Antigenic and structural differences in the catalytic subunits of the molecular forms of acetylcholinesterase

(monoclonal antibodies/basal lamina/tryptic peptides)

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ABSTRACT A mixture of the 5.6S hydrophobic dimer and the asymmetric, tail-containing (17 + 13)S forms of acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from *Torpedo californica* was used to immunize mice, and spleen cells from these mice were used to produce nine hybridoma lines secreting antibodies against acetylcholinesterase. Antibodies from one of the lines showed a 100-fold greater affinity for the 5.6S species when compared with the catalytic subunits of the (17 + 13)S species. This difference in specificity was retained after denaturation of the two acetylcholinesterase species. Another line produced antibody directed only to structural subunits of the (17 + 13)S species, whereas the remaining seven antibodies exhibited nearly equivalent crossreactivity for all of the forms of acetylcholinesterase. Tryptic peptides were generated from the catalytic subunits of the 5.6S and tail-containing acetylcholinesterase species, and high-pressure liquid chromatographic profiles show at least two distinct peptides in the catalytic subunits for each enzyme species. Some of these peptides exhibit retention times different from those of the identified glycopeptides. Thus, it is likely that the catalytic subunits of two molecular forms of acetylcholinesterase differ in primary structure and sites of antigenicity.

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7) from the *Torpedo* electric organ exists as two distinct structural forms: a hydrophobic dimer of catalytic subunits with a sedimentation coefficient of 5.6 S and an elongated, dimensionally asymmetric form that sediments as discrete 17S and 13S entities (1–5). The elongated or tail-containing species contain between 8 and 12 catalytic subunits disulfide-linked to distinct collagenous and noncollagenous structural subunits (2). The structural subunits of the 17S and 13S species can be removed by proteolysis, yielding an 11S species that retains full catalytic activity. The 11S form proves to be of value for comparing the structure of catalytic subunits of the elongated form with the hydrophobic form of acetylcholinesterase. The hydrophobic 5.6S acetylcholinesterase is devoid of structural subunits and appears to be a simple dimer of the catalytic subunits.

Based on a variety of studies of the structure and location of the acetylcholinesterase species and their reassociation with various membrane fractions, the elongated forms all appear to be exported to and deposited in the basal lamina of the synapse (6–8), whereas the hydrophobic globular forms are associated with plasma membrane of the cell (3, 9). The hydrophobic form associates with membranes and other hydrophobic proteins and even the purified protein in the absence of detergent exists as an aggregate. Both the elongated and globular hydrophobic species are found also in mammalian systems. The distribution of the various molecular forms appears under precise cellular

regulation. Developmental factors, innervation, and muscle activity are known to influence the disposition of acetylcholinesterase species (cf. ref. 10).

The asymmetric and globular forms in *Torpedo* exhibit indistinguishable catalytic behavior and susceptibilities to inhibitors (4, 5). More recent characterization has revealed different electrophoretic behaviors of the catalytic subunits of the two forms and possible differences in their compositions (3).

A fundamental question that underlies the above considerations and future studies on the regulation of acetylcholinesterase is whether the multiple molecular forms of this enzyme simply arise from alternative processing and assembly steps for a common catalytic subunit or whether separate genes encode for each form of the enzyme. To approach this question we have used hybridoma techniques and peptide separations to examine structural differences in the catalytic subunits from the various molecular forms of acetylcholinesterase.

MATERIALS AND METHODS

Immunization, Fusion, Antibody Screening, and Cloning. The (17 + 13)S, 11S, and 5.6S forms of acetylcholinesterase have been isolated and purified by previously described affinity chromatographic techniques (1–3). A 75%/25% mixture of purified (17 + 13)S and 5.6S forms of the enzyme was used as the immunogen. On day 1, BALB/CJ mice were injected subcutaneously with 100 μ g of purified acetylcholinesterase in Freund's complete adjuvant; on day 25, 100 μ g of enzyme in Freund's incomplete adjuvant was injected intraperitoneally; on day 28, 100 μ g of enzyme was administered intravenously; and on day 31, the spleens were harvested for fusion.

