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Selective Labeling of Anionic Binding Sites of the Acetylcholinesterase from *Torpedo californica* with a Photoaffinity Label

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SUMMARY

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The synthesis of 4-azido-2-nitrobenzyltrimethylammonium fluoroborate, a photoaffinity label for cholinergic ligand binding sites, is described. The effect of this compound on acetylcholinesterase from *Torpedo californica* electric tissue has been investigated. In the dark it inhibits the enzyme reversibly, with a mixed competitive-noncompetitive mechanism (K_i = 7 μM). After irradiation at a wavelength of about 350 nm, the compound binds irreversibly and highly specifically to anionic binding sites of the enzyme. This was shown by experiments with the radioactive label and with cholinergic ligands containing quaternary ammonium groups, which protect the enzyme against inactivation and incorporation of the label. Experiments with Tetram and its triethyl derivative indicate that mainly the anionic subsite within the active site is labeled. After protection of the active site with edrophonium or carbamoylcholine, a peripheral anionic site can be blocked and labeled selectively with the photoaffinity label. This labeling of the peripheral site alters the reversible inhibition by the photolabel in the dark from a mixed competitive-noncompetitive mechanism to a purely competitive pattern (K_i = 16 μM for competition of the photoaffinity label in the dark with the substrate acetylthiocholine).

INTRODUCTION

The photoaffinity label 4-azido-2-nitrobenzyltrimethylammonium fluoroborate has been used as a valuable tool for specifically labeling the acetylcholine receptor protein from *Torpedo californica* electric tissue (1, 2). With this reagent it was possible to obtain information about the quaternary structure and binding properties of the receptor subunits. In this paper we describe the synthesis of the label and its application to the investigation of acetylcholinesterase.

The properties of acetylcholinesterase with respect to its various binding sites still appear very complex. It is now widely accepted that the active center of the enzyme contains an esteratic and an anionic subsite (3). In addition to this, several authors assume the existence of a second anionic binding site outside the active center, the peripheral anionic site (4-8) (see Scheme 2, I). It is believed to have a regulatory function, as shown by kinetic stud-

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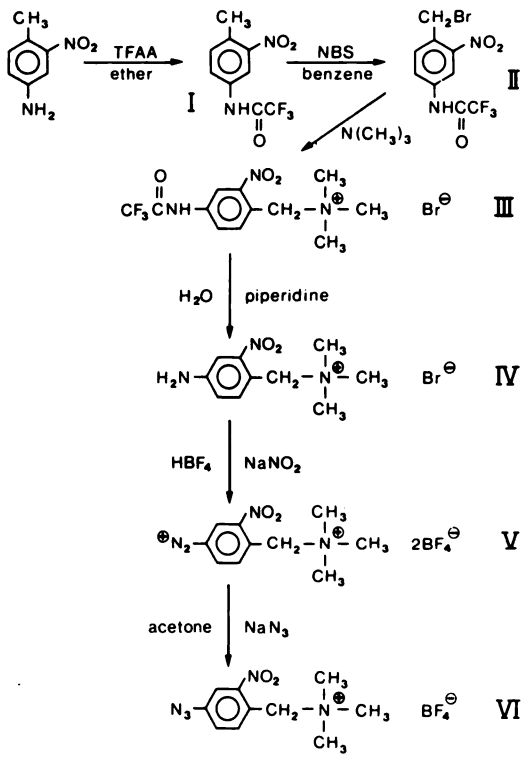
ies and fluorescence spectroscopic methods (9). Other models postulate three anionic binding sites (10), and some authors stress the importance of the ionic microenvironment (ionic strength and pH) rather than allosterism as a regulatory parameter (11).

We wished to learn whether it is possible to differentiate between the various functional sites with the aid of a covalent (and radioactive) marker. Selective blocking of these sites will facilitate the interpretation of kinetic measurements. [A successful attempt in this direction with a water-soluble carbodiimide has been published recently (12).] Furthermore, selective blockade should open the way for isolation and sequencing of the peptides involved.

