Proc. Nat. Acad. Sci. USA
Vol. 70, No. 9, pp. 2473-2476, September 1973

Molecular Structures of Acetylcholinesterase from Electric Organ Tissue of the Electric Eel

(electron microscopy/analytical ultracentrifugation/maleylation/protein subunits/membrane enzymes)

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Communicated by David Nachmansohn, May 23, 1973

ABSTRACT **Three purified molecular forms of acetylcholinesterase (EC 3.1.1.7) with sedimentation coefficients of 18 S, 14 S, and 11 S were studied by analytical ultracentrifugation and electron microscopy. The three species have molecular weights of $(1.1 \pm 0.1) \times 10^6$, $(7.5 \pm 1.5) \times 10^5$, and $(3.35 \pm 0.25) \times 10^5$, respectively. Electron micrographs reveal that the 18S and 14S forms are asymmetric, composed of a head, containing a large number of subunits, and an elongated tail. The 11S form of acetylcholinesterase is apparently a tetrameric structure devoid of the tail. Maleylation of 18S and 14S acetylcholinesterases abolishes their tendency to aggregate at low ionic strength.**

Acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) in extracts of fresh electric organ tissue of electric eels consists of several components distinguished by their sedimentation coefficients (1, 2). The major component is an 18S form, which aggregates at low ionic strength (2, 3). A 14S component, which also aggregates at low ionic strength, is present in smaller amounts. Treatment of electric organ tissue with proteolytic enzymes or autolysis converts all the acetylcholinesterase to an 11S form, which does not aggregate at low ionic strength and is not present in fresh tissue (2, 3).

We have purified, by affinity chromatography, the different molecular forms of acetylcholinesterase present in fresh tissue or obtained after proteolysis (3, 4). Purified 18S and 14S acetylcholinesterases retain their tendency to aggregate at low ionic strength, and the 18S, 14S, and 11S acetylcholinesterases all display similar patterns on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol (4). Massoulié *et al.* (5) have suggested, on the basis of gel-filtration studies, that 18S and 14S acetylcholinesterases have an asymmetric structure, in contrast with 11S acetylcholinesterase, which is globular.

In the following we will show, by electron microscopy and ultracentrifugation, that 18S and 14S acetylcholinesterases differ markedly in their quaternary structure from the 11S form. Similar electron microscopic observations have been recently reported by Rieger *et al.* (6). We will also show that the tendency of 18S and 14S acetylcholinesterases to aggregate at low ionic strength can be prevented by chemical modification. The relevance of these observations to the relationship of acetylcholinesterase with the electroplax membrane will be discussed.

METHODS

18S, 14S, and 11S forms of acetylcholinesterase were purified by affinity chromatography (3, 4). The 18S and 14S enzymes were separated from each other by sucrose gradient centrifugation as follows: aliquots of 1.8 ml of a mixture of purified 18S and 14S enzymes (containing about 1 mg/ml of enzyme) were layered on a 5–20% linear sucrose gradient in 1.0 M NaCl–0.01 M phosphate (pH 7.0) of total volume of 28 ml, with a 5-ml cushion of 60% sucrose in the same buffer at the bottom. Centrifugation was performed in an SW27 rotor in an L2 65-B Beckman preparative ultracentrifuge at 27,000 rpm at 4° for 20 hr. The 18S and 14S samples thus obtained were dialyzed against 1.0 M NaCl–0.01 M phosphate (pH 7.0).

Analytical ultracentrifugation was performed by the high-speed meniscus depletion method of Yphantis (7), with a Beckman model E ultracentrifuge equipped with an electronic speed-control unit. Aluminum-filled epon double-sector cells (12 mm) were used in an An-F aluminum rotor with four cell holes (for speeds higher than 10,000 rpm) or an An-J aluminum rotor with four cell holes (for speeds less than 10,000 rpm). Filling of the cells, the criteria for equilibrium, running of blanks, and analysis of the Rayleigh patterns were as described by Godfrey and Harrington (8).

Electron microscopy was performed with a Jeol 100 B electron microscope, with an accelerating voltage of 80 kV, magnifications of 25,000–60,000, and an objective aperture of of 40 μm. Negatively stained samples of acetylcholinesterase were prepared by the technique of Huxley and Zubay (9). The best results were obtained when the enzyme was stained with a 1% solution of uranyl acetate (pH 3.5) over holes.

