Human serum cholinesterase was digested with pepsin under conditions which left disulfide bonds intact. Peptides were isolated by high pressure liquid chromatography, and those containing disulfide bonds were identified by a color assay. Peptides were characterized by amino acid sequencing and composition analysis. Human serum cholinesterase contains 8 half-cystines in each subunit of 574 amino acids. Six of these form three internal disulfide bridges: between Cys58, Cys82, Cys225-Cys383, and Cys106-Cys518. A disulfide bond with Cys58 rather than Cys82 was inferred by homology with Torpedo acetylcholinesterase. Cys871 forms a disulfide bridge with Cys771 of an identical subunit. This interchain disulfide bridge is four amino acids from the carboxyl terminus. A peptide containing the interchain disulfide is readily cleaved from cholinesterase by trypsin (Lockridge, O., and La Du, B. N. (1982) J. Biol. Chem. 257, 12012-12018), suggesting that the carboxyl terminus is near the surface of the globular tetrameric protein. The disulfide bridges have exactly the same location as in Torpedo californica acetylcholinesterase. There is one potential free sulfhydryl in human cholinesterase at Cys886, but this sulfhydryl could not be alkylated. Comparison of human cholinesterase, and Torpedo and Drosophila acetylcholinesterases to the serine proteases suggests that the cholinesterases constitute a separate family of serine esterases, distinct from the trypsin family and from subtilisin.

Human serum cholinesterase (EC 3.1.1.8 acetylcholine acetylhydrolase, butyrylcholinesterase, pseudocholinesterase, non-specific cholinesterase) is distinguished from acetylcholinesterase by substrate preferences and differential sensitivity to certain inhibitors (1). Nevertheless, the two enzymes hydrolyze many of the same esters and are inhibited by chemically similar compounds. Their structures are organized similarly into globular and asymmetric collagen-tailed forms (2). The globular form in human serum is a tetramer of four identical subunits assembled as a dimer of dimers (3). Similarly a tetrameric dimer of dimers structure is also found in the subunits assembled as a dimer of dimers activity and structure, antibodies to human cholinesterase do not cross-react with human acetylcholinesterase, and vice versa (6). The lack of recognition by antibodies aroused suspicion that the amino acid sequences and the protein folding might be significantly different in the two kinds of cholinesterases. Therefore, it was a surprise to find that the amino acid sequence of human serum cholinesterase (7) is 53.8% identical with the amino acid sequence of acetylcholinesterase from Torpedo californica (8). The chain lengths are nearly the same at 574 and 575 amino acids/subunit.

In this report we locate the disulfide bonds in human cholinesterase and compare the results with the disulfide bonds in Torpedo acetylcholinesterase (9). We found that the disulfide bonds in human cholinesterase are in the same location as in Torpedo acetylcholinesterase and have the same number of amino acids within each disulfide loop. This suggests that protein folding in the two enzymes is similar.

To explain the lack of recognition by antibodies, amino acid sequence differences could still be invoked. The human cholinesterase but not the human acetylcholinesterase sequence is known; the Torpedo acetylcholinesterase but not the Torpedo cholinesterase sequence is known. Therefore, it is not yet possible to compare the two enzymes from a single species. Drosophila acetylcholinesterase is the only other cholinesterase which has been sequenced to date (10), but Drosophila has only one type of cholinesterase which has specificities intermediate between acetylcholinesterase and cholinesterase. Another factor which may affect antibody recognition is the number and location of carbohydrate chains. Human cholinesterase has nine asparagine-linked carbohydrate chains (7), whereas Torpedo acetylcholinesterase has four potential asparagine-linked carbohydrate chains (9), only two of which have the same location in cholinesterase.

**EXPERIMENTAL PROCEDURES**

**Purification of Cholinesterase**—Outdated human plasma was a gift from the Michigan Department of Public Health, Lansing, MI. 7.5 liters of plasma were used for each cholinesterase purification procedure, which consisted of three steps: ion-exchange chromatography at pH 4.0, followed by affinity chromatography on procamidine-Sepharose 4B, and finally by ion-exchange chromatography at pH 7.0 (3, 11, 12). The yield was 8-12 mg of highly purified cholinesterase.

**Digestion with Pepsin**—Cholinesterase disulfides were not reduced and alkylated prior to digestion with pepsin, because we wanted the disulfide bands intact. 25 mg of cholinesterase in a volume of 6.5 ml was used for the digestion. The pH was adjusted to 1.3 by adding 1.6 ml of 88% formic acid. Then, 0.5 mg of pepsin (porcine mucous, Sigma No. P6887) was added. Digestion was at 37 °C for 48 h.

