The inter- and intrasubunit disulfide bridges for the 11 S form of acetylcholinesterase isolated from Torpedo californica have been identified. Localized within the basal lamina of the synapse, the dimensionally asymmetric forms of acetylcholinesterase contain either two (13 S) or three (17 S) sets of catalytic subunits linked to collagenous and noncollagenous structural subunits. Limited proteolysis of these molecules yields a tetramer of catalytic subunits that sediments at 11 S. Each catalytic subunit contains 8 cysteine residues which were identified following tryptic digestion of the reduced, \(^{14}C\)-carboxymethylated protein. The tryptic peptides were purified by gel filtration followed by reverse-phase high performance liquid chromatography (HPLC) and then sequenced. The disulfide bonding profile was determined by treating the native, nonreduced 11 S form of acetylcholinesterase with a fluorescent, sulfhydryl-specific reagent, monobromobimane, prior to tryptic digestion. Peptides again were resolved by gel filtration and reverse-phase HPLC. One fluorescent cysteine-containing peptide was identified, indicating that a single sulfhydryl residue, Cys\(^{291}\), was present in its reduced form. Three pairs of disulfide-bonded peptides were identified. These were localized in the polypeptide chain based on the cDNA-deduced sequence of the protein and were identified as Cys\(^{87}\)-Cys\(^{244}\), Cys\(^{304}\)-Cys\(^{366}\), and Cys\(^{402}\)-Cys\(^{621}\). Hence, three loops are found in the secondary structure. Cys\(^{572}\), located in the carboxyl-terminal tryptic peptide, was disulfide-bonded to an identical peptide and most likely forms an intersubunit crosslink.

Since the 6 cysteine residues in acetylcholinesterase that are involved in intrachain disulfide bonds are conserved in the sequence of the homologous protein thyroglobulin, it is likely that both proteins share a common folding pattern in their respective tertiary structures. Cys\(^{291}\) and the carboxyl-terminal cysteine residue Cys\(^{572}\) are not conserved in thyroglobulin.

The dimensionally asymmetric or elongated forms of acetylcholinesterase, purified from the electric organ of Torpedo californica, contain two or usually three tetrameric sets of catalytic subunits disulfide-linked to structural subunits of collagenous and noncollagenous composition (Lwebuga-Mukasa et al., 1976; Reiger et al., 1976; Vir.tellte and Bernhard, 1980; Lee et al., 1982a; Lee et al., 1982b). These forms sediment at 13 S and 17 S, respectively. Each catalytic subunit of 68,000 daltons contains both intrachain and interchain disulfide bonds, and the tetramer furthermore is linked by disulfide bonds to the collagen-like, filamentous subunit (Lee et al., 1982b; Lee et al., 1982a; Lee and Taylor, 1982). This collagenous subunit, which has been estimated to be a 500 x 20 Å filament based on electron microscopy, is thought to contain three strands, each of which may terminate at a tetramer of catalytic subunits (cf. Cartaud et al., 1975). Limited proteolysis of the 17 S and 13 S species cleaves the structural subunits yielding a predominant 11 S form. This 11 S enzyme exists as a tetramer of catalytic subunits and has identical catalytic activity to the elongated species. Localization of the 17 S and 13 S species of acetylcholinesterase at the basal lamina, usually in synaptic regions, has been demonstrated histochemically (Sanes et al., 1975). The relative abundance of this form of acetylcholinesterase in muscle and in the electric organ of Torpedo can be correlated with the processes of development and synaptogenesis (cf. Massoulie and Bon, 1982).