Purified 18S + 14S acetylcholinesterase was treated with maleic anhydride according to Butler *et al.* (10). A sample of 300 μg of purified 18S + 14S enzyme in 0.6 ml of 0.5 M NaCl–0.11 M phosphate (pH 7.5) was treated with 20 μl of a 7.5% (w/v) solution of maleic anhydride in redistilled dioxane, and the reaction mixture was kept for 3 hr at room temperature (25°). Control aliquots of acetylcholinesterase were treated as above, but the maleic anhydride solution was replaced by 20 μl of redistilled dioxane or 20 μl of H_2O. The samples were then dialyzed overnight at 4° against 1 M NaCl–0.01 M phosphate (pH 7.0). Acetylcholinesterase activity of the samples

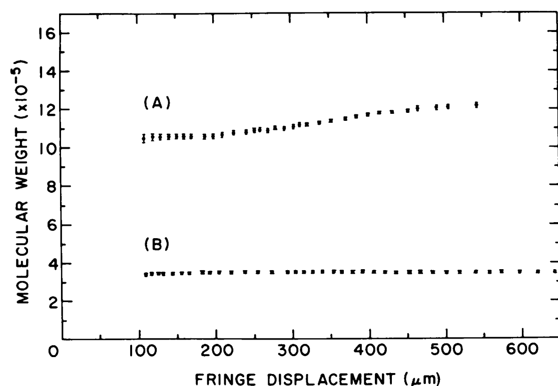

FIG. 1. The weight-average molecular weight of 18S and of 11S acetylcholinesterase plotted against fringe displacement as computed by the high-speed equilibrium ultracentrifugation program of Roark and Yphantis (11). (*A*) 18S acetylcholinesterase, centrifuged at 6000 rpm; (*B*) 11S acetylcholinesterase, centrifuged at 10,000 rpm.

was assayed before and after maleylation by the pH-stat method (3). Sucrose gradient centrifugation and enzymic assay of the fractions from the gradient were performed as described (3).

RESULTS

Equilibrium sedimentation measurements on purified 11S and 18S acetylcholinesterases yield the results shown in Fig. 1. The molecular weight of the 18S form is $(1.1 \pm 0.1) \times 10^6$, assuming a $\bar{v}$ of 0.72, as computed from amino-acid analysis of purified acetylcholinesterase (Dudai and Silman, unpublished results). The molecular weight for 11S acetylcholinesterase according to Fig. 1 is 350,000 ± 10,000, again from a $\bar{v}$ of 0.72. This preparation was obtained by tryptic digestion of fresh tissue (acetylcholinesterase A in ref. 3). Another preparation of 11S acetylcholinesterase, obtained from toluene-treated tissue (acetylcholinesterase C in ref. 3), displayed similar homogeneity on equilibrium sedimentation, and had a molecular weight of 320,000 ± 10,000.

A preparation of 14S acetylcholinesterase, prepared as described in *Methods*, appeared to be rather heterogeneous on equilibrium sedimentation, although on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate it displayed a pattern similar to those observed for 18S and 11S acetylcholinesterase (4). From the ultracentrifugation measurements, a molecular weight of 750,000 ± 150,000 could be estimated.

Electron micrographs of the different molecular forms of acetylcholinesterase are shown in Fig. 2. The 18S form (Fig.

