nanomolar range; 3) affinity is reduced 3-fold by 1 M NaCl; 4) binding of enzyme from animals other than the rabbit is poor. It has not yet been determined whether the antibody is directed against conformational or sequential determinants, but preliminary experiments have shown no loss of immunoreactivity for heat-inactivated AChE (100°, 3 min) adsorbed to polystyrene.

For a detailed study of the inhibitory effects of F3-43 on rabbit AChE, we prepared immunopurified dimeric (G2) AChE from rabbit brain and exposed the enzyme for 1 hr at 23° to antibody in various concentrations. As Fig. 1 shows, the maximal degree of inhibition was about 90%, reached at an IgG concentration of 300 nM, while half-maximal inhibition occurred at an IgG concentration of approximately 1 nM.

To measure binding of AChE *per se*, different experimental conditions were required. In particular, to minimize interference owing to enzyme inhibition in the fluid phase, the antibody was preadsorbed to insolubilized protein A (see "Materials and Methods"). Binding of AChE under these conditions was half-maximal at an IgG concentration of 1 nM and was essentially complete at 100 nM (Fig. 1). Computer modelling, based on the assumption of two antibody binding sites on the dimeric AChE, indicated an apparent k_d of 5 ± 0.4 nM. Since the *effective* concentration of solid phase antibody was unknown, the binding and inhibition curves are not directly comparable. Nevertheless, the shallower slope of the

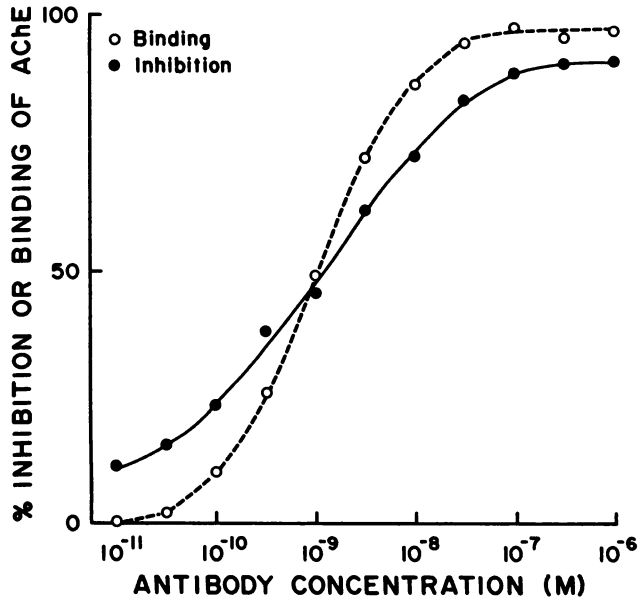

FIG. 1. *Inhibition and binding of immunopurified dimeric rabbit brain AChE by monoclonal antibody F3-43*

Measurements of enzyme activity and precipitation were made after 1 hr of exposure at 23° to antibody in solution or adsorbed to a solid phase, respectively (see "Materials and Methods," *Binding assay*). The estimated concentration of AChE active sites was 0.1 nM. Values shown are means of duplicate observations. The two curves are not directly comparable because of differences in the reactivity of solid phase and fluid phase antibodies, and because binding did not require antibody attachment to both catalytic subunits of the enzyme.

latter suggests that the relation between binding and inhibition was complex.

The inhibitory properties of F3-43 were highly species selective: no inhibition could be detected with AChE in crude homogenates of brain from guinea pig, rat, cat, or man. F3-43 likewise lacked the ability to bind or inhibit butyrylcholinesterase. Conversely, the antibody was able to inhibit AChE from rabbit red blood cells as well as brain, and was as effective against enzyme *in situ* as against enzyme in solution. For example, surface AChE activity in washed rabbit red blood cells incubated with 1 μM F3-43 for 1 hr at 23° was 84% less than in cells exposed to noninhibitory antibody or no antibody.