The catalytic properties of acetylcholinesterase and its role in synaptic transmission are well documented (see Rosenberry, 1975 and Taylor, 1985 for reviews). Each catalytic subunit contains a reactive serine residue at the active center (MacPhee-Quigley et al., 1985) as well as a peripheral anionic site (Taylor and Lappi, 1975). The immediate sequence surrounding the catalytic serine exhibits some homology with the extensively studied trypsin-based family of serine hydrolases, and, until recently, it seemed likely that the cholinesterases might be evolutionarily related to this serine protease family of enzymes. However, a cDNA clone for acetylcholinesterase has been isolated, and from the DNA sequence the complete amino acid sequence of the mature enzyme has been deduced (Schumacher et al., 1986). This sequence, which has been largely confirmed by protein sequencing (MacPhee-Quigley et al., 1986), has shown unambiguously that acetylcholinesterase is not homologous to any of the known serine proteases or esterases.

With the deduced amino acid sequence in hand, one of the next steps in the structural analysis of this extracellular protein was to establish the pattern of the inter- and intrasubunit disulfide bonding. The arrangement of the disulfide bonds is reported here. Since the recently reported primary structure of bovine thyroglobulin exhibits substantial sequence homology in its COOH-terminal domain with acetylcholinesterase (Schumacher et al., 1986), the potential disulfide bonding patterns of these two proteins are compared.

**EXPERIMENTAL PROCEDURES**

*Purification of Acetylcholinesterase*—The 11 S species of acetylcholinesterase was purified from T. californica using affinity chromatography (Taylor and Jacobs, 1974; Lee et al., 1982a). Both specific activities and staining intensities following polyacrylamide gel elec-
trophoresis were used to verify purity.

Isolation of the Cysteine-containing Tryptic Peptides from Reduced Acetylcholinesterase—Twenty mg of the 11 S form of acetylcholinesterase at a concentration of 3-4 mg/ml in 50 mM NH$_4$HCO$_3$, pH 8.1, were denatured in 6 M guanidine-HCl in 0.1 M Tris-HCl, pH 8.1. Dithiothreitol was added to a 2-fold molar excess over the total number of cysteine residues. This sample was incubated at 50 °C for 3 h under nitrogen. The cysteines were labeled with $^{[14]C}$iodoacetic acid (specific activity 10-50 mCi/mmole) by incubating for 1 h at 25 °C in the dark under nitrogen at a 2-fold molar excess over total thiols. This reaction was stopped with a 10-fold excess of dithiothreitol and the mixture dialyzed against 50 mM NH$_4$HCO$_3$, pH 8.1. Trypsin (1%, w/w) was added and incubation was continued for 8 h at 37 °C. The digested protein was applied to a Sephadex G-50 (superfine) column (1.5 x 200 cm) which had been equilibrated in 50 mM NH$_4$OH. Three-ml fractions were collected at a flow rate of 30 ml/h. Absorbance at 219 nm and $^{14}$C radioactivity were monitored. The fractions were pooled and lyophilized to approximately 1 ml. The pooled peptides were separated by reverse-phase HPLC on a Vydac C-4 or C-18 µBondapack column using an aqueous 0.1% trifluoroacetic acid or 10 mM sodium phosphate, pH 6.9, acetonitrile gradient.

Isolation of the Cysteine-containing Tryptic Peptides from Nonreduced Acetylcholinesterase—Acetylcholinesterase was denatured as described for the reduced peptides, but the dithiothreitol and $^{[14]C}$iodoacetic acid were not added. Free cysteines were covalently modified with monobromobimane (Thiolyte) obtained from CalBiochem (Fahy and Newton, 1983). The monobromobimane was dissolved in a small volume of acetonitrile, then added to a final concentration of 10 mM in the presence of guanidine-HCl and allowed to incubate at 20 °C under nitrogen for 1 h. The protein was then dialyzed against 50 mM NH$_4$HCO$_3$, pH 8.1. Tryptic digests were prepared as described for reduced peptides. Relative fluorescence was monitored with a Model A2-150 N Amino-Bowman spectrophotofluorometer, with an excitation wavelength of 390 nm and an emission wavelength of 480 nm. Reverse-phase HPLC was performed as for the reduced samples. The bimane absorbance/fluorescence was followed at 390 nm or with a Gilson Model FLIA fluorometer.

