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Acetylcholinesterase

I. A Study by Nuclear Magnetic Resonance of the Binding of Inhibitors to the Enzyme

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SUMMARY

A nuclear magnetic resonance method has been used to study the binding of atropine and eserine to purified squid acetylcholinesterase (EC 3.1.1.7). The dissociation constant, K_D, and the linewidth of the acetylcholinesterase-inhibitor complex, $\Delta\nu_{bound}$, for atropine and eserine were estimated from the linewidth changes of the *N*-methyl and phenyl group resonances of atropine and from the *N*-methyl and *C*-methyl group resonances of eserine resulting from association with the enzyme. The results indicate that there is at least one binding site on the enzyme surface for atropine and one for eserine. Further evidence that the two sites are distinct is demonstrated by the fact that gallamine displaces atropine from its site without competing with eserine.

INTRODUCTION

Since Changeux *et al.* (1, 2) found that acetylcholinesterase (EC 3.1.1.7) has binding sites for acetylcholine, gallamine, and *d*-tubocurarine which are distinct from the active center, this enzyme has attracted renewed interest as a possible physiological receptor for acetylcholine. Others (3, 4) have confirmed the existence of noncatalytic sites on the enzyme which are capable of binding certain cholinergic ligands. The aim of the present work was to investigate the binding of atropine and eserine to a highly purified squid enzyme, using a high-resolution nuclear magnetic resonance (NMR) method.

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The binding of inhibitors to enzymes is usually studied by kinetic methods or by equilibrium dialysis. Both techniques, however, have inherent limitations. The nature of the interaction can be studied in more detail by NMR methods (5).

With NMR spectroscopy, two types of change are commonly observed in the spectrum of a small molecule when it binds to a macromolecule. The linewidths of the protons of a small molecule give information about the degree of restriction of motion of its specific parts, the degree of saturation of binding sites, and rates of exchange. In addition, chemical shift changes for some protons of a small molecule give information on its magnetic environment when it is bound. Such an effect could result from nearby aromatic systems or paramagnetic ions. A recent review summarizes the application of NMR spectroscopy to biochemical problems (5).

THEORY

For the equilibrium $E + S \rightleftharpoons ES$, describing enzyme–small molecule association:

$$K_D = \frac{[E][S]}{[ES]} \tag{1}$$

where $[E] = E_0 - [ES]$ and $[S] = S_0 - [ES]$. $[ES]$ is the concentration of the enzyme–small molecule complex, $[S_0]$ is the total concentration of the small molecule and $[E_0]$ is the total concentration of enzyme.

For the situation in which a small molecule binds reversibly to an enzyme, the observed linewidth of a proton on the small molecule is given by

$$\Delta\nu_{obs} = P_{ES}\,\Delta\nu_{ES} + P_S\,\Delta\nu_S + \left(\frac{1}{\pi T_2}\right)_{ex} \tag{2}$$

where P_{ES} and P_S are the fractions of the small molecule bound and free in solution, $\Delta\nu_{ES}$ and $\Delta\nu_S$ are the line widths of the bound and free species, respectively, and $(1/\pi T_2)_{ex}$ is the contribution to the observed linewidth due to exchange (6).

If the exchange lifetime is much less than $1/\Delta\nu_{ES}$, then

$$\Delta\nu_{obs} = P_{ES}\,\Delta\nu_{ES} + P_S\,\Delta\nu_S \tag{3}$$

or

$$\Delta\nu_{obs} = \left(\frac{[ES]}{[S_0]}\right)\Delta\nu_{ES} + \left(\frac{S_0 - [ES]}{S_0}\right)\Delta\nu_S \tag{4}$$

In all experiments $S_0 \gg [E_0]$ so that $(S_0 - [ES])/S_0 = 1$ and

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\,\Delta\nu_{ES} + \Delta\nu_S \tag{5}$$

All values of $\Delta\nu$ were corrected by subtracting from the measured values of $\Delta\nu$ the values of $\Delta\nu_S$. Therefore

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\,\Delta\nu_{ES}, \qquad [ES] = \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}\,[S_0] \tag{6}$$

Substitution of Eq. 1 leads to Eq. 7:

$$K_D = E_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] - \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}\,S_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] \tag{7}$$

If $\Delta\nu_{obs} \ll \Delta\nu_{ES}$,

$$S_0 = \frac{E_0\,\Delta\nu_{ES}}{\Delta\nu_{obs}} - K_D \tag{8}$$

A plot of S_0 vs. $1/\Delta\nu_{obs}$ gives a line whose intercept is $-K_D$ and whose slope is used to calculate $\Delta\nu_{ES}$ (7).