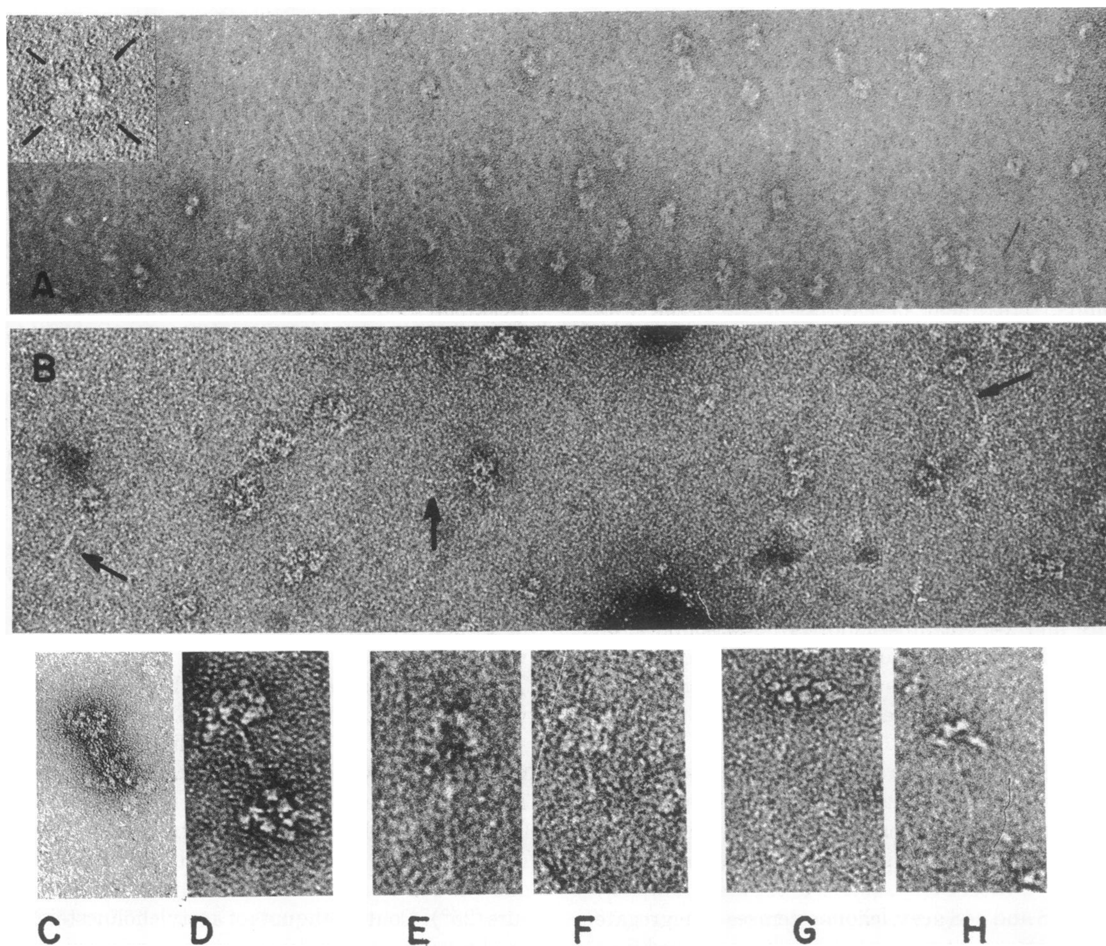

FIG. 2. Electron micrographs of various molecular forms of acetylcholinesterase, stained with 1% uranyl acetate. (*A*) 11S acetylcholinesterase, × ≃ 225,000; *insert* shows a single molecule, × ≃ 650,000. (*B*) 18S enzyme, × ≃ 225,000; *arrows* indicate tails. (*C*) Dumbbell form of acetylcholinesterase, × ≃ 225,000. (*D*) Dumbbell form of acetylcholinesterase, × ≃ 340,000. (*E* and *F*) 18S acetylcholinesterase, × ≃ 340,000. (*G* and *H*) 14S acetylcholinesterase, × ≃ 340,000.

2*B*, *E*, and *F*) resembles a bush with a long stem. The head is probably composed of at least 10 subunits, each measuring about 50 ± 10 Å across. The stem (or tail) is long, up to 500 Å, and narrow (about 20 Å across). The 14S form of acetylcholinesterase (Fig. 2*G* and *H*) resembles the 18S form, but the number of subunits in the head is fewer (at least six). We have also observed, in fields of both 18S and 14S forms, clusters of subunits without tails and separated tails.

When preparations of acetylcholinesterase purified by affinity chromatography (4) were used for isolation of the separate forms by sucrose gradient centrifugation, a small amount of enzyme sedimented to the bottom of the sucrose gradient even in the presence of 1 M NaCl. Examination of such fractions revealed the presence of dumbbell-like structures, in which two clusters of subunits seemed to be attached to the ends of one or several tail fibers (Fig. 2*C* and *D*).