Onset and offset of enzyme inhibition. The rates of onset and offset of enzyme inhibition were measured as indices of the rates of formation and dissociation of the enzyme-antibody complex (see "Materials and Methods"). As shown in Fig. 2, antibody at high concentration (500 nM) inhibited the enzyme too quickly for an accurate measurement of the onset time, but diluted antibody (5 nM) gave an inhibition that appeared to follow simple exponential kinetics. Since antibody was in excess and since dissociation was much slower than association, we can approximate the rate of formation of immune complex, C, by the simple differential equation:

$$dC/dt = k_1A(E - C)$$

where A and E are the molar concentrations of antibody and enzyme, t is time in seconds, and k_1 is the forward rate constant. The solution for the initial condition, $C = 0$, is:

$$C = E(1 - e^{-k_1At})$$

Computer fitting of the data in Fig. 2 gives an estimate of $2 \pm 0.2 \times 10^6$ M^{-1} sec^{-1} for k_1, corresponding to an onset half-time of about 50 sec in the presence of diluted antibody.

After a rapid 10,000-fold dilution of preformed immune complex, it was possible to follow the offset of enzyme inhibition with precision. During this period the level of inhibition dropped exponentially from an estimated starting value of 80% toward an an equilibrium value of 35%. Evidently the inhibition was largely reversible, although there may have been an irreversible component (prediluted antibody lowered AChE activity only 20%). The half-time of offset was 13.1 ± 0.4 min and the rate constant (k_2) was $9 \pm 0.3 \times 10^{-4}$ sec^{-1} (Fig. 2).

From the measured rates of onset and offset of enzyme inhibition, one can derive an alternative estimate of the dissociation constant for the F3-43·AChE complex: $k_2/k_1 = (9 \times 10^{-4}\ sec^{-1})/(2 \times 10^6\ M^{-1}\ sec^{-1}) = 0.45$ nM. This "dynamic k_d" is approximately 10% as large as the k_d derived under equilibrium conditions with the solid phase binding assay.

Effect on substrate kinetics. The effect of F3-43 on the substrate kinetics of immunopurified rabbit brain AChE was examined for clues to the nature of the enzyme inhibition. The AChE activity of a control preparation plotted as a function of log substrate concentration gave a bell-shaped curve with an apex near a concentration of 1 mM (Fig. 3). This typical behavior, resulting from substrate inhibition, was greatly altered in the presence of 1 μM F3-43, which shifted the optimum substrate concentration at least 50-fold to the right while generally reducing reaction velocity. As a result, the concentration-velocity curves determined in the presence and absence of antibody nearly intersected at a substrate concentration of 50 mM (Fig. 3). A rigorous kinetic analysis was precluded by the slow dissociation rate of the antigen-antibody complex and the consequent difficulty in establishing equilibrium conditions. It was deemed unlikely, however, that F3-43 was acting as a purely competitive AChE inhibitor.

In order to test whether the inhibitory effects of F3-43 were due to the sheer bulkiness of the IgG molecule, we examined the effect of Fab fragments on AChE activity. The fragments were prepared from F3-43 by papain digestion and were demonstrated to be free of intact IgG (see "Materials and Methods"). These fragments retained more than 90% of the anticholinesterase activity of native F3-43, when compared in equivalent protein concentration. Furthermore, the effect of Fab fragments on the substrate kinetics of AChE (data not shown) were essentially the same as those of the whole molecule.

Interaction with propidium iodide. A partly noncompetitive inhibition of AChE could result from effects on the so-called peripheral anionic site of this enzyme. It was therefore of interest to determine whether F3-43 would affect the inhibition of AChE by propidium iodide, which is believed to act through this secondary site. An experiment showed that, with 1 mM acetylthiocholine as the substrate, 1 μM F3-43 increased the IC_{50} concentration of propidium from 30 μM to 1 mM (Fig. 4).

