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SHORT COMMUNICATION

Purification and Fractionation of Acetylcholinesterase into Subspecies by Affinity Chromatography on a *d*-Tubocurarine–Sepharose Column

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SUMMARY

A method is described for the covalent attachment of *d*-tubocurarine to Sepharose. The preparation was successfully used for the rapid purification of acetylcholinesterase by allosteric site affinity chromatography. Selective elution of the enzyme with NaCl solutions of various concentrations was readily achieved. Selective interference with adsorption by bisquaternary effectors, such as decamethonium, gave even better results. Of considerable interest was the observation that selective interference with adsorption of the enzyme on a tubocurarine-Sepharose column by β-D-methylsuccinyldicholine iodide led to complete separation into two subspecies in a ratio of approximately 1:1.

As part of our efforts to elucidate the physiological role of the regulatory curare-binding site of acetylcholinesterase (1, 2), the need arose for a rapid and convenient method for purification of the enzyme and especially its resolution into molecularly distinct constituents. Recently, affinity chromatography based on esteratic site-directed anchoring reagents was utilized successfully in purification (3–6), whereas ion-exchange chromatography was reported to achieve partial resolution of the enzyme from human erythrocytes into two active components, although in low yields (7, 8). Much evidence has accumulated that acetylcholinesterase from various sources consists of isozymes (8–11). The question thus arises whether these catalytically similar but isomeric species are allosterically distinguishable. Our recent demonstration through anionic site labeling studies (1, 12) that *d*-tubocurarine binds exclusively on an allosteric site of acetylcholinesterase suggested that covalent attachment of this drug to a suitable support such as Sepharose might be useful as an allosteric site-anchoring reagent for the purpose of purifying and fractionating the enzyme into molecularly distinguishable species. It was reasoned that quantitative fractionation into distinct molecular species might be readily accomplished by controlled elution with allosterically selective bisquaternary effectors of the kind recently synthesized in our laboratories[2] (12). This communication reports our success in attaining these goals.

The tubocurarine-derivatized Sepharose gel was prepared as follows. A suspension (100 ml) of Sepharose 4B (Pharmacia) in an equal volume of water was activated in the usual manner with 30 g of cyanogen bromide (13) and then treated with 100 ml of 2 mM aqueous ethylenediamine (Fisher, reagent

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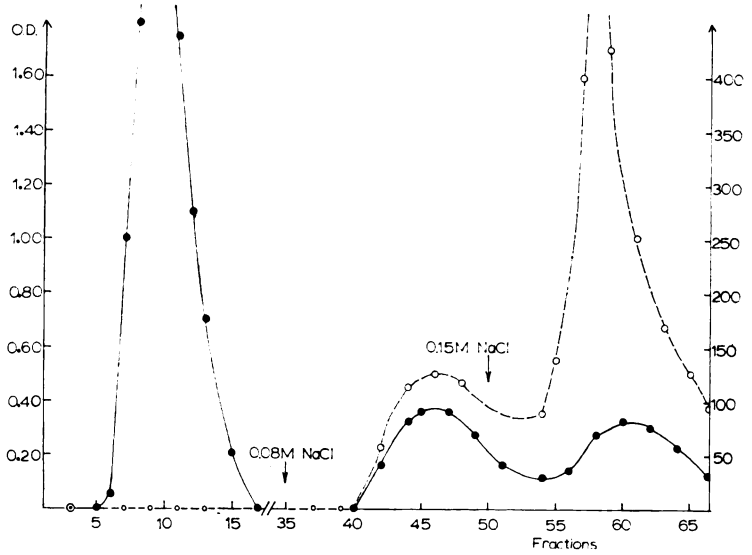

FIG. 1. *Controlled elution of erythrocyte acetylcholinesterase from a tubocurarine-Sepharose column* O- - -O, enzyme activity (units per 5.5-ml fraction), ●——●, protein concentration (O.D. = absorbance at 280 nm). From fractions 35 through 50, the NaCl concentration was gradually increased to 0.15 M.