METHODS

Enzyme. Acetylcholinesterase was prepared from the head ganglia of squid (*Loligo opalescens*) obtained frozen from the U. S. Freezer Company, Monterey, Cal. The enzyme was purified by the method of Kremzner and Wilson (8). It was dialyzed against double-distilled water and stored frozen as a powder. The enzyme had a specific activity of 200 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein. The K_m value of acetylthiocholine was 58 μM.

Assuming that squid acetylcholinesterase is similar to the eel enzyme [specific activity of crystalline eel acetylcholinesterase = 730 mmoles of acetylcholine hydrolyzed per hour per milligram of protein (9)], the enzyme used in this study would be about 30% pure. Protein concentrations were determined by the method of Lowry *et al.* (10).

Assay method. In some experiments the enzyme was assayed according to Ellman *et al.* (11) on a Cary 14 spectrophotometer at 25°.

Chemicals. Chemicals were obtained from the following sources: atropine sulfate, atropine hydrochloride, and eserine (physostigmine) hydrochloride, Mann Research Laboratories; eserine sulfate, Nutritional Biochemicals Corporation; neostigmine bromide, Pierce Chemical Company; edrophonium chloride, gift from Hoffmann-La Roche; gallamine triethiodide, gift from Poulenc Pharmaceutical Company; car-

bamylcholine chloride, Aldrich Chemical Company; and deuterium oxide (D_2O), Merck Pharmaceutical Company.

Magnetic resonance techniques. Solutions were made up volumetrically from D_2O stocks of known pH and concentration. The buffer used in most experiments was 0.1 M sodium phosphate, pH 7.4. All pH measurements were made on a Radiometer model 26 pH meter. The pH values given correspond to actual meter readings and were not corrected for the deuterium isotope effect.

Acetylcholinesterase was prepared in 0.1 M phosphate buffer and brought to pH 7.4 by the addition of small amounts of 0.1 M NaOD. For the inhibitor linewidth measurements, a stock inhibitor solution was prepared (atropine or eserine sulfate = 1.0 M in D_2O) and added to a solution of acetylcholinesterase (specific activity, 200 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein), and the individual solutions were brought to the desired pH by the addition of small amounts of 0.1 M NaOD. The final inhibitor concentration ranged from 4 to 14 mM in a total volume of 0.5 ml.

NMR spectra were obtained with a Varian A-60D high-resolution spectrometer. Chemical shifts are expressed in Hertz from tetramethylsilane as external standard. Inhibitor binding studies were made at a sweep scan of 2 Hz/cm and a sweep rate of 0.2–0.4 Hz/sec. Linewidths reported are averages for a total of at least six up- and downfield scans.

All measurements were carried out at a probe temperature of 39°. Every sample was equilibrated at 39° for at least 5 min in a water bath before introduction into the probe. The time between preparation of samples and determination of spectra did not exceed 2 hr, to minimize decomposition of labile compounds.

RESULTS

The NMR spectrum of atropine hydrochloride (40 mM) in D_2O containing 0.1 M phosphate buffer, pH 7.4, exhibits resonances at 444 and 161 Hz, which can be assigned to the phenyl and *N*-methyl group protons,