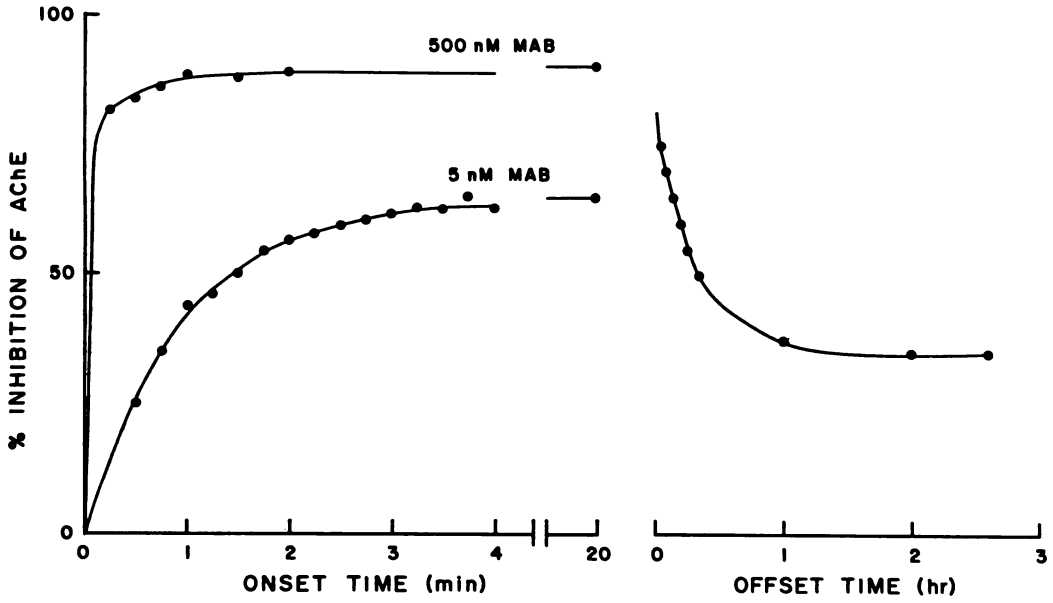

FIG. 2. *Onset and offset of AChE inhibition*

Left, enzyme (~0.1 nM active sites) mixed with inhibitory antibody in a concentration of 500 nM or 5 nM. AChE activity is shown as a function of time after mixing. *Right*, enzyme (~100 nM active sites) preincubated for 30 min with antibody (500 nM). AChE activity is shown as a function of time after a rapid, 10,000-fold dilution. The *solid lines* were computer-fitted by a least squares nonlinear regression method.

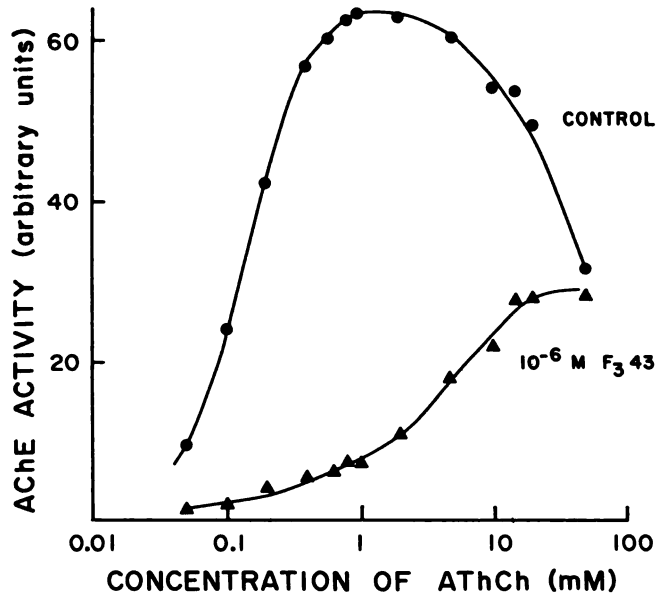

FIG. 3. *Effect of monoclonal antibody on substrate inhibition of AChE*

Enzyme activity (~0.1 nM active sites) is plotted as a function of acetylthiocholine concentration on a logarithmic scale. The bell-shaped control curve is typical. Note the marked depression and rightward shift induced by antibody (1 μM).