Chymotryptic Digestion of Tryptic Peptides—Approximately 10 nmol of peptide from reverse-phase HPLC were adjusted to pH 8.0 with 1 M Tris-HCl. Chymotrypsin was added (approximately 3%, w/w) and incubated at 37 °C for 4 h. This digest was purified by again injecting onto a Vydac C-18 reverse-phase HPLC column utilizing the same conditions as described for the tryptic peptides.

Amino Acid Analysis—Amino acid analyses were performed using an LKB 4400 amino acid analyzer. Approximately 1-5 nmol of peptide was dissolved in 6 N HCl and hydrolyzed in vacuo at 110 °C for 20 h.

Sequencing—Sequencing was done by gas-phase methods employing an Applied Biosystems Protein Sequencer (Model 470A).

RESULTS

Amino acid analysis of the 11 S form of acetylcholinesterase indicated that each subunit contained a total of 8 unique cysteine residues. In order to identify all of these cysteine residues, the protein was denatured with guanidine-HCl, reduced with dithiothreitol, and alkylated with $^{[14]C}$iodoacetate acid. The $^{14}$C-carboxymethylated protein then was digested with trypsin. The resulting tryptic peptides were initially fractionated by gel filtration on Sephadex G-50. The eluted peptides shown in Fig. 1 were divided into 10 fractions as indicated and further resolved by reverse-phase HPLC. The two largest fractions, I and II, were chromatographed on a C-4 µBondapack column. The elution profile of fraction II is shown in Fig. 2. Two unique radioactive peaks were isolated from this fraction. The remaining fractions from the Sephadex column were resolved by HPLC on a C-18 µBondapack column and yielded 5 additional cysteine-containing peptides. A summary of the elution of these radioactive peaks following HPLC and the amino acid sequences of each of the cysteine-containing sequences is given in Fig. 3 and Table I.

Having identified all of the cysteine-containing tryptic pep-

1 The abbreviations used are: HPLC, high performance liquid chromatography; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.
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only this peptide in the protein was found to contain cysteine in its reduced state. Fraction III also contained a fluorescent peak that was recovered following lyophilization and HPLC; however, the fluorescence did not correlate with a major peptide based on absorbance at 219 nm (Fig. 5). The identification of this peak is still under investigation. Preliminary scans indicate an excitation maximum of 385 nm for this bimane peak versus an excitation maximum of 380 nm for the bimane peak fraction V. The emission maximum, 480 nm, was identical for both fractions.

In order to facilitate the identification of cystine-containing peptides, the elution profiles of fractions I and II (Fig. 4) from the HPLC runs were compared to the profiles that were obtained for the fully reduced carboxymethylated protein. This comparison revealed that the previously identified carboxymethylated peptides were missing. At the same time, several new peaks were apparent based on comparison of the

![Figure 3](image-url)  
**FIG. 3. Localization of the 14C-carboxymethylated peptides.** The bar graph depicts the elution of the tryptic peptides containing 14C-carboxymethylated cysteine. Each of the pooled Sephadex fractions from Fig. 1 was lyophilized and separated by reverse-phase HPLC as described under "Experimental Procedures." Each peak was numbered as indicated. Fractions I, II, and III were separated on a Vydac C-4 column with a trifluoroacetic acid to CH3CN gradient of 0-20% in 20 min, then 20-50% in 120 min, while fractions IV, V, and VI were separated on a Vydac C-18 column with a trifluoroacetic acid to CH3CN gradient of 0-50% in 120 min.

![Figure 4](image-url)  
**FIG. 4. Gel filtration profile of the nonreduced tryptic peptides from the catalytic subunit of the 11 S form of Torpedo acetylcholinesterase.** The enzyme was denatured, treated with monobromobimane, and digested with trypsin. The resulting peptides were eluted from Sephadex G-50 as described in Fig. 1. The bimane-modified peptides were determined by relative fluorescence, (---), and absorbance was monitored at 280 nm (---). Solid arrows indicate pooled fractions. The inset shows the amount of relative fluorescence remaining in pools I, III, and V after lyophilization.