grade). Differential titration indicated that about 12 μmoles of basic residues per milliliter of Sepharose were introduced. This "aminoethyl"-Sepharose was allowed to react at 4° with 150 mmoles of succinic anhydride at pH 6 (14), followed by washing. The acidic gel was coupled again with ethylenediamine (as above), using water-soluble 7-cyclohexyl-3(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (Aldrich) (14). The resulting amino residues were allowed to react with *p*-nitrobenzoyl azide, and the bound nitro functions were subsequently reduced at pH 9–10 with sodium dithionite at 40° (15). The *p*-aminobenzoyl residues were then diazotized in 0.5 N hydrochloric acid (15), followed by coupling with *d*-tubocurarine (Nutritional Biochemicals) (1% solution in saturated borate buffer, pH 9.5). The resulting tubocurarine-derivatized Sepharose was colored deep red and contained 7 ± 2 μmoles of bound *d*-tubocurarine per milliliter of gel [differential assay at 285 nm (16)].

Acetylcholinesterase was purified as follows from bovine erythrocytes. A commercial preparation (Winthrop Laboratories) stabilized with 300 mg of serum albumin and containing 251 units of activity[3] in 330 mg of total protein [as determined by the method of Lowry *et al.* (17)] was applied at 5° in 3 ml of distilled water to a Pharmacia column containing 50 ml of tubocurarine-derivatized Sepharose. The column was washed with water and then with NaCl solutions not exceeding 0.05 M. Washing was continued until the eluate was protein-free. Each fraction was simultaneously assayed for acetylcholinesterase activity by the pH-stat method (18). In this manner, 80% of the applied inactive proteins were eluted (70 ml). Subsequent washings with 0.08–0.15 M NaCl (170 ml) eluted 211 units of activity (87% yield) in about 15% of the applied total proteins. The results of this partial purification are summarized graphically in Fig. 1.

Elution in the presence of decamethonium bromide was carried out as follows. The same quantity of crude enzyme as above, in 3 ml of 0.01 M NaCl solution containing decamethonium bromide at 0.5 mM, was applied to a tubocurarine-Sepharose column (50 ml). The elution profile using 0.01 M

[3] One unit of enzyme hydrolyzes 1 μmole of acetylcholine per minute at 25° and pH 7.4.

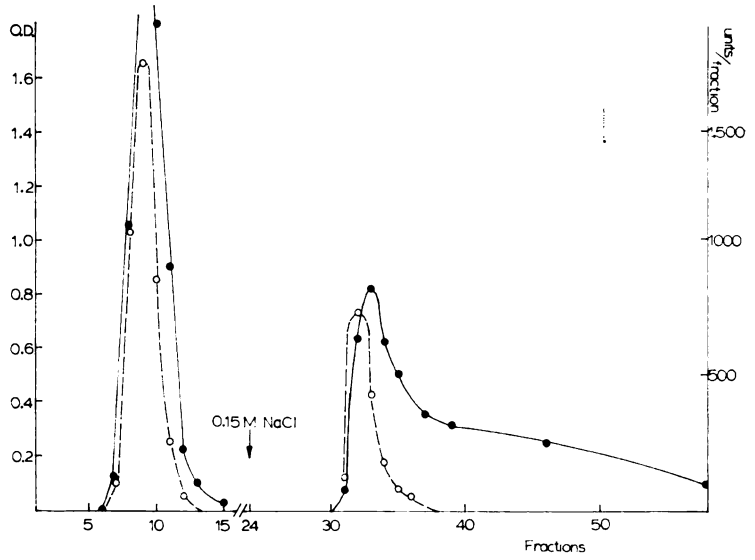

FIG. 2. *Elution of acetylcholinesterase in the presence of decamethonium bromide*
Fractions 1–24, 0.01 mM NaCl + 0.5 M decamethonium bromide; fractions 30–60, 0.15 M NaCl. O- - -O, enzyme activity; ●——●, protein concentration (O.D. = absorbance at 280 nm).