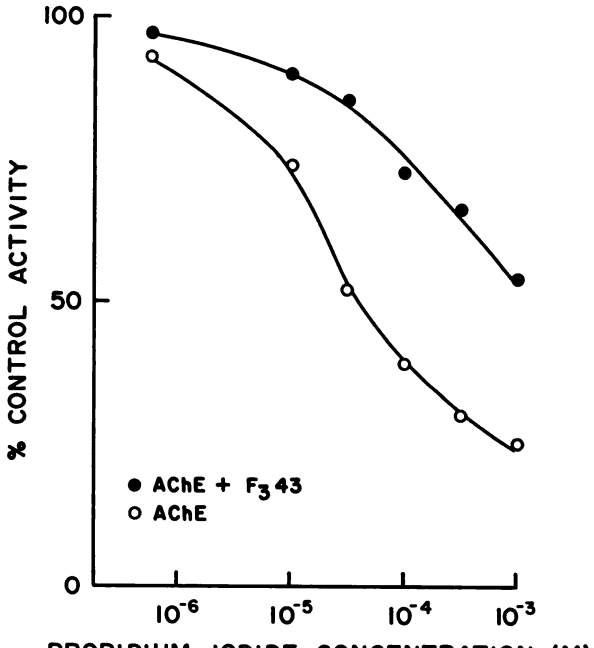

FIG. 4. *Effect of antibody on the inhibition of AChE by propidium iodide*

Enzyme (~0.1 nM active sites) was preincubated for 30 min at 23° in the presence or absence of antibody (1 μM). Propidium iodide was then added in the indicated final concentrations, and enzyme activity was measured after a subsequent 30-min incubation. Activities are expressed as percentages of control values obtained without propidium: 0.12 and 1.9 μmol/min in the presence and absence of antibody, respectively.

Because propidium iodide is a fluorescent compound whose emission properties change on binding to AChE, fluorescence spectroscopic methods were used to examine further the ternary system of AChE, propidium, and F3-43. An aqueous solution of propidium (1 μM) fluoresced maximally at 600 nm on excitation at 535 nm. When AChE was added, the fluorescence intensity of the solution was approximately doubled, with little change in the shape or position of the spectrum (Fig. 5). F3-43 reduced the fluorescence of the propidium-AChE mixture, but this result was complicated by a direct effect on the fluorescence of propidium at certain wavelengths. To avoid this complication we used only the data recorded at the isoemissive point (645 nm), where the fluorescence intensity of the ligand was unaffected by antibody. These data indicated that F3-43, at concentrations of 1, 3, and 10 μM, displaced the enzyme-bound propidium by 9%, 26%, and 43%, respectively. The displacing ability was as great when antibody was added after propidium as it was when antibody and enzyme were allowed to react before the fluorophore was added.

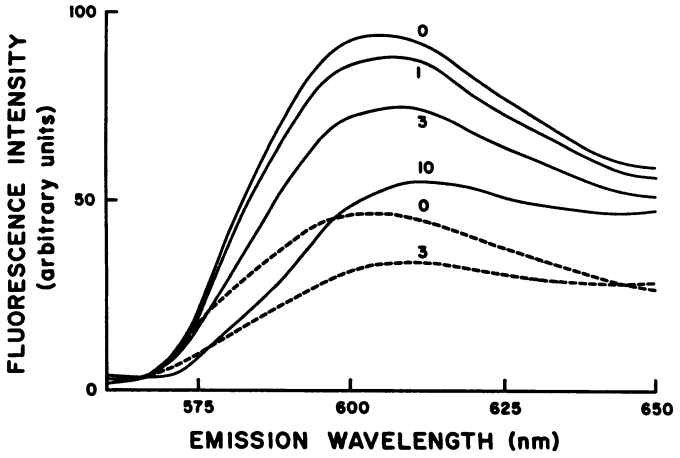

FIG. 5. *Effects of inhibitory monoclonal antibody on binding of propidium iodide to AChE*

Fluorescent probe (1 μM) and electrophoretically homogeneous enzyme (estimated concentration of active sites, 1 μM) were mixed. Excitation was at 535 nm. *Solid lines*, corrected fluorescence emission spectra obtained in the presence of enzyme. *Dashed lines*, spectra obtained in the absence of enzyme. The accompanying numbers indicate the concentration of F3-43 antibody (μM).