The 11S form of acetylcholinesterase (Fig. 2*A*) appeared different from the 14S and 18S forms in the electron microscope. Structures composed of three to four subunits, and sometimes of only two subunits, can be seen. No tail-like structures appear in the field. The subunits again measure, on the average, 50 ± 10 Å across.

Treatment of a mixture of 18S and 14S acetylcholinesterases with maleic anhydride under the conditions of Butler *et al.* (10) abolishes the tendency of the enzyme to aggregate at low ionic strength, as shown by the sucrose gradient centrifugation experiments illustrated in Fig. 3. A control sample of the enzyme aggregated completely in 0.05 M NaCl (Fig. 3*A*) and displayed a major 18S peak in 1 M NaCl (Fig. 3*C*). In contrast, the same enzyme, subsequent to maleylation, displays major peaks of 18 S and 22 S, with no aggregated component (Fig. 3*B*), while in 1 M NaCl a major peak of 18 S is seen (Fig. 3*D*). The maleylated enzyme retained more than 90% of its initial activity on acetylcholine.

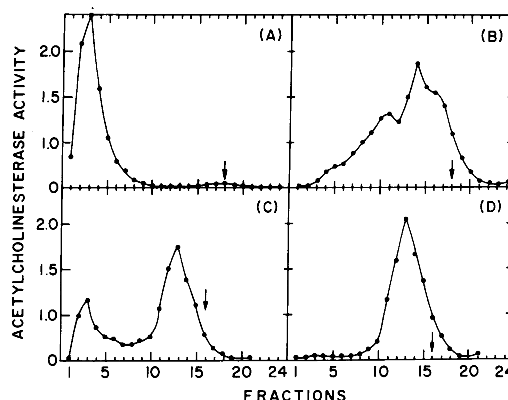

FIG. 3. Sucrose gradient centrifugation of acetylcholinesterase treated with maleic anhydride. (*A*) Control in 0.05 M NaCl–0.01 M phosphate (pH 7.0); (*B*) maleylated acetylcholinesterase, run under the same conditions as *A*; (*C*) control in 1.0 M NaCl–0.01 M phosphate (pH 7.0); (*D*) maleylated acetylcholinesterase, run under the same conditions as *C*. *Arrows* indicate the position of the catalase marker (11.4 S) on the gradient.

DISCUSSION

Strong evidence has accumulated that acetylcholinesterase is a membrane-bound enzyme. The evidence is based on electron microscopy combined with histochemical studies, and on subcellular fractionation (12–14). However, the exact relationship of the enzyme to the membrane has not been clarified (15, 16). It seems that electrostatic forces play a major role in the interaction of the enzyme with the membrane (17), and that Ca^{+2} ions may play a role in such association (18).

Acetylcholinesterase with a sedimentation coefficient of about 11 S, earlier purified from the electric organ of electric eels (19, 20), is a product of autolysis or proteolysis (2, 3, 21). The main soluble molecular form of acetylcholinesterase found in extracts of fresh electric organ tissue has a sedimentation coefficient of about 18 S (2) and a tendency to aggregate at low ionic strength (1, 2, 4). This 18S acetylcholinesterase can be converted to the 11S form by trypsin treatment (2).

Our ultracentrifuge results show that the 18S acetylcholinesterase has a molecular weight of more than 10^6. In shape it resembles a bush. The head appears to be composed of at least 10 subunits, as judged by electron microscopy, and the stem (or tail) is an elongated structure.

If it is assumed that the tail of the enzyme is composed of protein (see below) with a density of about 1.3 g/cm^3 (22), then from its average dimensions (about 500 × 20 Å) one can calculate a molecular weight of about 120,000. Our earlier data (4) indicated a molecular weight of about 80,000 for the major polypeptide component of acetylcholinesterase based on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. If the head of the enzyme contains 12 such subunits (an assumption that is consistent with the number of subunits seen in electron micrographs), it would yield together with the tail a molecular weight of about 1,080,000. This value is in agreement with our ultracentrifuge data.

The 14S form of acetylcholinesterase is similar in its gross quaternary structure to the 18S enzyme, as it is also composed of a head and a tail, but the head contains fewer subunits (at least six, as revealed by electron microscopy). Thus both the 18S and 14S forms have an asymmetrical structure, as was suggested by gel filtration studies (5).