Spleen cells were fused by using polyethylene glycol 1000 with either the P3 $\times$ 63Ag8.653 mouse myeloma line (antibody-secreting cell lines 2C8, 2E6, 4E7, 4F3, 4F11, and 4G1) or the SP2/0-Ag14 mouse myeloma line (antibody-secreting cell lines 2G4, 9D1, and 11D5). The fusion and cloning procedures are detailed elsewhere (11). Antibody-secreting cell lines were identified by a solid-phase microtiter plate radioimmunoassay (12) utilizing 1.5 μ g of purified acetylcholinesterase per well. The second antibody was trivalent rabbit anti-mouse antiserum (IgG, IgA, IgM), affinity purified by the method of Ternzack and Avrameas (13) and labeled with ¹²⁵I (14).

Determination of the isotype of each monoclonal antibody was made by using tissue culture supernatants at concentrations that bound $\approx$ 50% of maximum counts in a radioimmunoassay. Details of the competition assay have been described (11).

Characterization of Anti-Acetylcholinesterase Antibodies. Culture supernatants from the cell lines were precipitated with

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Abbreviation: ELISA, enzyme-linked immunoadsorbent assay.

50% $(\text{NH}_4)_2\text{SO}_4$, resuspended in 5% of their original volume, and dialyzed against 50 mM sodium phosphate/150 mM NaCl, pH 7.4. For routine screening of specificity, an enzyme-linked immunoadsorbent assay (ELISA) was used. The particular species of acetylcholinesterase (0.5 μg) in 0.1 M NaHCO_3 (pH 9.5) was added to the individual microtiter wells and incubated for 3 hr at 37°C; then the wells were washed with 10 mM sodium phosphate/150 mM NaCl, pH 7.4/0.5% Tween 20/0.1% bovine serum albumin. Dilutions of monoclonal antibody in the same buffer were added to the wells and incubated for 24 hr at 4°C. The plates were again rinsed, and peroxidase-linked goat anti-mouse immunoglobulin was added, followed by incubation at 37°C for 1 hr. After another rinse, a solution of *o*-phenylenediamine and H_2O_2 in citrate/phosphate buffer (pH 5.2) was added, and color was allowed to develop for 5–30 min. The reaction was stopped with 2 M H_2SO_4 , and absorbance at 490 nm was monitored.

For the immunoprecipitation assay, 100 μg of either 5.6S or 11S acetylcholinesterase was iodinated by the glucose oxidase/lactoperoxidase coupling method with 1 mCi of ^{125}I . To ≈ 36 ng of ^{125}I -labeled acetylcholinesterase, antibody was added in a total volume of 0.25 ml. After incubation for 2 hr at room temperature, the reaction mixture was diluted five times, and protein A (Pansorbin, Calbiochem) was added and incubated for another 15 min at 4°C. After centrifugation the pellet was washed and counted. Total protein-associated ^{125}I radioactivity was determined by precipitation with 10% trichloroacetic acid. Results are expressed as the ratio of protein A-precipitated radioactivity to trichloroacetic acid-precipitated radioactivity.

To examine the sedimentation properties of acetylcholinesterase-antibody complexes, 400 ng of [^3H]diisopropyl fluorophosphate-labeled acetylcholinesterase was incubated with the designated quantity of antibody for 24 hr in a total volume of 0.25 ml. The complex was layered over a 5–20% sucrose density gradient and sedimented at 40,000 rpm for 16 hr in an SW-41 Ti rotor. Samples were collected and monitored for radioactivity.