MATERIALS AND METHODS

4-Azido-2-nitrobenzyltrimethylammonium fluoroborate (Scheme 1) was prepared as described below. 4-Methyl-3-nitroaniline was obtained from Chemicals Procurement Laboratories, College Point, N. Y. All chemicals were of the purest commercially available grade. All intermediates were characterized by NMR. The tritiated compound had a specific activity of 59.5 mCi/mmole. Starting from compound II (Scheme 1), synthesis of the radioactive compound VI was performed by NEN Chemicals, Dreieichenhain, Germany.



SCHEME 1. *Synthesis of 4-azido-2-nitrobenzyltrimethylammonium fluoroborate*

TFAA, trifluoroacetic acid; NBS, *N*-bromosuccinimide.

Synthesis of 4-Azido-2-nitrobenzyltrimethylammonium Fluoroborate (VI, Scheme 1)

4-Methyl-3-nitrotrifluoroacetanilide (I). 4-Methyl-3-nitroaniline (45 g, 296 mmoles) was dissolved in 500 ml of dry ether, filtered, and added slowly with stirring to a cooled solution of 63 g (0.3 mole) of trifluoroacetic anhydride in 200 ml of ether. The ether solution was treated with activated charcoal, and the trifluoroacetylated product was precipitated by the addition of petroleum ether (b.p. 30–60°): light yellow needles, m.p. 126–128°, recrystallized from ether–petroleum ether; yield, 70 g (95%).

4-Bromomethyl-3-nitrotrifluoroacetanilide (II). *N*-Bromosuccinimide (35.6 g, 0.2 mole) and 4-methyl-3-nitrotrifluoroacetanilide (45 g, 0.18 mole) were dissolved in 500 ml of dry benzene and heated to reflux under nitrogen. The reaction was initiated with 200 mg of dibenzoyl peroxide and sustained under a 275-W G. E. sunlamp for 2–5 hr, depending on the purity of the starting materials. To determine the completion of the reaction, 1 ml of the reaction mixture was removed, ether was added, and the melting point of the precipitate was determined (succinimide, m.p. 124–126°). The benzene was then evaporated under vacuum and ether was added. Upon cooling the mixture, succinimide precipitated and was filtered off. The ether solution was then evaporated, and the product was recrystallized from chloroform. Yield, 32 g (54%); m.p. 99–100°; almost colorless fluffy needles.

4-Trifluoroacetamide 2-nitrobenzyltrimethylammonium bormide (III). To 50 ml of dry ether containing 3.0 g (9.16 mmoles) of 4-bromomethyl-3-nitrotrifluoroacetanilide, 1.2 g (20 mmoles) of dry trimethylamine in 10 ml of dry ether were added.

After standing at room temperature for 2 hr, the precipitated product was filtered off, dissolved in hot methanol, treated with charcoal, evaporated, and recrystallized from absolute ethanol. Yield, 3.5 g (91%) of light yellow needles, m.p. 220–221°.

4-Amino-2-nitrobenzyltrimethylammonium bromide (IV). To remove the trifluoroacetyl group, 5.0 g of III were heated on a steambath in 5 ml of a 1 M aqueous piperidine solution for 5 min. The free base crystallized from the reaction mixture upon cooling in ice and was filtered and recrystallized from water. Yield, 3.1 g (81%) of yellow leaflets, m.p. 200°.

4-Diazo-2-nitrobenzyltrimethylammonium difluoroborate (V). Compound IV (1.2 g, 4.14 mmoles) was dissolved in 10 ml of cold (0–5°) 50% HBF_4 and stirred in an ice bath. A cold solution of 285 mg (4.13 mmoles) of $NaNO_2$ in 2 ml of water was added dropwise to the stirred solution of the amine. The reaction mixture was stirred for 15 min, filtered, and washed with cold ethanol. Yield, 1.5 g (25%), m.p. 164° with decomposition.