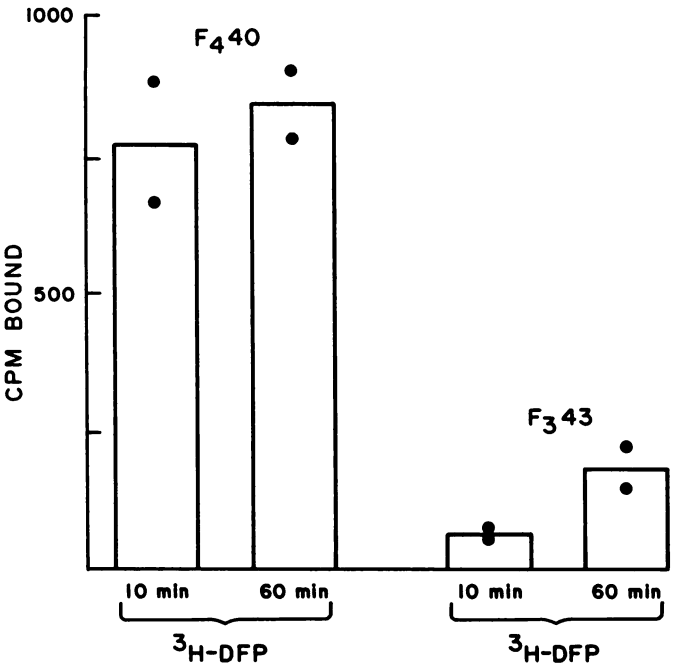

FIG. 6. *Effects of inhibitory and noninhibitory antibodies on labeling of AChE by DFP*

Samples of immunopurified rabbit brain AChE (final concentration of active sites about 40 nM) were incubated for 16 hr at 4° with monoclonal antibodies F3-43 (inhibitory) or F4-40 (noninhibitory) at an IgG concentration of 1 μM. The enzyme was then exposed to [^{3}H] DFP for 10 or 60 min and was immunoprecipitated with solid phase rabbit antimouse IgG (see "Materials and Methods"). The *circles* represent radioactivity precipitated in separate experiments done on different days (mean values indicated by bar heights).

Similar experiments were performed with the active site-directed fluorescent probe, *N*-methylacridinium (data not shown). It was noteworthy that F3-43 caused no measurable displacement of acridinium from the enzyme during a 1-hr observation period, when antibody and probe were both present at a 1 μM concentration.

Effect on phosphorylation of enzyme. To test the possibility that F3-43 affected AChE by interfering with the access of substrate to the catalytic site, we examined the effects of antibodies on the phosphorylation of enzyme by DFP. Immunopurified AChE was incubated overnight at 4° in the presence of 1 μM F3-43 or F4-40 (a high affinity, noninhibitory antibody). Next, 1 μCi of [^{3}H] DFP (~2 μM) was added to each sample and incubation was continued at 37° for 10 min ("short term") or 60 min ("long term"). The reactions were stopped with a reversible cholinesterase inhibitor, the samples were rinsed with buffer, and the specific incorporation of radiolabel into AChE was determined.

As compared with the noninhibitory antibody, F3-43 reduced the "short term" incorporation of DFP by over 90% and reduced the "long term" incorporation by over 75% (Fig. 6). These results imply that the effect of F3-43 is exerted at some stage before the hydrolysis of substrate and regeneration of the enzyme.

Effect of AChE ligands on antibody binding. We wished to determine whether the binding of AChE by F3-43 was sensitive to the presence of other ligands. Accordingly, we labeled immunopurified AChE with [^{3}H]DFP and compared the binding of labeled and native antigen by F3-43 in various concentrations. The experiment showed that the binding curve for DFP-labeled AChE was parallel to the binding curve for native enzyme, but shifted about 3-fold to the right (Fig. 7). Thus, the antibody displayed a reduced affinity for enzyme with active sites occupied by DFP.