Purification of the Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins G, M, and A (Cappel Laboratories, Cochranville, PA; 100 mg) were coupled with 56 ml of CNBr-activated Sepharose 4B; ≈ 50 ml of culture supernatant from the cell lines was mixed with a 10-ml slurry of the immunoglobulin-coupled Sepharose 4B. After incubation overnight at 4°C with gentle agitation, the slurry was poured into a column and rinsed with 10 column volumes of 10 mM NaPO_4 /150 mM NaCl, pH 7.4. Anti-acetylcholinesterase antibody was eluted by passing 0.1 M glycine-HCl/0.2 M NaCl, pH 2.8, through the column at a rate of 2 ml/hr. Samples were collected in tubes containing Tris-HCl (pH 8.5) to effect an immediate neutralization.

From this one-step purification procedure, the 4E7 and 4F3 antibodies were obtained in yields of 52% and 40%, respectively. Approximately a 100-fold purification was achieved. The antibody was estimated to be 80–90% pure based on the staining intensity of the light and heavy immunoglobulin chains on polyacrylamide gels. Titers obtained from purified antibody were used as a reference to estimate antibody concentrations in other experiments.

Preparation of Tryptic Peptides of Acetylcholinesterase. Either 5.6S or 11S acetylcholinesterase (12–25 mg) were allowed to react with a slight molar excess of [^3H]diisopropyl fluorophosphate (specific activity, 0.3 Ci/mmol) and allowed to age by standing overnight at room temperature. Completion of the aging process was assessed by the absence of reactivation by 2-pralidoxime chloride. The sample was exhaustively dialyzed, lyophilized, and suspended in 6 M guanidine-HCl/0.1 M Tris-HCl, pH 8.0. A 2-fold molar excess of dithiothreitol over total protein cysteine residues was added to reduce disulfide

bonds, and the sample was then incubated at 50°C for 3 hr under nitrogen. This was followed by reaction for 3 hr with [^{14}C]iodoacetate (specific activity, 3 mCi/mmol) at 25°C with a 2-fold molar excess of total thiols. The reaction was stopped with excess dithiothreitol, and the sample was dialyzed against 10 mM NH_4HCO_3 . Trypsin (1%, wt/wt) was added to 5 mg of aliquots of acetylcholinesterase, and the samples were lyophilized.

The sample was dissolved in 50 mM NH_4HCO_3 (pH 8.2) at 1 mg/ml. Trypsin (1%) was again added, followed by incubation at 37°C for 22 hr. The sample was then lyophilized and dissolved in 0.1% trifluoroacetic acid. Peptides were separated by HPLC on a $\mu\text{Bondapak C-18}$ column by using the following 0.1% trifluoroacetic acid (in H_2O)/acetonitrile gradient: 0% acetonitrile, 15 min; 0–20% acetonitrile, 60 min; 20–35% acetonitrile, 90 min; 35–45% acetonitrile, 45 min; and 45% acetonitrile, 15 min. The flow rate was 1.5 ml/min, and 1.5-ml fractions were collected. Absorbance at 219 nm and 280 nm along with ^3H and ^{14}C radioactivity were monitored.

RESULTS

Characterization of the Monoclonal Antibodies. Results of the specificity and immunoglobulin types of the monoclonal antibodies are summarized in Table 1. Most of the antibodies showed nearly equivalent reactivity to (17 + 13)S, 11S, and 5.6S forms of acetylcholinesterase (Fig. 1). However, two were of particular interest because of their selectivity. One, 4F3, reacted only with the (17 + 13)S enzyme. Because the 5.6S and the 11S species are devoid of either the 100-kilodalton noncollagenous or the collagen-containing tail subunits, it seemed likely that 4F3 is directed to these subunits. To confirm this, the subunits were cleaved by trypsin or collagenase treatment of the (17 + 13)S enzyme. After treatment with trypsin at 25°C or collagenase at either 25°C or 37°C, immunoreactivity was lost (Fig. 2). Because collagenase treatment at 25°C does not cleave the 100-kilodalton noncollagenous peptide or effect its removal from the catalytic subunits (2), it is likely that the immunogenic region is located on the collagenous tail units. Moreover, denaturation of the enzyme by guanidine-HCl in the presence of dithiothreitol also resulted in a loss of immunoreactivity. Thus, 4F3 is likely to be directed to the native tail unit. The distal portion of the tail unit is believed to have a triple helical structure similar to collagenous sequences in basement membranes (15).