NaCl is shown in Fig. 2, where it can be seen that about 80% of the activity was desorbed when decamethonium bromide was present. The balance of 20% could eventually be eluted with stronger NaCl (0.15 M). The weak NaCl–decamethonium bromide fraction was dialyzed overnight at 5° against 0.01 M NaCl (to remove excess decamethonium bromide), and the solution was reapplied to 50 ml of tubocurarine-Sepharose. Washing with 0.01 M NaCl removed inactive proteins, but subsequent elution with 0.15 M NaCl removed all activity from the column. Thus 80-fold purification was readily achieved in about 60% over-all yield. The K_m and V_{max} characteristics of this purified enzyme (with acetylcholine as substrate) were not significantly different from those of the starting enzyme. However, whereas the latter was inhibited largely competitively by decamethonium bromide (with acetylcholine as substrate), the tubocurarine-Sepharose-purified form was inhibited noncompetitively (Fig. 3). It is clear that the allosteric site-anchoring reagent *d*-tubocurarine profoundly alters the molecular structure of the enzyme oligomers or removes a constituent controlling the binding properties and quaternary structure of the enzyme. The molecular parameters underlying this observtion are under investigation.

Fractionation into subspecies was accomplished as follows. To 1-ml aliquots of a solution, each containing 3.35 units of crude acetylcholinesterase in 0.01 M NaCl, were added gradually increasing concentrations of β-D-methylsuccinyldicholine iodide, and each mixture applied separately to 6 ml of tubocurarine-Sepharose. The columns were then washed with the same solution until all elutable activity was removed. The results are summarized in Fig. 4, where it can be seen that when β-D-methylsuccinyldicholine iodide reached concentrations ranging from 60 to 100 μM in the applied solutions, the quantity of absorbed enzyme (approximately 50% of the total) reached a plateau. The totality of this adsorbed portion was recovered in the eluates only at initial β-D-methylsuccinyldicholine iodide concentrations greater than 100 μM. The sum of the activities in the two fractions obtained from this column accounted for 90% of the initial activity. Disc electrophoresis on acrylamide gel [band visualization by the acetylthiocholine method (19) combined with protein staining] showed single sharp bands of mobility comparable to that produced by the

crude enzyme. However, the regulatory properties of these two species were readily distinguishable on the basis of their kinetic behavior toward methanesulfonyl fluoride (2). From the molecular point of view, it is clear that the presence of decamethonium bromide or β-D-methylsuccinyldicholine iodide on the enzyme alters the quaternary

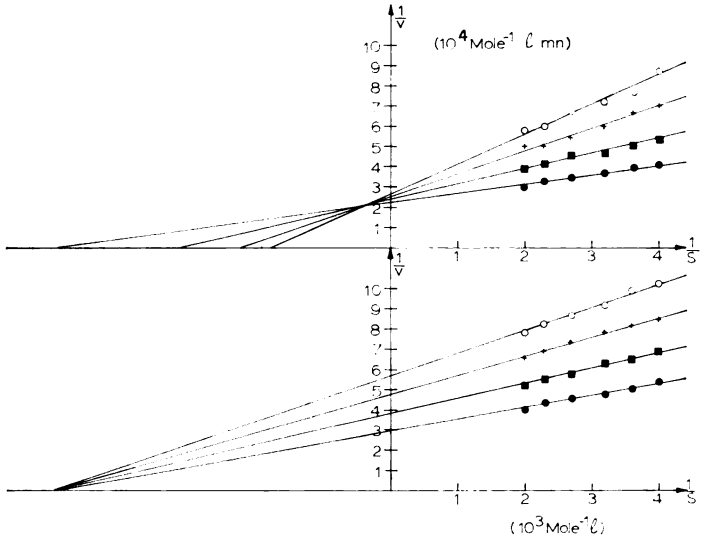

FIG. 3. *Reciprocal plots for decamethonium bromide inhibition of crude (upper graph) and purified acetylcholinesterase (lower graph) as in Fig. 1*

●——●, controls; ■——■, decamethonium bromide at 3.3 μM; +——+, decamethonium bromide at 6.6 μM; ○——○, decamethonium bromide at 10 μM. The assay was performed in 0.15 M NaCl at 25° and pH 7.4 by the pH-stat method.