![Figure 5](image-url)  
**FIG. 5. HPLC purification of Sephadex fractions III and V containing bimane-modified peptides.** The regions of the Sephadex profile indicated by the Roman numerals III and V in Fig. 4 were pooled, lyophilized, and separated by reverse-phase HPLC as indicated under "Experimental Procedures." Absorbance at 219 nm (---) and relative fluorescence (-----) were monitored.

**TABLE I**  
<table>
<thead>
<tr>
<th>Peptide number</th>
<th>Sequence position</th>
<th>NH2-terminal residue</th>
<th>Carbohydrate position</th>
<th>Carbohydrate</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ia(IIb)</td>
<td>67</td>
<td>Lys</td>
<td>No</td>
<td>Yes</td>
<td>KPW...</td>
</tr>
<tr>
<td>Ib(Vla)</td>
<td>521</td>
<td>Val (Leu)</td>
<td>(Yes)</td>
<td>(L.R)...</td>
<td></td>
</tr>
<tr>
<td>Ic(I1c,IIc)</td>
<td>94</td>
<td>Gix</td>
<td>No</td>
<td>E...</td>
<td></td>
</tr>
<tr>
<td>I1a(II1b)</td>
<td>254 and 265</td>
<td>Asx</td>
<td>No</td>
<td>N...</td>
<td></td>
</tr>
<tr>
<td>I1b(II1d)</td>
<td>402</td>
<td>Asx</td>
<td>(Yes)</td>
<td>D...</td>
<td></td>
</tr>
<tr>
<td>IIIa(Iva)</td>
<td>572</td>
<td>His</td>
<td>No</td>
<td>H...</td>
<td></td>
</tr>
<tr>
<td>IVb(Va)</td>
<td>231</td>
<td>Ala</td>
<td>No</td>
<td>A...</td>
<td></td>
</tr>
</tbody>
</table>
The region of the Sephadex profile indicated by Roman numeral II in Fig. 4 was pooled, lyophilized, and separated by reverse-phase HPLC as indicated under "Experimental Procedures." The peak labeled 1 contained cystine peptides Ia and Ic, while the peak labeled 2 contained cystine peptides Ib and IIb. The inset is a rechromatograph of peak J on the same HPLC column using a NaH₂PO₄/acetonitrile gradient.

Each of the purified cystine-containing peaks was reduced with 1 mM dithiothreitol and alkylated with [¹⁴C]iodoacetic acid. The resulting products were again separated by reverse-phase HPLC using a C-18 µBondapak column. The sample was injected but the gradient not started until 20 min after injection. This method allows excess reagents to flow through the column while the peptides are retained. These peptides were eluted utilizing a trifluoroacetic acid/acetonitrile gradient of 0–20% in 20 min and 20–50% in 120 min. The peptides containing [¹⁴C]carboxymethylcysteine were identified by their incorporated radioactivity, subjected to amino acid analysis, and again sequenced. Two of these [¹⁴C]iodoacetic acid-labeled samples contained two radioactive peptides, and the sequence of these peptides confirmed the earlier disulfide bonding assignments: Cys⁶⁷–Cys⁸⁴, and Cys⁴⁰⁵–Cys⁵ⁱ. This treatment was not performed on peptides containing Cys⁵⁷, Cys⁵⁷⁵, or the peptide containing the internal disulfide, Cys⁴⁰⁴–Cys⁵⁸⁵.