4-Azido-2-nitrobenzyltrimethylammonium fluoroborate (VI). One gram (2.0 mmoles) of diazonium fluoroborate (V) was dissolved in 40 ml of acetone cooled in an ice-salt mixture (−10°). Then 260 mg (4.0 mmoles) of NaN_3 dissolved in 1 ml of H_2O was added dropwise in the dark, followed by 0.5 ml of HBF_4 (50%) (2.85 mmoles). The reaction mixture was stirred for 30 min, evaporated on a rotary evaporator at room temperature to one-third its volume, and cooled in a refrigerator (5°) for 10 min. The precipitated $NaBF_4$ was filtered off, and absolute ethanol was added (5 ml). The azide crystallized from this solution in a freezer within 30 min; 600 mg (98%) of faintly yellow needles were obtained with m.p. 135° (decomposition) after recrystallization from ethanol–acetone.

The NMR spectrum showed a singlet at 3.02 ppm (9 Hz), a singlet at 4.80 ppm (2 Hz), and a multiplet centered at 7.60 ppm (3 Hz). The spectra were taken on a Jeolco PS-100 spectrometer at the Salk Institute for Biological Studies, La Jolla. Sodium 2,2-dimethyl-2-silapentanesulfonate was used as an internal standard in D_2O. Ultraviolet absorption maxima in water as a solvent were found at 251 nm (= 22,000 cm^2 $mole^{-1}$) and 324 nm (= 2200 cm^2 $mole^{-1}$). The infrared spectrum (KBr) showed an absorption for the N_3 group at 4.64 μm.

Effectors

Carbamoylcholine was purchased from Merck, Darmstadt; hexamethonium, from Sigma Chemical Company; gallamine (Flaxedil), from Boehringer, Ingelheim; and propidium, from Calbiochem. Edrophonium was synthesized from 3-hydroxyphenyldimethylamine (Fluka) by quaternization with ethyl iodide.

Preparation of Acetylcholinesterase

Five grams of electric tissue from *Torpedo californica* frozen in liquid nitrogen were cut in small pieces. After addition of 10 ml of water, the mixture was homogenized at 0° for 1 min at maximum speed with an Ultra Turrax homogenizer. The homogenate was centrifuged for 30 min at 30,000 × *g*. The supernatant contained acetylcholinesterase with a specific activity of 0.6–1.0 mmole/hr/mg.

Assay of Acetylcholinesterase

Activity was measured according to Ellman *et al.* (13), using acetylthiocholine as substrate. In the cuvette 2.8 ml of 0.1 M potassium phosphate buffer (pH 8.0) were mixed with 20 μl of 75 mM substrate, 100 μl of 10 mM dithiobisnitrobenzoate, 60 μl of effector solution at various concentrations (control, 60 μl of water), and 20 μl of enzyme solution. The change in optical density was measured at 405 nm in an Eppendorf photometer (20°). No photoaffinity labeling occurred in competition experiments with the photolabel present during the assay, as the label has its absorption maxima at 251 nm and 324 nm, i.e., below the wavelength used for the assay. Protein concentration was determined according to Lowry *et al.* (14).

Photoaffinity Labeling

Enzyme solution (450 μl in Ringer's solution: 0.16 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 3 mM phosphate

buffer, pH 7.0) was incubated with the appropriate amounts of photoaffinity label and effector (see legends to figures) and placed in a 10-ml beaker. Irradiation was performed at room temperature with an ultraviolet lamp (Sterisol F 1140, 5241, Quarzlampen GmbH, Hanau) mounted 5.5 cm above the surface of the sample. The sample was covered with a glass slide to protect it against ultraviolet light below 300 nm, which might affect the protein. The path length for the incident light in the sample was about 2 mm. Irradiation time was 10 min. The reaction was terminated by turning off the light. Excess photoaffinity label was removed by extensive dialysis.