11S acetylcholinesterase is presumably composed of the same major subunits found in the 14S and 18S forms (4). Our preparations seem to consist of three to four subunits, and sometimes structures appearing to contain two subunits appear in the electron micrographs. These observations are in agreement with those of Changeux *et al.* (23). We did not observe tail-like structures either bound to or detached from the enzyme.

The molecular weight we found for the 11S species, 335,000 ± 25,000, is consistent with a tetramer of four subunits of about 80,000 daltons, the value that we obtained from polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol for the 11S species (3, 4). However, there are marked discrepancies between our data and those of previous studies for the molecular weights both of the 11S enzyme (24–26) and of its subunits (25).

It may well be that autolysis or proteolysis can produce different preparations with different subunit sizes, depending on the exact purification procedures. Indeed a significant difference between the sedimentation coefficients of two 11S acetylcholinesterase preparations, from different laboratories, has been described (21). Moreover, recent observations (ref. 27; Dudai and Silman, unpublished results) show that, after prolonged autolysis of electric organ tissue, the purified acetylcholinesterase obtained displays various lower molecular

weight components on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. These smaller components appear to be derived from the component of molecular weight 80,000 by proteolytic cleavage.

The molecular structure of the tail in the 18S and 14S forms of acetylcholinesterase is not known. It is either detached or destroyed by various proteases, since it is not observed in the purified 11S form, which is obtained subsequent to proteolysis either of electric organ tissue (3) or of 18S enzyme. Collagenase detached acetylcholinesterase from synaptic structures (15, 28). We have indeed found that collagenase affects the molecular structure of 18S acetylcholinesterase, converting it to an 11S form and to heavier forms that do not aggregate at low ionic strength (Dudai and Silman, unpublished results). But even in highly purified preparations of collagenase we found residual proteolytic activity, so that we cannot be sure whether it is the collagenase that modifies the acetylcholinesterase. The purified forms of acetylcholinesterase do not seem to contain nucleic acids or a significant amount of lipids, although the presence of small amounts of such components in the enzyme could not be totally excluded (4). All three forms of the enzyme contain sugars, but no major quantitative difference has been found among them (4).

From the above it seems most plausible that the tail structure of 18S and 14S acetylcholinesterase is mainly protein. Indeed proteins with such an elongated structure are known, such as myosin (29), tail fibers from bacteriophage (30), and presumably transverse filaments from synaptonemal complexes (31). Elongated strands connecting subunits in a protein molecule have been observed recently in the C1q component of human complement (32, 33).

In our preparations we found that purified acetylcholinesterase can exist in forms larger than the 18S form even at high ionic strength. These forms appear as dumbbell-like structures in electron micrographs. Although such structures may represent a real entity found *in situ*, one must bear in mind the possibility that they may be artifacts formed during isolation and purification.

No role can be ascribed to the tail structure, nor is its orientation with respect to the membrane known. However, it is tempting to speculate that the tail structures (and in particular the dumbbell structures observed in Fig. 2*C* and *D*) play a role in fixing the acetylcholinesterase molecule within the framework of the membrane.

The experiments on maleylation of 14S and 18S acetylcholinesterase show that it is possible, by chemical modification of the native forms of the enzyme, to abolish their tendency to aggregate at low ionic strength. Since maleic anhydride probably modifies primarily ϵ-NH_2 groups of lysine (10), its effect is most likely achieved by increasing the net charge of the enzyme, which prevents aggregation, as a result of electrostatic repulsion between subunits, without affecting enzymic activity. We also found that by treatment of a homogenate of fresh electric organ tissue with maleic anhydride it is possible to bring about direct release into the medium of 18S acetylcholinesterase that does not aggregate at low ionic strength (Dudai and Silman, unpublished results). The above data further emphasize the role of electrostatic forces in the interaction of acetylcholinesterase with other molecules and, probably, with the membrane.

We thank Dr. Robert Josephs, Dr. Martin Hirshfeld, and Mr. Moshe Mevarech for valuable discussions and help with the ultracentrifuge experiments, Miss Esther Saksel for her skillful technical assistance, and Mr. Shmuel Engelstein for his assistance in processing the electron micrographs. M.H. was supported by a Grant DGRST from the French Government, and I.S. by a grant from the Volkswagen Foundation.

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