A second selective antibody was 4E7, which in the ELISA showed nearly a 100-fold preference for the 5.6S species over the 11S species (Fig. 3). Thus, this antibody recognizes a portion of the catalytic subunits unique to the 5.6S species. Denaturation of the enzyme with 6 M guanidine-HCl did not eliminate the selectivity in recognition by the antibody. To demonstrate that the reactivity actually occurred to the 11S species rather than to a 1% or 2% contaminant of 5.6S enzyme, im-

Table 1. Properties of the monoclonal antibodies

Hybridoma line	Antibody subtype	Estimated titer*
2C8	IgG1	—
2E6	IgG2a	1:750
4E7	IgG2b	1:1,500
4F3	IgG1	1:425
4F11	IgG1	—
4G1	IgM	1:350
7G4	IgG1	—
9D1	IgM	1:1,500
11D5	IgG1	—

* Determined from the ELISA (cf. Fig. 1A), 50% maximum absorbance at 490 nm.

FIG. 1. Specificity of five monoclonal antibodies raised against a mixture of (17 + 13)S and 5.6S forms of acetylcholinesterase. The monoclonal antibodies were obtained from the culture supernatant of the hybridoma cell lines and partially purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation. Assays were done by the ELISA method with peroxidase-coupled rabbit anti-mouse IgG/IgM as the second antibody. (A) Reaction with (17 + 13)S acetylcholinesterase. (B) Reaction with 5.6S acetylcholinesterase monoclonal antibodies: $\circ$, 4E7; $\bullet$, 4D1; $\triangle$, 4G1; $\blacktriangle$, 2C8; $\blacksquare$, 4F3. Note that 4F3 shows a selectivity for the asymmetric forms of acetylcholinesterase.

munoprecipitation of iodinated 5.6S and 11S enzymes also was monitored. Again about 2 orders of magnitude greater selectivity for the 5.6S enzyme was evident (Fig. 4). Lastly, the formation of a complex with the 5.6S enzyme could be demonstrated directly by sedimentation analysis after incubation of antigen and antibody. With the higher stoichiometric ratios of 4E7 antibody to acetylcholinesterase, formation of broadened peaks of higher sedimentation coefficients was observed (Fig. 5). These likely represent formation of multivalent complexes, as both the antibody and acetylcholinesterase would be expected to be bivalent. Larger complexes also may form, become impacted at the bottom of the centrifuge tube, and hence not be detected in our assay. This may account for the diminished recovery of radioactivity in the presence of antibody. The small

FIG. 2. Loss of reactivity of the monoclonal antibody 4F3 with (17 + 13)S acetylcholinesterase after treatment with collagenase and trypsin. Assay was carried out by the procedure described in the legend to Fig. 1, and the loss of reactivity probably reflects the inability of the low molecular weight peptides to adhere to the solid phase. $\circ$, Native acetylcholinesterase; $\bullet$, treatment with collagenase (1 $\mu\text{g}/\text{ml}$ for 1 hr) at 37°C; $\blacksquare$, treatment with collagenase (1 $\mu\text{g}/\text{ml}$ for 1 hr) at 25°C; $\blacktriangle$, treatment with trypsin (5 $\mu\text{g}/\text{ml}$ for 1 hr) at 25°C.

FIG. 3. Reactivity of monoclonal antibody 4E7 toward the 5.6S (hydrophobic dimer) and 11S (lytic form derived from the elongated species) forms of acetylcholinesterase. Denaturation of acetylcholinesterase was carried out in 6 M guanidine-HCl, and the sample was dialyzed. $\blacksquare$, Native 11S form; $\square$, denatured 11S form; $\bullet$, native 5.6S form; $\circ$, denatured 5.6S form. The assay procedure was identical to that of Figs. 1 and 2.

increases in S value are likely to be a consequence of the intrinsic dimensional asymmetry of the complex. The increase in mass would not be fully reflected in proportional changes in the sedimentation coefficients.