The reversible AChE inhibitors, *N*-methylacridinium

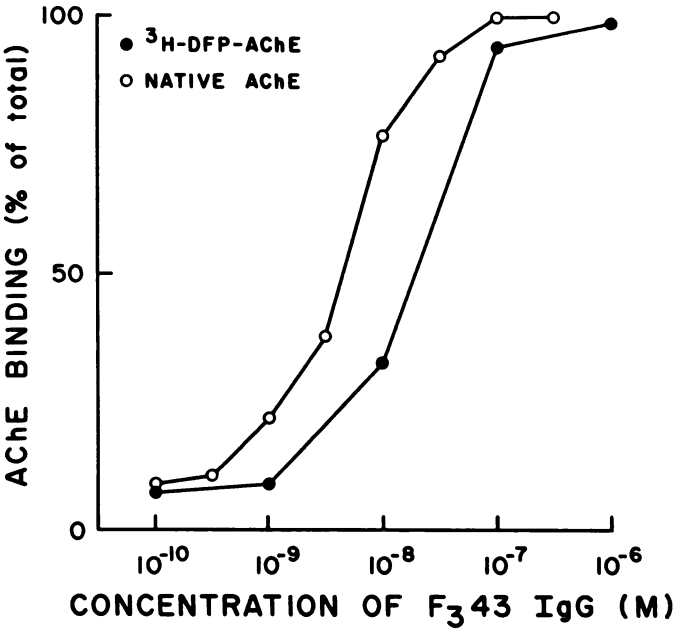

FIG. 7. *Relative affinity of inhibitory monoclonal antibody for native and DFP-labeled AChE*

Enzyme (estimated final concentration of active sites, 40 nM) was labeled as described in "Materials and Methods," and full IgG dilution, antigen-binding curves were constructed after 16 hr of incubation with antibody F3-43 at 4°.

iodide, decamethonium bromide, and *d*-tubocurare were also tested to determine their effects on the binding of enzyme by insolubilized F3-43 (we could not test propidium since the solid phase removed it all from solution, even in the absence of antibody). The inhibitors (1 mM) were preincubated with enzyme for 60 min before the exposure to antibody; binding was then determined as described in "Materials and Methods." Each inhibitor induced a parallel shift to the right in the curve of binding versus antibody concentration (Fig. 8A). The shift was smallest with curare (not quite 2-fold) and largest with decamethonium (about 4-fold). These effects were specific for F3-43, since AChE binding by the noninhibitory antibody, F4-40, was completely unaffected (Fig. 8B).

DISCUSSION

The inhibition of rabbit brain AChE by F3-43 presents some puzzling features. For example, there is the crossover of the curves describing enzyme binding and enzyme inhibition as functions of antibody concentration. A tentative explanation can be constructed if one assumes that inappropriate orientations of the solid phase antibody can lower its effective concentration (note that the dynamically determined k_d was smaller than the one calculated from the binding assay). The true binding curve may therefore lie to the left of the one represented in Fig. 1. Furthermore, since an enzyme molecule is "bound" when IgG attaches to *any* subunit, the binding curve of dimeric AChE is steeper than it would be otherwise. These effects could be at least partly responsible for the apparent discrepancies between binding and inhibition.

The mechanism of AChE inhibition might involve antibody binding to the catalytic or peripheral anionic sites of the enzyme. Also possible are conformational effects that interfere with catalysis. It must be acknowledged that the present results do not allow a clear choice among these alternatives. Evidence suggesting antibody binding near the catalytic site includes: 1) increases in apparent k_m; 2) inhibition of DFP binding; 3) reduced affinity for enzyme pretreated with DFP or *N*-methylacridinium. Evidence suggesting antibody binding near the peripheral anionic site includes: 1) interference with the binding and inhibition of AChE by propidium; 2) reduced affinity for enzyme pretreated with curare. In interpreting this evidence, however, one must avoid assuming that apparently competitive interaction between antibody and ligand implies enzyme binding at overlapping sites. In particular, the possibility of reciprocal allosteric inhibition of binding must be kept in mind.