**HPLC Purification of Peptides**—Pepsin-digested cholinesterase was injected onto a Synchronpak RP-P reverse-phase column on a Varian model 5060 HPLC equipped with UV and fluorescence detectors. The Synchronpak RP-P column, with its 300-Å pore size, gave high recoveries of even large peptides. Elution was with 0.1% heptafluorobutyric acid and acetonitrile. Further purification was achieved by using a phenyl mBondapak reverse-phase column (Waters Co.).

*This work was supported in part by the United States Army Medical Research and Development Command, Contract DAMD17-82-C-2271 (to O. L.) and by National Institutes of Health Grant GM 27028 (to B. N. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1The abbreviation used is: HPLC, high pressure liquid chromatography or high pressure liquid chromatograph.
eluted with 0.1% trifluoroacetic acid and acetonitrile, or the Synchropak RP-P column eluted with 0.1 M hexafluoroacetate-ammonia, pH 7.5, and acetonitrile. Hexafluoroaceton (Aldrich) is at present the only available neutral pH buffer that is volatile and UV transparent (15).

Identification of Disulfide-containing Peptides—Thannhauser et al. (14) have devised a rapid sensitive method for detecting disulfide bonds. 50 μl aliquots of HPLC fractions were mixed with 120 μl of Thannhauser’s cleavage buffer, incubated for 10 min, and then reacted with 5 μl of 25 mM disodium 2-nitro-5-thiosulfobenzoate assay solution. A yellow color indicated the presence of disulfide bonds.

Reduction and Alkylation with Vinylpyridine—Peptides containing a disulfide bond were dissolved in 0.5 ml of 6 M guanidine HCl (Pierce Chemical Co., Sequanul grade) containing 50 mM Tris-Cl, pH 8.0, and 1 mM EDTA. Dithiothreitol was added to a final concentration of 26 mM. The solution was blanketed with nitrogen. After 1-4 h, 4 μl of 4-vinylpyridine (Aldrich) was added to a final concentration of 72 mM. Alkylation with vinylpyridine was allowed to proceed under nitrogen for 2-24 h before the sample was injected into the HPLC. Vinylpyridine was used because it gives a derivative that is readily detected during sequencing and amino acid analysis (15).

Amino Acid Sequencing—Peptides were sequenced by the manual method of Edman (16). Tarr’s modifications of the Edman degradation allowed sequencing 5 to 50 peptides at the same time using 200 pmol to 2 pmol of each peptide. Phenylthiodyantoin was identified on a Waters HPLC using an Ultraspere ODS 5-μm column (Altex) at 50 °C. The column was equilibrated with 100 mM ammonium acetate, pH 4.4, containing 25% acetonitrile. Immediately after sample injection, the elution buffer was switched to 100 mM ammonium acetate, pH 4.5, containing 50% acetonitrile (17). One analysis was completed every 15 min.

Amino Acid Analysis—The University of Michigan Sequencing Facility under the direction of George Tarr performed amino acid composition analysis by the pico-tag method (15). Salt-free peptides were hydrolyzed for 4 h at 150 °C in 6 N HCl, the amino acids were derivatized with phenylisothiocyanate, and the phenylthiocarbamyl derivatives were identified by HPLC.

Disulfide bonds of cholinesterase—1 mg of cholinesterase in 1.5 ml of 6 M guanidine HCl, 50 mM Tris-Cl, 1 mM EDTA, pH 8.4, was made anaerobic by blowing nitrogen for 1.5 h. 28 μl of 6.66 mM [3H]iodoacetic acid (150 Ci/mol, Amersham Corp.) was added, and the reaction was allowed to proceed for 1 h. Dialysis was used to remove unbound iodoacetic acid. The stoichiometry of labeling was determined by analysis of aliquots of 5 to 50 peptides at the same time using HPLC. The HPLC chromatogram showed that digestion was extensive. All of the radioactivity eluted in the breakthrough volume.

Computer Analysis—The Protein Identification Resource, National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D. C. contains continuously updated databanks of protein and nucleic acid sequences in databanks NBRF, NEW, KABAT, JAPAN, PGTRANS, EMBL, and GENBANK. Cholinesterase was examined for the presence of tryptophan, tryptophan, growth factor, carrier, and vitamin B homologs by use of these databanks. The ALIGN program was used to compare homologies of three cholinesterase sequences. The PRPLOT program was used to plot the hydropathy index.