HPLC of the Tryptic Peptides of Acetylcholinesterase. To compare the tryptic peptides of the catalytic subunits from the hydrophobic dimer and the asymmetric species, we used 5.6S and 11S enzymes after denaturation, reduction, and carboxymethylation. To form the 11S enzyme, the collagenous and noncollagenous subunits were cleaved by trypsin. This procedure solubilized an 11S species, leaving small residual peptide(s) disulfide-linked to the catalytic subunits (1, 7, 15). These peptides should be dialyzable and removed in the preparation of the enzyme for digestion. Proteolysis of the structural subunits, which yielded the 11S enzyme, did not appear to affect

FIG. 4. Precipitation of 11S ($\bullet$) and 5.6S ($\circ$) acetylcholinesterase by the 4E7 monoclonal antibody. The respective enzymes were labeled with ^{125}I and precipitated by incubation for 2 hr with the specified dilution of antibody (undiluted antibody, 16.5 μg per assay; total acetylcholinesterase, 37 ng). Precipitation was achieved with protein A and values are compared with the total amount of radioactivity precipitated with trichloroacetic acid.

FIG. 5. Sedimentation characteristics of various 5.6S acetylcholinesterase-antibody complexes. Acetylcholinesterase (400 ng) labeled with [^3H]diisopropyl fluorophosphate was added to antibody 4E7 in a volume of 250 μl and incubated for 24 hr. The material was layered over a 5–20% sucrose gradient in 10 mM Tris chloride/0.1 M NaCl/0.1% Triton X-100, pH 7.4. Samples were centrifuged in an SW-41 rotor at 40,000 rpm for 16 hr. $\bullet$, Acetylcholinesterase alone; $\blacktriangle$, acetylcholinesterase with 5.5 μg of 4E7; $\blacktriangledown$, acetylcholinesterase with 55 μg of 4E7. The left and right arrows denote the sedimentation positions of catalase (11 S) and carbonic anhydrase (3.3 S).

the electrophoretic behavior of the reduced and dissociated catalytic subunits of the enzyme in NaDodSO₄ (2).

From the amino acid compositions, we would expect to obtain around 60 tryptic peptides from the catalytic subunits of acetylcholinesterase; in the gradient elution, approximately 55 peptides could be identified (Fig. 6). Diisopropyl fluorophosphate is an inhibitor of acetylcholinesterase; as in the case of other serine hydrolases, it can be expected to conjugate with the active center serine on the enzyme. We found that [^3H]diisopropyl fluorophosphate was conjugated to a single peptide (number 38) that was clearly resolved in each profile. The same relative positions of this peptide derived from the two enzymes would indicate that these active center peptides are very similar if not identical in the two acetylcholinesterase forms. Amino acid analysis showed identical compositions for this peptide in the two enzyme forms. Seven cysteine-containing peptides in the 11S and in the 5.6S forms were identified (peptides 13, 31, 33, 35, 41, 44, and 48). Although this is about two to three fewer than predicted from the composition (3), labeling with either N -[^3H]ethylmaleimide or iodo[^{14}C]acetate in other experiments gave less than the predicted stoichiometry (unpublished observations).

Although the profiles of the two enzymes showed a general similarity, we identified at least four peptides that differ in the two profiles. Peptides 10 and 42 were absent in 5.6S acetylcholinesterase, whereas peptides 45 and 47 appeared to be ab-

sent in 11S acetylcholinesterase. In addition, qualitative differences appeared in peaks 6, 14, 17, 27, and 48, but further characterization would be necessary to establish these as different peptides. The glycopeptides were provisionally located by comparing paired HPLC profiles run prior to and after chromatography over separate columns of wheat germ agglutinin and ricin linked to Sepharose-4B. By the criterion of lectin adsorption, the majority of peaks that differed between the two enzymes did not contain oligosaccharide residues. Thus, the differences in tryptic peptides between the 5.6S and 11S enzymes cannot solely be attributed to post-translational alterations in the oligosaccharide residues. Hence, we would expect the catalytic subunits of the two forms of acetylcholinesterase to have different amino acid sequences.

