Primary Structures of the Catalytic Subunits from Two Molecular Forms of Acetylcholinesterase

A COMPARISON OF NH$_2$-TERMINAL AND ACTIVE CENTER SEQUENCES*

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Two distinct classes of acetylcholinesterase exist in near equal amounts in the electric organ of *Torpedo californica*. A globular 5.6 S form is a dimer which possesses a hydrophobic region. The second form is present as elongated species that sediment at 17 and 13 S and contain structural subunits disulfide-linked to the catalytic subunits. Removal of the structural subunits by mild proteolysis yields a tetramer of catalytic subunits which sediments at 11 S. To compare the primary structures of the catalytic subunits of the 5.6 S and 11 S forms of acetylcholinesterase, amino acid sequences from the active sites and from the amino-terminal regions have been elucidated. Active site serines were labeled with $[^{3}H]$isopropyl fluorophospho-

The electric organ of *Torpedo californica* contains two distinct classes of molecular forms of acetylcholinesterase (acetylcholine acetylhydrolase EC 3.1.1.7) in nearly equal abundance (1–3). The dimensionally asymmetric or elongated forms, which are located within the basal lamina of the synapse, are comprised of two or usually three sets of catalytic subunits linked to collagenous and noncollagenous structural subunits. These forms sediment, respectively, as discrete 13 S and 17 S entities (4–6). Limited proteolytic treatment releases the catalytic subunits from the structural subunits to which they are disulfide-linked. This cleavage yields an 11 S species which is a tetramer of catalytic subunits and remains catalytically active. The other molecular form is a hydrophobic, globular species which in the presence of neutral detergent sediments at 5.6 S (1–3). This form is a simple dimer of catalytic subunits being devoid of structural subunits (1–3).

In mammalian muscle and brain, these classes of acetylcho-

ExPERIMENTAL PROCEDURES

Purification of Acetylcholinesterases—Affinity chromatographic procedures for purification of the 5.6 S and 11 S species of acetylcho-

Preparation of Tryptic Peptides—Either 5.6 S or 11 S acetylcholin-

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react with a slight molar excess of [3H]DFP¹ (specific activity, 4 Ci/ mmol) in the presence of 0.02% NaCl for approximately 24-48 h to achieve complete inhibition and aging. The aging process which results in loss of one of the isopropoxy groups was necessary to ensure that a single species conjugated to the serine. Completion of aging was evaluated by the absence of reactivation by pyridine-2-aldoxime methiodide. After removing the unreacted DFP by dialysis, the sample was lyophilized and resuspended in 6 M guanidine HC1 in 0.1 M Tris. Further Fragmentation of the Active Site Tryptic Peptide—Approximately 40 nmol of the [3H]diisopropyl fluorophosphate-labeled tryp- tic peptide were lyophilized and redisolved in 0.4 ml of 10 mM NH4HCO3 at pH 8.1. Chymotrypsin was added at approximately 1% (w/w) and incubated at 37 °C for 4 h. This digest was injected onto a reverse-phase HPLC Vydac C18 column under the same conditions as described for the trypptic peptides.

Amino Acid Analyses—Amino acid analyses were carried out using an LKB 4400 amino acid analyzer. Peptides dissolved in 6 N HCl were hydrolyzed in vacuo at 110°C for 20-24 h.

¹The abbreviations used are: DFP, diisopropyl fluorophosphate; HPLC, high performance liquid chromatography.
Acetylcholinesterase Primary Structures

TABLE I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid compositions</th>
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<tbody>
<tr>
<td></td>
<td>DFP-labeled tryptic</td>
</tr>
<tr>
<td></td>
<td>5.6 S</td>
</tr>
<tr>
<td>Axx</td>
<td>0.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Thr</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Serine</td>
<td>3.7 (4)</td>
</tr>
<tr>
<td>Glycine</td>
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</tr>
<tr>
<td>Proline</td>
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<tr>
<td>Valine</td>
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</tr>
<tr>
<td>Methionine</td>
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<td>Isoleucine</td>
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<tr>
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<tr>
<td>Tyrosine</td>
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</tr>
<tr>
<td>Phenylalanine</td>
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</tr>
<tr>
<td>Histidine</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>NH$_2$-terminal</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
</tr>
</tbody>
</table>

* Acetylcholinesterase form.