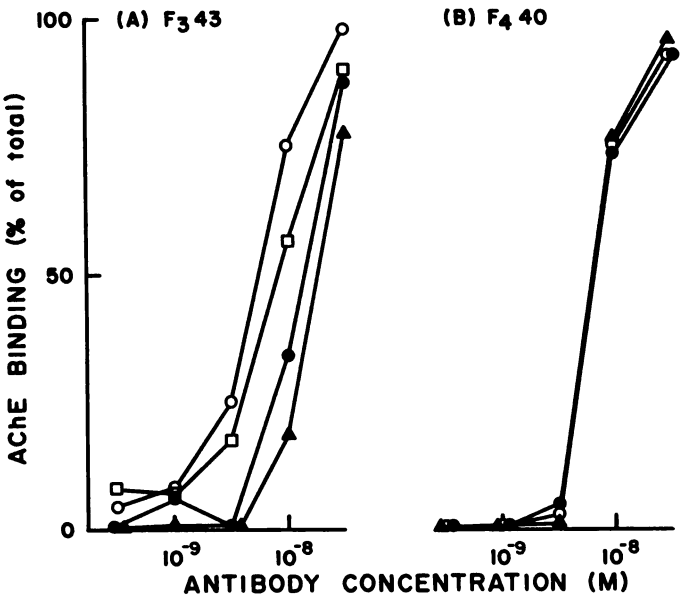

FIG. 8. *Effect of reversible AChE-ligands on the affinity of antibodies for the enzyme*

A, ligands tested with the inhibitory antibody, F3-43. B, ligands tested with the noninhibitory antibody F4-40. Binding was determined by enzyme assays of the unbound fractions after extensive dialysis (see "Materials and Methods"). Estimated final concentration of AChE active sites was 0.1 nM in both cases.

Given that F3-43 causes incomplete enzyme inhibition and interacts only weakly with *N*-methylacridinium, it seems unlikely that the antibody binds tightly to the active site of AChE. Likewise, if F3-43 binds to the peripheral anionic site, it is surprising that the competition with propidium was not stronger. The affinity of propidium for rabbit AChE was not measured but can be roughly estimated from the IC_{50} for enzyme inhibition: 30 μM. Since the affinity of F3-43 is at least 3 orders of magnitude greater, a one-to-one competition of equimolar concentrations should have led to near complete displacement of probe by antibody. The modest effects actually observed in the fluorescence experiments are perhaps better explained on the hypothesis that antibody binding indirectly reduces the affinity of AChE for propidium.

One could consider the possibility that F3-43 binds between the peripheral anionic site of the enzyme and the catalytic site, thereby partially blocking the access of substrate to both locations. This possibility is consistent with the observation that decamethonium, which interacts at both sites (20), was more effective than other reversible ligands in reducing the binding of AChE by F3-43. However, structural data on *Torpedo* AChE suggest that a single IgG molecule would have difficulty in reaching both substrate-binding sites. Taylor and Lappi (20) first suggested an intersite distance of 14 Å, in a study of the interaction between propidium iodide and various bis-quaternary ligands, but a later analysis of fluorescence energy transfer indicated a distance of 25 Å (21). There is no proof that *Torpedo* AChE is a good model for the rabbit enzyme, but such a span is larger than that of a typical antigenic determinant (22). At present, we lean toward the view that the inhibition of AChE by F3-43 is due to conformational effects.

A full understanding of the effects of F3-43 on AChE requires detailed knowledge of the enzyme's antibody-combining site. It should be feasible to obtain some information from a chemical analysis of the peptide fragments recognized by the antibody after proteolytic digestion of AChE. Since it has recently been shown that certain lectins are effective anticholinesterases (23), the possibility should be considered that sugar moities on the enzyme surface participate in antibody binding. Test-

ing this possibility will require experiments on the interaction of the antibody with enzymatically deglycosylated AChE. Meanwhile, F3-43 joins the ranks of proteins whose powerful and specific anticholinesterase activities can be expected to be useful in exploring the relation between enzyme structure and function.