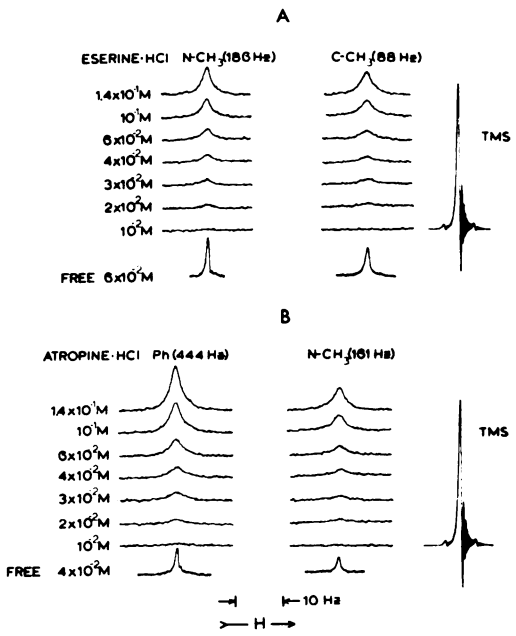

FIG. 1. *NMR spectra of eserine and atropine*
A. NMR spectra of the *N*-methyl and *C*-methyl resonances of eserine HCl, both free (lower trace) and in the presence of acetylcholinesterase (17 μM) (upper traces). B. NMR spectra of the phenyl and *N*-methyl resonances of atropine HCl, both free (lower trace) and in the presence of acetylcholinesterase (17 μM) (upper traces). The intense resonances at right are those of protons of an external tetramethylsilane (TMS) standard. Samples were dissolved in 0.1 M phosphate buffer, pH 7.4, in D_2O, and spectra were recorded at 39°.

respectively. The spectrum of eserine hydrochloride (60 mM) consists of a resonance at 186 Hz due to the *N*-methyl group and a resonance at 88 Hz due to the *C*-methyl group in the fused ring system. Because these were the most intense resonance in the spectra, they were best suited for our present study. Figure 1 shows these resonances at 60 MHz with tetramethylsilane as an external standard. The addition of purified acetylcholinesterase (specific activity, 200 mmoles of acetylthiocholine hydrolyzed per hour per milligram of protein; final concentration, 17 μM to a sample of eserine or atropine hydrochloride at various concentrations (10–140 mM) resulted in the spectra shown in Fig. 1A and B, respectively. The resonances of both atropine and eserine

were extensively broadened without a change in their shifts.

As proof that the effects observed in the NMR spectra of atropine and eserine in the presence of acetylcholinesterase were due to association between the inhibitors and the enzyme rather than to increased viscosity effects in the protein solution, the NMR spectra of atropine and eserine in similarly concentrated solutions of albumin were also recorded. It was found that the addition of bovine serum albumin (5–10 mg/ml) to atropine hydrochloride (20 mM) or eserine hydrochloride (20 mM) produced no linewidth changes. The solution of albumin (10 mg/ml) had the same viscosity as a solution of acetylcholinesterase (4 mg/ml) used in these studies. The concentration of atropine or eserine itself did not affect the linewidths of the respective resonances. No concentration-dependent broadening was observed over the concentration range of 5 to 100 mM.

Figure 1 illustrates that the *N*-methyl and aromatic resonances of atropine and the *N*-methyl and *C*-methyl resonances of eserine become progressively broader as the concentration of inhibitor is decreased. It is evident from these observations that the chemical exchange of both inhibitors between free and enzyme-bound species is rapid.

As described under THEORY, if the enzyme-inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of enzyme, a plot of S_0 with respect to $1/\Delta\nu_{obs}$ should yield a straight line with a slope of $E_0\,\Delta\nu_{ES}$ and an intercept equal to $-K_D$, provided that the conditions described above are met.

The linewidth data obtained for atropine and eserine are plotted in this manner in Fig. 2. In these experiments E_0 was held fixed at 17 μM. Variations of S_0 with $1/\Delta\nu_{obs}$ are linear for both peaks of atropine and eserine over the range of conditions used.