FIG. 4. Sequence analyses of the active site tryptic peptides and chymotryptic peptide. The arrow indicates the [3H]isopropylphosphoryl-labeled serine. The consensus sequence is indicated in circles at the top. Residues in parentheses indicate placement only by amino acid composition. Residues indicated by capital letters are positions identified unambiguously, whereas sequences in lower case letters were tentatively identified. AChE, acetylcholinesterase.

Sequencing—Dansyl-Edman degradations were performed manually. Solid phase sequencing was done on an automated sequenator modeled after the instrument described by Doolittle et al. (15), employing standard methods (16). Peptides (10–40 nmol) were coupled to phenyl diisothiocyanate-glass beads (30 mg) in a buffer containing 0.1 M NaHCO$_3$, 10% 1-propanol, pH 9.3, and incubated overnight at 25 °C (17). Thiazolinones were converted to phenylthiohydantoins by incubating at 80 °C for 10 min with 0.2 ml of 1.5 N HC1 in methanol and drying under N$_2$. The phenylthiohydantoins were identified by HPLC according to the method of Bhown et al. (18) with Buffer A at pH 3.9. Liquid phase sequencing was done using a Beckman Spinning Cup Sequencer (Model 890C). Gas phase sequencing employed an Applied Biosystems Protein Sequencer (Model 470A).

RESULTS

Both the 5.6 S and 11 S forms of acetylcholinesterase were covalently labeled with [3H]diisopropyl fluorophosphate and aged in order to identify the reactive serine residue. The DFP-labeled protein was subsequently denatured, reduced, and alkylated with [14C]iodoacetic acid. After digestion with trypsin, the resultant peptides were initially resolved by gel filtration. The elution profile of the tryptic peptides from the 5.6 S form of the enzyme (Fig. 1A) showed the [3H]radioactivity
migrating as a single major peak that was well retained in the gel. When the tryptic peptides from the 11 S enzyme were eluted in an analogous manner from the same Sephadex G-50 column, a similar tritium-labeled peak was observed (Fig. 1B). The elution profiles, based on absorbance at 219 and 14C radioactivity, also were similar although not identical for the 5.6 S and 11 S forms of the enzyme. In order to further resolve the peptides, individual fractions were pooled and subjected to HPLC.

The 3H-labeled peak was pooled as Fraction IV for both the 5.6 S and 11 S enzymes. The elution from the HPLC reverse-phase column of the peptides contained in Fraction IV is shown in Fig. 2, A and B. The tritium was associated with a single well resolved peptide and eluted at an identical position for both proteins. The amino acid compositions of both peptides were identical (Table I). For both peptides, the NH2-terminal residue was identified as threonine by dansylation.

The 3H-labeled tryptic peptide from the 11 S species was further characterized by chymotryptic digestion. HPLC separation of the digestion products is shown in Fig. 3. All of the 3H label was associated with peak A, which had an NH2-terminal threonine similar to the parent peptide. The remainder of the tryptic peptide was contained in peak B and the compositions of both chymotryptic peptides are included in Table I.

The sequences of the two tryptic peptides and of the chymotryptic peptides were determined by a combination of manual dansyl-Edman degradation and automated sequencing using solid phase, spinning cup, and gas phase methodologies. The results of this sequencing are summarized in Fig. 4.

The first 7 residues of chymotryptic peak A were identified by stepwise manual Edman degradation, as well as the entire 7 residues of chymotryptic B. Because the 3H isopropylphospho-modified active site serine could not be identified from either the dansyl-Edman degradations or a phenylthiohydantoin, it was determined by the appearance of radioactivity following sequential degradation of protein coupled to the solid phase. The DFP-labeled residue was localized at position 8 for both the 11 S (Fig. 5) and 5.6 S enzymes. The peptide containing the reactive serine was isolated three times from the 5.6 S species and twice from the 11 S species.

The amino-terminal sequence of both enzyme forms of acetylcholinesterase was determined initially by spinning cup sequencing of the whole protein (Fig. 6). Since the first 4 residues of the 11 S enzyme form showed two amino acids at each step, to elucidate and further confirm the NH2-terminal sequences, the tryptic peptides following HPLC separation were screened by dansylation for peptides having an amino-terminal aspartic acid. One such peptide having the appropriate amino acid composition was found in Fraction IV for the 11 S enzyme and is identified in Fig. 2B. This peptide was sequenced by solid-phase and manual dansyl-Edman methods and is included in Fig. 6. The amino-terminal tryptic peptide of the 5.6 S form was not found in Fraction IV but was eventually recovered in Fraction III eluting at a similar position. Its sequence is also indicated in Fig. 6. The compositions of both peptides are shown in Table I.