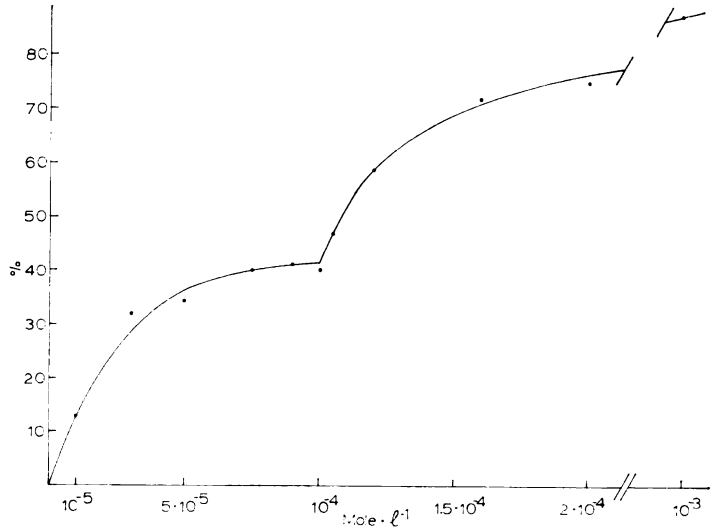

FIG. 4. *Fractionation of acetylcholinesterase on tubocurarine Sepharose with increasing concentrations of β-D-methylsuccinyldicholine iodide*

Each point represents the percentage of enzyme retained on the column at each initial concentration of β-D-methylsuccinyldicholine iodide followed by washing with the same solution until no more activity was elutable by that solution. Between 60 and 100 μM a constant proportion remains absorbed.

structure in such a manner that the affinity of the curare-binding site (or sites) for the regulatory center is drastically modified. The fact that acetylcholinesterase initially adsorbed on tubocurarine-Sepharose was hardly elutable by the same effectors supports this conclusion. Parallel experiments with acetylcholinesterase from *Electrophorus electricus* will be reported.

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REFERENCES

1. B. Belleau and V. DiTullio, *Can. J. Biochem.* **49**, 1131 (1971).
2. B. Belleau, V. DiTullio and Y. H. Tsai, *Mol. Pharmacol.* **6**, 41 (1970).
3. J. D. Berman and M. Young, *Proc. Nat. Acad. Sci. U. S. A.* **68**, 395 (1971).
4. N. Kalderon, I. Silman, S. Blumberg and Y. Dudai, *Biochim. Biophys. Acta* **207**, 560 (1970).
5. T. I. Rosenberg, H. W. Chang and Y. T. Chen, *J. Amer. Chem. Soc.* **94**, 1555 (1972).
6. Y. Dudai, I. Silman, K. Kalderon and S. Blumberg, *Biochim. Biophys. Acta* **268**, 138 (1972).
7. T. Shafai and J. A. Cortner, *Biochim. Biophys. Acta* **236**, 612 (1971).
8. T. Shafai and J. A. Cortner, *Biochim. Biophys. Acta* **250**, 117 (1971).
9. J. Massoulie and F. Rieger, *Eur. J. Biochem.* **11**, 441 (1969).
10. A. R. Main, *J. Biol. Chem.* **244**, 829 (1969).
11. M. E. Edelfrawi, R. K. Tripathi and R. D. O'Brien, *Biochim. Biophys. Acta* **212**, 308 (1970).
12. B. Belleau and V. DiTullio *in* "Drugs and Cholinergic Mechanisms in the CNS" (E. Heilbronn and A. Winter, eds.), p. 441. Research Institute of National Defence, Stockholm, 1970.
13. R. Axen, J. Porath and S. Ernback, *Nature* **214**, 1302 (1967).
14. P. Cuatrecasas, *Nature* **228**, 1327 (1970).
15. P. Cuatrecasas, *J. Biol. Chem.* **245**, 3059 (1970).
16. J. D. Dutch, *J. Amer. Chem. Soc.* **74**, 2221 (1952).
17. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
18. B. Belleau and H. Tani, *Mol. Pharmacol.* **2**, 411 (1966).
19. J. M. Karnovsky and L. Roots, *J. Histochem. Cytochem.* **12**, 219 (1964).