RESULTS

Binding Properties of 4-Azido-2-nitrobenzyltrimethylammonium Fluoroborate

Covalent binding of photoaffinity label. 4-Azido-2-nitrobenzyltrimethylammonium fluoroborate in the absence of irradiation is a reversible inhibitor of the acetylcholinesterase (Fig. 1). The double-reciprocal plot shows a mixed inhibition pattern, indicating that the compound does not simply compete with the substrate for the active center of the enzyme. The inhibitor constant (K_i) determined from a Dixon plot (not shown) is 7 μM. After irradiation of the enzyme–photoaffinity label complex the enzyme becomes inactive. This inactivation cannot be reversed by dialysis. Experiments with tritium-labeled reagent resulted in the irreversible incorporation of radioactivity. At 75% inactivation, for example, the amount of incorporated label was 3–6 nmoles/mg of protein. This radioactivity could not be removed by prolonged dialysis, even under denaturing conditions.

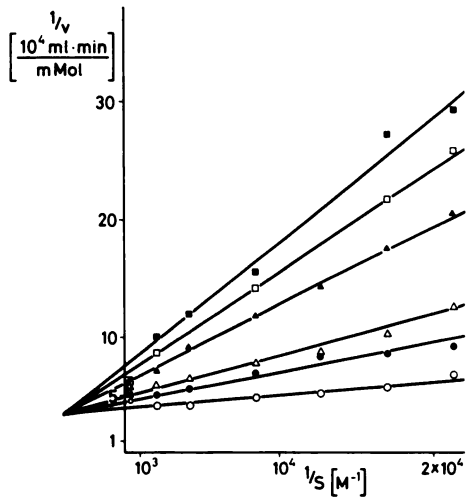


FIG. 1. *Lineweaver-Burk plot of 4-azido-2-nitrobenzyltrimethylammonium fluoroborate inhibition of acetylcholinesterase activity, using acetylthiocholine as substrate*

The protein concentration in the test cuvette was 6.3 μg/ml. For other assay conditions, see MATERIALS AND METHODS. ○——○, no photoaffinity label; ●——●, 3.16 μM; △——△, 6.32 μM; ▲——▲, 15.8 μM; □——□, 29.3 μM; ■——■, 31.6 μM photoaffinity label.

Specificity of photoaffinity label for acetylcholinesterase. To test the specificity of the photoaffinity label, we applied different cholinergic effectors to protect the esterase against binding of the label to acetylcholine binding sites (Table 1). With carbamoylcholine as protecting agent, even at very high concentrations, the protective effect against incorporation of radioactivity was relatively low (29%), although protection against inactivation was almost complete. All other reversible esterase inhibitors showed high protective efficacy. Up to 88% protection against radioactive labeling could be achieved, indicating that nonspecific reaction of the label does not exceed 12%. This result suggests a high specificity of the label, because the experiments were performed with a crude enzyme preparation, i.e., in the presence of a large excess of proteins other than the esterase.

Further proof for the specificity of the label comes from control experiments with enzymes and membrane proteins with no obvious functional relationship to the label. For example, irradiation of pure glutamate dehydrogenase from beef liver mitochondria or of cytochrome b_{559} from chloroplast membranes in the presence of 0.4 mM photolabel did not result in any significant incorporation of radioactivity. Similar results were obtained with acetylcholine receptor or esterase preparations denatured by heating or trypsin digestion prior to irradiation in the presence of label.

TABLE 1

Protection against photoaffinity labeling

The concentration of photoaffinity label was 0.1 mM in experiments 1–3 and 7–10 and 0.67 mM in experiments 4–6. The acetylcholinesterase activity in the unprotected sample remained up to 75% inhibited after dialysis (depending on the photolabel and protein concentrations), whereas activity in the protected samples was completely restored after dialysis (with the exception of the irreversible inhibitors in experiments 5 and 6).