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REFERENCES

1. Adamson, E. D. Acetylcholinesterases in mouse brain, erythrocytes and muscle. *J. Neurochem.* **28**:605–615 (1977).
2. Fambrough, D. M., A. G. Engel, and T. L. Rosenberry. Acetylcholinesterase of human erythrocytes and neuromuscular junctions: homologies revealed by monoclonal antibodies. *Proc. Natl. Acad. Sci. USA* **79**:1078–1082 (1982).
3. Doctor, B. P., S. Camp, M. K. Gentry, S. S. Taylor, and P. Taylor. Antigenic and structural differences in the catalytic subunits of the molecular forms of acetylcholinesterase. *Proc. Natl. Acad. Sci. USA* **80**:5767–5771 (1983).
4. Marsh, D. M., J. Grassi, M. Vigny, and J. Massoulié. An immunological study of rat acetylcholinesterase: comparison with acetylcholinesterases from other vertebrates. *J. Neurochem.* **43**:204–213 (1984).
5. Brimijoin S. Molecular forms of acetylcholinesterase in brain, nerve and muscle; nature, localization and dynamics. *Prog. Neurobiol.* **21**:291–322 (1983).
6. Williams, R. M. Antibodies to acetylcholinesterase. *Proc. Natl. Acad. Sci. USA* **62**:1175–1180 (1969).
7. Holmes, M. J., D. Michaeli, and H. U. Fudenberg. Immunoenzymology of acetylcholinesterase. IV. Studies on an enzyme-inhibiting antibody. *Immunochemistry* **10**:639–644 (1973).
8. Gurari, D., I. Silman, and S. Fuchs. Immunochemical and enzymatic properties of the electric eel acetylcholinesterase-antiacetylcholinesterase system. *Eur. J. Biochem.* **43**:179–187, (1974).
9. Greenburg, A. J., K. K. Parker, and A. J. Trevor. Immunochemical studies of mammalian brain acetylcholinesterase. *J. Neurochem.* **29**:911–917 (1977).
10. Abe, T., M. Sakai, and H. Saisu. A monoclonal antibody against catalytic subunits of acetylcholinesterase in the electric organ of an electric ray, *Narke japonica*. *Neurosci. Lett.* **38**:61–66 (1983).
11. Mintz, K. P., and S. Brimijoin. Monoclonal antibodies to rabbit brain acetylcholinesterase: selective enzyme inhibition, differential affinity for enzyme forms and cross reactivity with other mammalian cholinesterases. *J. Neurochem.* **45**:289–292 (1985).
12. Bruck, C., D. Portetelle, C. Glineur, and A. Bollen. One step purification of mouse monoclonal antibodies from ascitic fluid by DEAE Affi-Gel Blue chromatography. *J. Immunol. Methods* **53**:313–319 (1982).
13. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. J. Sussdorf. *Methods in Immunology*. W. A. Benjamin, Inc., New York, 224–234, (1970).
14. Mintz, K. P., and S. Brimijoin. Two-step immunoaffinity purification of acetylcholinesterase from rabbit brain. *J. Neurochem.* **44**:225–232 (1985).
15. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **92**:248–254 (1976).
16. Ellman, G. L., K. D. Courtnay, V. Andres, Jr., and R. M. Featherstone. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**:88–95 (1961).
17. Wilkinson, G. N. Statistical estimation in enzyme kinetics. *Biochem. J.* **80**:324–332 (1961).
18. Mooser, G., H. Schulman, and D. S. Sigman. Fluorescent probes of acetylcholinesterase. *Biochemistry* **11**:1595–1602 (1972).
19. Brimijoin, S., K. P. Mintz, and M. C. Alley. Production and characterization of separate monoclonal antibodies to human acetylcholinesterase and butyrylcholinesterase. *Mol. Pharmacol.* **24**:513–520 (1983).
20. Taylor, P., and S. Lappi. Interaction of fluorescence probes with acetylcholinesterase. The site and specificity of propidium-binding. *Biochemistry* **14**:1989–1997 (1974).
21. Berman, H. A., J. Yguerabide, and P. Taylor. Fluorescence energy transfer on acetylcholinesterase: spatial relationship between peripheral site and active center. *Biochemistry* **19**:2226–2235 (1980).
22. Bach, J. F. Antigens, in *Immunology* (J. F. Bach, ed.). John Wiley and Sons, New York, 129–162, (1982).
23. Meflah, K., S. Bernard, and J. Massoulié. Interactions with lectins indicate differences in the carbohydrate composition of the membrane-bound enzymes acetylcholinesterase and 5′-nucleotidase in different cell types. *Biochimie* **66**:59–69 (1984).

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