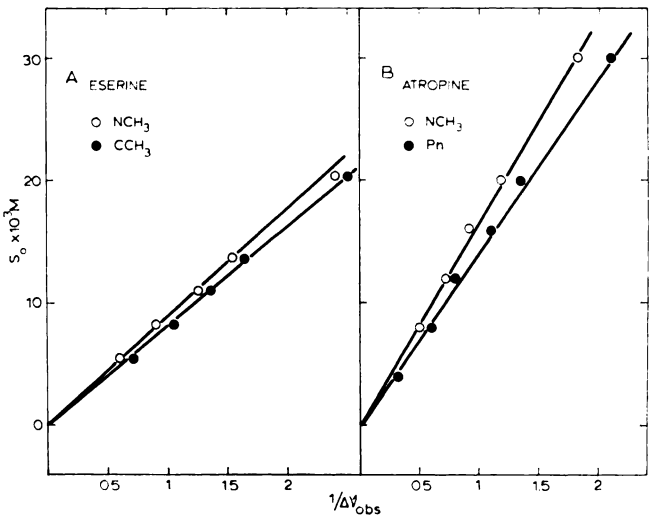

FIG. 2. *Linewidth data for eserine and atropine at fixed enzyme concentration with substrate concentration varied.*

A. Plot of the reciprocal of the observed linewidth ($\Delta\nu_{obs}$) for the *N*-methyl and *C*-methyl group resonances of eserine with respect to various concentrations of eserine sulfate (S_0) in the presence of a constant concentration of acetylcholinesterase (17 μM). B. Plot of the reciprocal of the observed linewidth ($\Delta\nu_{obs}$) for the phenyl and *N*-methyl group resonances of atropine with respect to various concentrations of atropine sulfate (S_0) in the presence of a constant concentration of acetylcholinesterase (17 μM). Samples were dissolved in 0.1 M phosphate buffer, pH 7.4, in D_2O, and spectra were recorded at 39°. In these experiments the linewidth of the free inhibitor, $\Delta\nu_S$, was subtracted from the observed linewidth, $\Delta\nu_{obs}$.

The following linewidths for the enzyme-inhibitor complexes and dissociation constants are calculated from the least-squares lines: for eserine, $\Delta\nu_{ES,NCH_3} = 496 \pm 26$ Hz; $K_D = 5.9 \pm 2.4 \times 10^{-4}$ M; $\Delta\nu_{ES,CCH_3} = 488 \pm 16$ Hz; $K_D = 4.3 \pm 4.0 \times 10^{-4}$ M. For atropine, $\Delta\nu_{ES,NCH_3} = 984 \pm 52$ Hz; $K_D = 1.2 \pm 5.8 \times 10^{-4}$ M; $\Delta\nu_{ES,ph} = 858 \pm 32$ Hz; $K_D = 3.0 \pm 3.9 \times 10^{-4}$ M.

The *N*-methyl and *C*-methyl groups of eserine are immobilized to the same extent when eserine binds to the enzyme, as might be expected, since both groups are on the same fused ring system. With atropine the *N*-methyl group is also immobilized to approximately the same extent as the phenyl group.

Since $K_D \ll S_0$, K_D cannot be obtained with great precision. Nevertheless it is clear that atropine and eserine bind with high affinity ($K_D < 1$ mM). The K_i values for atropine and eserine were then determined from kinetic studies, using acetylthiocholine as the substrate (11). Whereas eserine sulfate was an effective anticholinesterase ($K_i = 1.2$ μM), atropine sulfate inhibited poorly ($K_i = 6.0$ mM). If both compounds bind to the active site, both should inhibit substrate hydrolysis. Yet atropine is an ineffective anticholinesterase but binds to the enzyme with high affinity. This indicates that eserine may bind to the active site, and atropine to another.

That atropine and eserine do not bind to the same site on the enzyme surface was confirmed by adding gallamine triethiodide to a solution of acetylcholinesterase containing either atropine or eserine; gallamine reduced the amount of bound atropine but not eserine (Table 1). Gallamine and atropine therefore compete for the same site on the enzyme, but gallamine and eserine do not.

Carbamylcholine chloride, which structurally resembles acetylcholine but is not hydrolyzed by acetylcholinesterase, competes with eserine binding (Table 2). Carbamylcholine also competes with the binding of atropine, but only with the *N*-methyl group; the binding of the phenyl group remains unchanged.