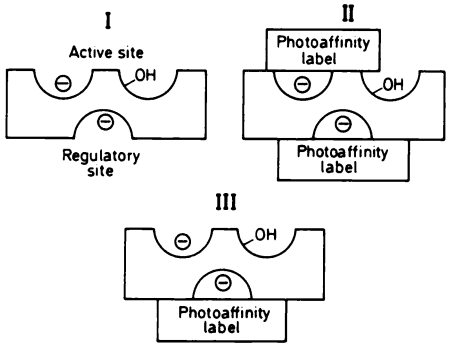
	Effector	Decrease in incorporation of radioactive photolabel
		%
1.	Carbamoylcholine (38 mM)	29
2.	Hexamethonium (38 mM)	88
3.	Benzyltrimethylammonium bromide (38 mM)	75
4.	Gallamine (0.465 mM)	77
5.	Tetram (0.465 mM)[a]	26
6.	"Quaternary" Tetram (0.465 mM)[a]	47
7.	Edrophonium (0.8 mM)[b]	58
8.	Propidium (0.8 mM)	75
9.	Propidium (0.01 mM)	0
10.	Edrophonium (0.8 mM) + propidium (0.8 mM)	81.5

[a] Both these compounds blocked the esteratic subsite irreversibly.

[b] This compound was a reversible competitive inhibitor.

Binding site specificity: protection with Tetram, carbamoylcholine, and edrophonium. Tetram, an organophosphate reacting covalently with the serine hydroxyl group of the esteratic subsite in the catalytic center of acetylcholinesterase (3), decreases the incorporation of the photoaffinity label by 26%. After quaternization of the amino group of this drug with ethyl iodide, the protective effect becomes more pronounced (47%). These experiments suggest that the photoaffinity label may react preferentially with an area remote from the serine hydroxyl group, i.e, with the anionic subsite. According to this model the slight protection of this site by Tetram could arise from steric hindrance (Scheme 2, II). This steric hindrance would be enhanced with the quaternary ammonium derivative of Tetram because of the interaction of its positive charge with the anionic subsite.

On the other hand, carbamoylcholine alos diminished incorporation of radioactivity, by only 29% (Table 1), but preserved almost completely the activity of the enzyme during photoaffinity labeling. This and the mixed competitive-noncompetitive inhibition pattern of our photoaffinity label (without irradiation) suggested binding of the label to more than one binding site (Scheme 2). The protection experiments with carbamoylcholine and the quaternary ammonium derivative of Tetram indicate that about half the label reacts with the active site, and the other half, with a peripheral site. This conclusion was confirmed by further experiments with edrophonium (3-hydroxyphenyldimethylethylammonium bromide). Its effect is quite similar (Fig. 2) to that of the corresponding trimethyl derivative (11): it acts as a nearly competitive inhibitor of the acetylcholinesterase. Assuming that this means preferential binding to the active center, we investigated the protective effect of this reagent. We allowed the enzyme to react with the photoaffinity label in the presence of edrophonium and dialyzed it after irradiation to remove the edrophonium and unbound



SCHEME 2. *Photoaffinity labeling of acetylcholinesterase*

I. Native enzyme (with unmodified subsites). II. Enzyme after photoaffinity labeling without protection. III. Enzyme with blocked peripheral (regulatory) site (photoaffinity labeling with protection of the active site by edrophonium or carbamoylcholine).

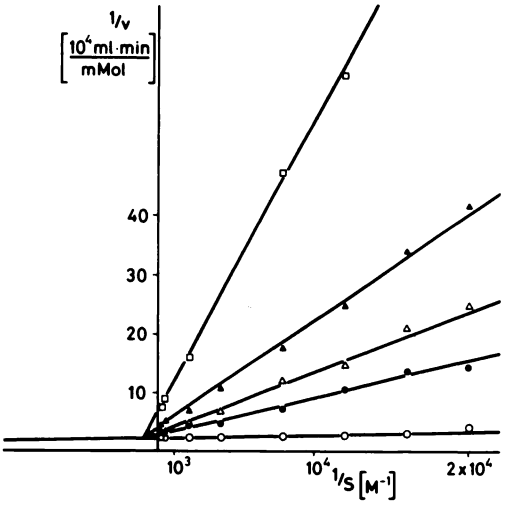


FIG. 2. *Edrophonium inhibition of acetylcholinesterase*

Protein concentration was 6.3 μg/ml. For other assay conditions see MATERIALS AND METHODS. ○——○, no edrophonium; ●——●, 1 μM; △——△, 2 μM; ▲——▲, 3.3 μM; □——□, 10 μM edrophonium.