DISCUSSION

The two general classes of acetylcholinesterase found in *Torpedo* and mammalian muscle can be categorized as asymmetric and globular. The asymmetric species contain a variable number of catalytic subunits linked to a collagenase-sensitive tail unit. They are localized primarily in synaptic regions (16) and generally increase in content upon synaptogenesis (10). After damage to the nerve and muscle membranes, plasma membranes are degraded, yet the basal lamina is retained along with its associated acetylcholinesterase (8). Based on studies in electric organ of *Torpedo* and *Electrophorus*, it would appear that the tail unit is actually a component of the basal lamina (6–8, 15). *Torpedo* acetylcholinesterase forms differ from those in *Electrophorus* in that a substantial percentage of a hydrophobic, globular species can be identified in *Torpedo* (3–5).

The globular forms found in mammalian muscle appear to be both soluble and membrane-associated and are found as monomers through tetramers (17). The globular forms precede the formation of synapses in development and do not show a preferential localization to synaptic regions. After irreversible inactivation of acetylcholinesterase in myotubes, active globular forms precede the appearance of the asymmetric forms (18). The small quantities of acetylcholinesterase in mammalian muscle complicate detailed structural studies. However, the abundance of acetylcholinesterase in the *Torpedo* electrocyte allows isolation from an alternative source. The globular forms also appear first during the development of the *Torpedo* electrocyte (19).

Pertinent to the considerations of the biosynthesis and assembly of the complex species of acetylcholinesterase is whether a common catalytic subunit prevails in the diverse molecular forms. As suggested previously (20), should there be a common catalytic subunit, regulation of the distribution of acetylcholinesterase species would occur entirely at a post-translational stage, presumably during assembly of the catalytic and structural subunits. Under these circumstances one would expect both structural and immunologic identity of the catalytic subunits. Fambrough *et al.* (21) have isolated seven hybridoma lines secreting antibodies to mammalian erythrocyte acetylcholinesterase that show similar antibody specificity to the globular and asymmetric enzymes from muscle.

From our hybridoma cell lines, we have been able to isolate an antibody that shows a 100-fold difference in crossreactivity of the catalytic subunits from the hydrophobic dimer (a globular form) and the asymmetric form. These data, when coupled with an analysis of the tryptic peptides, suggest that the catalytic subunits differ both antigenically and in primary structure. Because most of the different peptides appear as non-glycopeptides, it is likely that either separate genes encode for acetylcholinesterase or alternative RNA processing (22) prevails. Thus, regulation of the distribution and disposition of

FIG. 6. HPLC fractionation of tryptic peptides. (Upper) 5.6S (hydrophobic dimer) form of acetylcholinesterase. (Lower) 11S form of acetylcholinesterase. Tryptic peptides were prepared from the reduced, denatured, and iodo ^{14}C acetate-alkylated enzyme. Prior to reduction the enzymes were inhibited with ^3H diisopropyl fluorophosphate and allowed to age by incubation at 37°C for 18–24 hr. The tryptic hydrolysate was placed on a reverse-phase $\mu\text{Bondapak C}_{18}$ column and eluted with a programmed 0–50% acetonitrile gradient in aqueous 0.1% trifluoroacetic acid. —, Absorbance at 219 nm; ----, absorbance at 280 nm; ●, ^{14}C carboxymethyl-labeled peptides; ◇, ^3H diisopropylphosphoryl-labeled peptides.

acetylcholinesterase upon synapse formation would extend to the control of transcriptional and translational events.

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