![Fig. 6. Sequence analysis of the NH2-terminal regions. Letter designations are the same as described in the legend for Fig. 4. AChE, acetylcholinesterase.](image-url)

![Fig. 7. Sequence comparison of the active sites and NH2-terminal regions of Torpedo acetylcholinesterase and human serum butyrylcholinesterase.](image-url)

The sequences from Torpedo acetylcholinesterase are circled. Large arrows indicate identical sequences between the Torpedo acetylcholinesterase and the human butyrylcholinesterase. Small arrows denote conservative changes where functionally related amino acids are retained and codons potentially differ by a single nucleotide. The triangle above serine at residue 8 indicates the active site. The CHO on residue 19 of the active site of human butyrylcholinesterase indicates a carbohydrate attachment site. The butyrylcholinesterase sequences were taken from Lockridge (23).
Fig. 8. Comparison of active site region sequences with other serine proteinases and esterases (see Refs. 23 and 24). The arrow indicates [3H]isopropylphosphoryl-labeled or active site serine. Residues included in boxes are identical with the Torpedo acetylcholinesterase sequence.

DISCUSSION

The molecular forms of acetylcholinesterase found in Torpedo are characteristic of the individual forms identified in mammalian muscle and nerve (7). Although it is evident that the two forms can be distinguished by the nature of the post-translational processing events, it is unclear whether separate genes and/or separate mRNAs control the synthesis of the catalytic subunits of the individual forms. An examination of the primary structures of the two enzyme forms should resolve this issue and, for this reason, sequencing studies on the two enzyme forms were initiated. Acetylcholinesterase from the electric organ in Torpedo seems well suited for this undertaking since the individual enzyme forms appear in nearly equal proportions and several milligrams can be isolated from a kilogram of electric organ.

The globular species in Torpedo exist as simple dimers of 5.6 S which can be dissociated to a 3.3 S species following disulfide bond reduction in the presence of neutral detergent (3). Recently, evidence has accrued that the 5.6 S species contains a hydrophobic region near either the NH2-terminal or COOH-terminal end of the polypeptide chain (19–21). Digestion with proteases eliminates the tendency of the species to aggregate or associate with lipids, yet only a small reduction in molecular weight (~2000) can be detected on gel electrophoresis. The similarity in NH2-terminal sequences that we observe for the two forms of the enzyme and the absence of a blocked NH2-terminal group indicate that the hydrophobic region is not associated with the NH2-terminal end of the molecule.

The other form of acetylcholinesterase contains two structural subunits disulfide-linked to the catalytic subunits; thus, its biosynthesis involves post-translational assembly of dis-similar subunits. The entire assembly appears to occur intracellularly (cf. Ref. 22) and is subsequently transported across the cell membrane to its location in the basal lamina. Hence, both classes of acetylcholinesterase undergo unique post-translational changes which dictate the eventual disposition of the enzyme. It remains unestablished whether distinct differences in the primary structures of the nascent peptide chains provide the means for initiating the divergent post-translational steps. However, distinguishing peptide maps and antibody reactivity are suggestive of either separate messages or genes controlling the biosynthesis of the two forms of acetylcholinesterase (13).

The peptides sequenced to date are identical, and in the case of the active center peptide, considerable homology is evident with the corresponding peptide from human butyrylcholinesterase (23). This is particularly noteworthy because considerable evolutionary distance separates Torpedo and human and because the two enzymes exhibit different acyl group specificities (Fig. 7). More limited homology can be seen with several other esterases and proteinases that have a reactive serine at the active site (24, 25, and Fig. 8). The esterases are more homologous as a group and show conservation of sequence on both sides of the reactive serine. In all cases, an acidic residue immediately precedes the reactive serine. The amino-terminal sequences of the Torpedo enzymes show some homology with the amino-terminal sequence from human butyrylcholinesterase (23, Fig. 7), but do not exhibit the striking similarities found in the active site region. One noteworthy difference in this region is the absence of a glycosylation site in the Torpedo enzymes to match the site found in the human enzyme. Obviously there have been fewer evolutionary constraints to conserve the sequence in this region of the molecule.

REFERENCES