**DISCUSSION**

Our understanding of the protein folding for acetylcholinesterase has been facilitated greatly by the recent sequencing of the gene that codes for the enzyme. This sequence also has been confirmed in many regions by protein sequencing. The protein sequence deduced from the cDNA has been used to place all of the cysteine residues in the linear sequence as indicated in Table I. Although the detailed three-dimensional structure of acetylcholinesterase can only be ascertained from x-ray diffraction analysis of crystals of the protein, disulfide bonding assignments should place constraints on the enzyme’s folding pattern.

Eight cysteine residues have been identified and sequenced in the 11 S form of acetylcholinesterase. The disulfide assignments for these 8 residues include three pairs of intramolecular disulfide bonds, Cys⁵⁷–Cys⁸⁴, Cys⁴⁰⁵–Cys⁵⁷⁵, and Cys⁴⁰⁴–Cys⁵⁸⁵. This assignment of disulfide bonds creates three secondary loop structures in the linear molecule, one short loop (B) and two long loops (A and C), as shown in Fig. 8. Of the 2 remaining cysteines, Cys⁴⁷⁷ was alkylated with monobromobimane in the absence of a reducing agent and thus is identified as the only free sulfhydryl group. Free sulfhydryl groups are rarely found in secreted proteins, and whether this cysteine has a unique function remains to be determined. The remaining cysteine residue, Cys⁴⁰⁴, presumably participates in an intramolecular disulfide bond, since it is disulfide-bonded to itself. This residue then links the carboxyl-terminal tryptic peptide of one monomeric subunit to another in the 11 S form of acetylcholinesterase. Since in the absence of reduction, the 17 S tetrameric species can be dissociated into a free dimer and a tail-associated dimer by treatment with sodium dodecyl
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FIG. 9. Schematic representation of the homology and common secondary structure between acetylcholinesterase (residues 1–575) and thyroglobulin (residues 2182–2750). Numbering is based on the deduced sequence of acetylcholinesterase. Loops created by disulfide bonds are indicated by A, B, and C. ● indicates identical residues; ○ indicates conservative changes; @ indicates the active site serine; SH, the free sulfhydryl group; [C(CHO)], AChE carbohydrate attachment sites; (S)₈, disulfide link to a secondary AChE monomer. Homologous residues with thyroglobulin are as indicated. Arrows denote residue gaps in acetylcholinesterase; the number indicates the number of residues missing; – indicates residue gaps in thyroglobulin sequence. @, thyroglobulin carbohydrate attachment sites. Conservative changes reflect amino acid substitutions that could be encoded by a single base difference.

sulfate (Lee and Taylor, 1982), it would appear that the free dimer at least is connected at this point. This assignment of Cys₅⁶² as the intermolecular disulfide linkage is consistent with data which indicate limited proteolysis of the dimeric species of human butyrylcholinesterase produces monomers without an apparent reduction in molecular weight (Lockridge and La Due, 1982).

The collagen-like filamentous tail is also thought to be disulfide-bonded to the catalytic subunit. However, in the present study we did not identify the site of this covalent linkage. The two most probable candidates would be Cys²³¹ and Cys⁵⁶², since neither of these residues appears to play a crucial role in the folding of the linear polypeptide chain. Disulfide bonding to the tail need not be stoichiometric since an intersubunit bond to only one of the four tetramers could be sufficient. In any case, should one or even two of the four subunits in the tetramer be disulfide-bonded to small peptides from the collagen-like tail, the identification has eluded us so far.

An examination of the primary structure of acetylcholinesterase (Schumacher et al., 1986) indicated that there was only very limited homology with the trypsin family of serine proteases in the active site region (MacPhee-Quigley et al., 1985). This serine protease family contains a uniquely reactive serine residue which catalyzes the hydrolysis of peptide or ester substrates (Holbrook and Wolfe, 1972). Studies from the crystal structure of chymotrypsin revealed a catalytic triad, designed to increase the nucleophilicity of the serine (Davie et al., 1979), the “charge relay system” (Blow et al., 1970). In chymotrypsin, this system involved His⁵⁷, Asp¹¹⁷⁶, and Ser¹⁹⁵, and the histidine residue was localized next to a cysteine in the histidine loop (Hartley, 1970). However, when the first bacterial serine protease crystal structure (subtilisin from Bacillus amyloliquifaciens) was solved, the folding of the polypeptide chain was found to be completely different from the previously solved mammalian serine proteases (Coulson and Rabin, 1969).