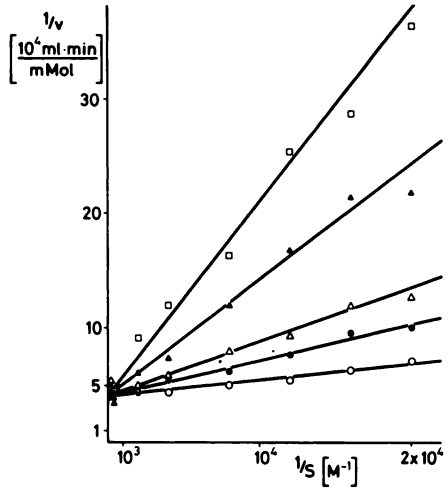


FIG. 3. *Reversible inhibition by 4-azido-2-nitrobenzyltrimethylammonium fluoroborate (in the dark) after blockade of peripheral site*

The sample was photolabeled under edrophonium-protected conditions and then dialyzed. Protein concentration was 8.7 μg/ml. For other assay conditions, see MATERIALS AND METHODS. ○——○, no photoaffinity label; ●——●, 10 μM; △——△, 33 μM; ▲——▲, 100 μM; □——□, 200 μM photoaffinity label.

photoaffinity label. The reversible inhibition by the photoaffinity label applied in the dark had now shifted from a mixed competitive-noncompetitive pattern (Fig. 1 and Scheme 2, I) to a purely competitive one (Fig. 3 and Scheme 2, III), with a K_i of 16 μM. This result may indicate that edrophonium protected the active center and the photoaffinity label covalently blocked the peripheral binding site.

A similar shift to a competitive inhibition pattern was observed after photoaffinity labeling in the presence of carbamoylcholine (Fig. 4). This compound also appears to bind preferentially to the active site and leaves the peripheral site unprotected.

The converse experiment, i.e., covalent labeling of the active center anionic site with protection of the peripheral site, proved to be more difficult. Gallamine was applied in attempts to protect the peripheral site selectively and to label the active site. Irradiation of the enzyme-photolabel complex in the presence of gallamine resulted in a 77% decrease in incorporated radioactivity (Table 1). Since the enzyme lost its activity in this experiment, one can conclude that the remaining 23% of the radioactivity is bound to the active site.

Propidium (10 μM), another ligand specifically binding to a peripheral anionic site (9, 15), did not protect the enzyme against incorporation of radioactivity (Table 1), possibly because at the high ionic strength of our solution it is not bound tightly enough. At higher concentrations it prevented photoaffinity labeling of the enzyme. However, this effect was at least partially due to its strong absorbance of the light necessary for the photoreaction.

DISCUSSION

The photoaffinity label 4-azido-2-nitrobenzyltrimethylammonium fluoroborate appears to be specific for protein sites binding quaternary ammonium groups. This has been shown by its selective reaction with the acetylcholine receptor (2) and in the present investigation with acetylcholinesterase. The specificity is shown by the observation that even in the presence of large excesses of other proteins very little label was incorporated into protein when

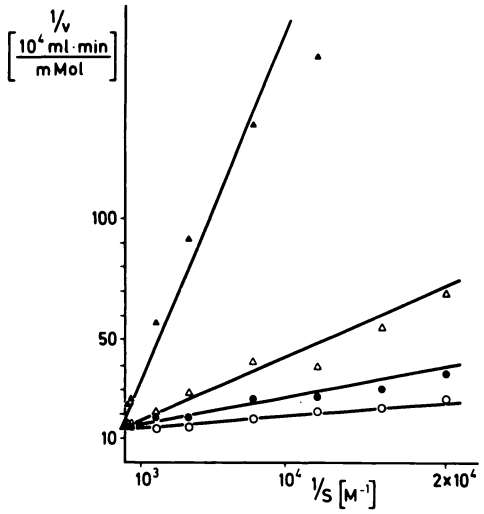


FIG. 4. *Reversible inhibition by 4-azido-2-nitrobenzyltrimethylammonium fluoroborate after blockade of peripheral site*