TABLE 1

Linewidth data for N-methyl and phenyl protons of atropine and N-methyl protons of eserine, obtained from their association with acetylcholinesterase in the absence and presence of gallamine triethiodide

Measurements were made in 0.1 M phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer.

Inhibitor	Concentration		$\Delta\nu_{obs}$[a]	$\Delta\Delta\nu$
	Inhibitor	Acetylcholinesterase		
	M	*mg/ml*	*Hz*	*Hz*
Atropine	0.02	0	1.5	
N-methyl	0.02	4	3.9	2.4
	0.02	4	2.8	1.3
+ Gallamine	0.04			
Atropine phenyl	0.02	0	1.0	
	0.02	4	3.1	2.1
	0.02	4	2.2	1.2
+ Gallamine	0.04			
Eserine	0.02	0	1.2	
N-methyl	0.02	4	2.8	1.6
	0.02	4	3.0	1.8
+ Gallamine	0.04			

[a] The values given are measured linewidths corrected for instrumental broadening (0.4 Hz).

DISCUSSION

The results of this work indicate that acetylcholinesterase has groups outside its active site for binding certain inhibitors, such as atropine. The most decisive evidence that the atropine-binding site is distinct from the active site comes from the following reasoning and observations. If we assume that atropine and eserine bind at the same site, then any compound able to compete with atropine at its binding site should compete with eserine. If, on the other hand, the atropine and eserine sites are distinct, one may expect to find compounds able to displace atropine from its site without necessarily competing with the substrate or eserine. The latter possibility is confirmed by the finding that gallamine inhibits atropine binding without inhibiting eserine binding.

TABLE 2

Linewidth data for N-methyl and phenyl protons of atropine and N-methyl and C-methyl protons of eserine, obtained from association with acetylcholinesterase in the absence and presence of carbamylcholine chloride (carbachol)

Measurements were made in 0.1 M phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer.

Inhibitor	Concentration		$\Delta\nu_{obs}$[a]	$\Delta\Delta\nu$
	Inhibitor	Acetylcholinesterase		
	M	*mg/ml*	*Hz*	*Hz*
Atropine	0.02	0	1.5	
N-methyl	0.02	4	3.5	2.0
	0.02	4	2.7	1.2
+ Carbachol	0.10			
Atropine phenyl	0.02	0	1.0	
	0.02	4	2.5	1.5
	0.02	4	2.5	1.5
+ Carbachol	0.10			
Eserine	0.02	0	1.2	
N-methyl	0.02	4	3.2	2.0
	0.02	4	2.3	1.1
+ Carbachol	0.10			
Eserine *C*-methyl	0.02	0	1.4	
	0.02	4	3.8	2.4
	0.02	4	3.0	1.6
+ Carbachol	0.10			

[a] See Table 1.

Gallamine decreased the linewidth of both phenyl and *N*-methyl groups of atropine in the presence of enzyme, as expected under the present conditions, since both gallamine and atropine have a phenyl and a charged quaternary ammonium group. The phenyl group of gallamine competes with the phenyl group of atropine, and the *N*-ethyl group of gallamine displaces the *N*-methyl group of atropine.

The results with carbamylcholine are less clear. One explanation is that there are two different binding sites for atropine. In this case the atropine molecule binds via the *N*-methyl group at one site and via the phenyl group at the other. Carbamylcholine may interfere with the binding of atropine at the first site without affecting the second. In the presence of carbamylcholine, therefore, atropine would remain bound at the second site via the phenyl group. This is not unlikely, since carbamylcholine does not contain an unsaturated ring. The second possibility is that atropine binds via both the *N*-methyl and phenyl groups and that carbamylcholine interferes with the binding of the *N*-methyl group only, leaving the phenyl group bound. This is consistent with the idea that both groups of atropine are necessary for maximum binding to the enzyme (12, 13).

The use of NMR provides information regarding the extent of immobilization of a small molecule through association with a macromolecule. Assuming a molecular weight of 260,000 for acetylcholinesterase (14), the predicted linewidth of various types of protons would be 260 Hz (15). The values of the linewidth for bound atropine (858–984) and eserine (488–496) are larger than would be predicted, but are within the same order of magnitude.

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