In spite of limited homology surrounding the active site serine and similarities in catalytic mechanism of acetylcholinesterase and the trypsin-like serine proteases, the elucidation of the cDNA sequence established that the two structures were distinct. Not only are the residues for a conserved charge relay system clearly missing from their predicted locations, but the disulfide bonding pattern is quite different in the two proteins. All of the serine proteases that are homologous to trypsin have conserved the disulfide bonding pattern even though several of them such as prothrombin contain large insertions and are glycosylated. With the active site serines of acetylcholinesterase (Ser²⁰⁰) and chymotrypsin (Ser¹⁹⁵) at similar positions, the catalytic histidine would be expected to be near position 57, yet we find no histidine in acetylcholinesterase between positions 26 and 158. The B-loop in acetylcholinesterase actually is similar to the histidine loop in trypsin with the histidine immediately adjacent to the disul-
fide bond. This histidine, however, is quite distal to the reactive serine.

Serinolism also contains a catalytic triad that participates in a charge-relay system (Asp, His, and Ser) (Alden et al., 1970; Wright et al., 1969) even though its overall structure is unrelated to trypsin. Although the rank order of residues contributing to the triad in serinolism differ from the aforementioned serine proteases, this family of enzymes also shows no homology with acetylcholinesterase. Thus, while the pH dependence of acetylcholinesterase activity is consistent with a role of histidine in catalysis, its assignment in the sequence is not evident. Acetylcholinesterase may represent an additional example of convergent evolution to achieve the appropriate three-dimensional disposition of critical residues and a common catalytic mechanism.

A search for related proteins in Bovet (Doolittle, 1981), NBRF, and Genbank (Bionet) did, in fact, identify a homology of approximately 31% between acetylcholinesterase and the last 594 amino acids of the carboxyl-terminal portion of thyroglobulin. Thyroglobulin becomes iodinated and is stored in the thyroid gland as a precursor for the thyroid hormones, thyroxine and triiodothyronine. Thyroglobulin is a dimeric glycoprotein with molecular mass of 600,000. Each monomer of 2,769 amino acids contains a pattern of cysteine-rich imperfect repeats. The carboxyl-600 residues of thyroglobulin do not manifest this internal homology, but do contain 3 of the 4 tyrosine residues which are iodinated and thus involved in hormonogenesis. The 1 residue that is known to be involved in catalysis, the active site serine from acetylcholinesterase, is not conserved in thyroglobulin. The 3 tyrosines in the carboxyl-terminal portion of thyroglobulin, which can be iodinated, also are not conserved in acetylcholinesterase. The glycosylation sites, likewise, are not conserved in the two proteins.

On the other hand, common structural characteristics are evident in the two proteins. For example, when the cysteine positions in the asymmetric form of acetylcholinesterase (Schumacher et al., 1986) are compared to the cysteine positions in the homologous portion of thyroglobulin (Mercen et al., 1985), it is apparent that these two proteins share a similar folding pattern (Fig. 9). All of the cysteine residues that participate in disulfide bonding in acetylcholinesterase are conserved in both proteins and the three loops are of similar size. Furthermore, Cys that remains as a free sulfhydryl in acetylcholinesterase and Cys that is involved in an intermolecular disulfide bond are not conserved in thyroglobulin, which supports the idea that these 2 residues play a role that is not related directly to stabilizing the folded form of the enzyme. The conservation of global structure in conjunction with the divergence in functional residues suggests that this structure may be a prototype for a family of high molecular weight secreted proteins which have evolved with totally different functions.

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