The sample was photolabeled under carbamoylcholine-protected conditions and then dialyzed. Protein concentration was 4.5 μ/g. For other assay conditions, see MATERIALS AND METHODS. ○——○, no photoaffinity label; ●——●, 1 μM; △——△, 3.3 μM; ▲——▲, 100 μM photoaffinity label.

the enzyme was protected by ligands specific for acetylcholinesterase. Furthermore, proteins with no functional relationship to cholinergic ligands or denatured cholinergic proteins did not react with the label even after prolonged irradiation in the presence of high concentrations of the compound and in the absence of protective ligands. The label therefore appears to be generally applicable to the detection or selective blocking of proteins binding cholinergic ligands. It must be emphasized, however, that these investigations were performed with proteins in solution. There is evidence that selectivity might be less pronounced when the label is applied to membrane-bound proteins (16), perhaps because of interactions with membrane lipids.

The mechanism of the reaction of the photoaffinity label has been discussed elsewhere (16). The possibility exists that during its lifetime the reactive nitrene intermediate which is formed from the azide by photolysis diffuses from its initial binding site and reacts with amino acid residues farther away. For similar reasons it is unlikely that the photoaffinity label reacts at its binding site with only one specific amino acid residue.

For a final determination of the exact site of the covalent modification, it will be necessary to isolate and determine the sequence of the labeled peptides. Only preliminary conclusions can be drawn from kinetic studies of the enzyme labeled under different protective conditions. The mixed competitive-noncompetitive inhibition (Fig. 1) by the photoaffinity label in the dark might indicate simultaneous binding to active and peripheral sites. However, the appearance of a noncompetitive component could also mean that the inhibitor interferes with the deacylation step of the reaction after binding solely to the active site (9, 17). Therefore more convincing evidence concerning the assumption that the photolabel also binds to a peripheral site is furnished by the observations with active site-specific ligands. Saturation of the enzyme with edrophonium or carbamoylcholine preserves the activity of the enzyme but only partly prevents incorporation of radioactive photoaffinity label. The simultaneous shift to a competitive inhibition pattern (Fig. 3) may be further proof of an irreversible blockade of the peripheral site by the label.

Conversely, it appears possible to label the active site preferentially by carrying out the photoreaction in the presence of gallamine, a ligand binding to peripheral sites of the enzyme. Gallamine greatly reduced the incorporation of the radioactive photolabel but did not prevent inactivation. This suggests that gallamine protects a peripheral site but not the active site. Further detailed kinetic and binding studies are necessary to confirm this conclusion. Furthermore, at this state of the investigation it is not possible to specify how many and which of the peripheral sites are attacked by the label. Different peripheral binding sites have been postulated for gallamine and decamethonium, for example (10).

Propidium, another ligand with a high specificity for a peripheral site (9, 15), is difficult to apply for the purpose of photoaffinity labeling the active site during protection of the peripheral site. This is

due mainly to a fundamental drawback of all photoaffinity labels: the protecting reversible ligand has to compete during a relatively long period of irradiation with a covalently binding reagent. For full protection very high ligand concentrations are required. These are often unphysiologically high, or—as appears to be the case with propidium—absorb the light necessary for the photoreaction.

In conclusion, we think that selective radioactive labeling appears to be possible, although final proof has to await sequencing of the peptides carrying the radioactivity. Aside from the importance of analysis of the binding areas of the enzyme for elucidation of the catalytic and regulatory mechanisms, the structure of these sites appears to be especially interesting from the standpoint of evolution. Acetylcholine is bound by several proteins, among others by the acetylcholine receptor of the postsynaptic membrane and acetylcholinesterase. Sequence homologies between binding site peptides from these proteins may indicate a common evolutionary origin.

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