RESULTS

Our strategy for identifying disulfide-linked peptides was based on knowledge of the complete amino acid sequence of cholinesterase (7) and on our observation that the protein contains no detectable free sulfhydryls. The protein was digested under conditions which left the disulfide bonds intact. HPLC fractions containing disulfide were identified by a color assay (14). After peptide purification and sequencing, a disulfide link could be inferred by reference to the complete amino acid sequence. To have additional proof of disulfide linkage the purified disulfide peptides were reduced, the products alkylated with vinylpyridine and rechromatographed on HPLC, and finally subjected to amino acid composition analysis. This additional proof was considered necessary because neither sequencing nor amino acid composition analysis detected cystine or nonalkylated cysteine. Finding the expected number of alkylated cysteines and the expected amino acid composition led to the conclusion that two cysteine residues were disulfide-linked. The possibility that peptides contained free sulfhydryls rather than disulfide bonds was tested by alkylation experiments with radiolabeled iodoacetic acid or with vinylpyridine.

Pepsin was chosen for digestion of cholinesterase because the optimum pH for pepsin activity is pH 1-2. Disulfide bonds are stable at acid pH. Disulfide interchange, a possible occurrence in denatured protein at alkaline pH (18), was thereby avoided. We found that pepsin preferred to cleave at the carbonyl side of leucine and phenylalanine when digestion was carried out at pH 1.3. When digestion was at pH 2.0, pepsin was less specific. Fig. 1 shows HPLC separation of peptides produced by digestion of cholinesterase with pepsin at pH 1.3. The Thannhauser color reaction (14) showed that disulfide-containing peptides eluted at 41, 43, 45, 47, 49, 51, and 59 min. Fractions were purified by additional HPLC runs and the identity of the peptides determined by amino acid sequencing. The HPLC fractions in Fig. 1 are mixtures of nondisulfide peptides as well as one or more disulfide-containing peptides. The four disulfide-containing peptides obtained in highest yield are discussed below. Other disulfide-containing peptides were subfragments of the four peptides in Fig. 2.
was with vinylpyridine. The peptide peaks are identified as disulfide-containing peptides. The solvent in which peptides were dissolved, HPLC for side products. In solvent B (acetonitrile containing 0.075% trifluoroacetic acid) at 20% for 5 min is formic acid, the solvent in which peptides were dissolved. HPLC for A1, A2, B2, B3, and B4 used a phenyl column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with a gradient increasing in solvent B (acetonitrile containing 0.075% trifluoroacetic acid) at 1%/min. For A2 the phenyl column was equilibrated with 80% solvent A, 20% solvent B, and eluted with a gradient increasing in solvent B at a rate of 0.3%/min. For A3 and A4 a Synchronex RP-P column was equilibrated with 75% solvent C (0.1 M hexafluoracetone/ammonia, pH 7.0), 25% acetonitrile, and eluted with a gradient increasing in acetonitrile at a rate of 1%/min.

**FIG. 2.** HPLC purification of disulfide-containing peptides and of the same peptides after reduction and alkylation. Panels A1, A2, A3, and A4 show HPLC chromatograms which yielded purified disulfide-containing peptides. Panels B1, B2, B3, and B4 show HPLC chromatograms of the same peptides after reduction with dithiothreitol in the presence of 6 M guanidine chloride and alkylation. In B1 alkylation was with iodoacetic acid. In B2, B3, and B4 alkylation was with vinylpyridine. The peptide peaks are Ib, IIb, IIIb, and IVb; all other peaks in panel B are due to excess reagent and alkylation side products. In panel A the large peak between 2 and 5 min is formic acid. The sequence of both peptides was obtained simultaneously when the purified disulfide peptide IIa was subjected to amino acid sequencing. After reduction and alkylation of IVa, the peptide separated into IIb and IIIc as shown in panel B of Fig. 2. Both fragments were sequenced and subjected to amino acid composition analysis. Results agreed with the sequence shown and led to the conclusion that the disulfide bond is between Cys61 and Cys62.

Peptide IVa of Fig. 2 contained Phe-Ile-Cys-Pro-Ala-Leu-Glu and Asn-Thr-Glu-Ser-Thr-Arg-Ile-Met-Thr-Lys-Leu-Arg-Ala-Glu-Gln-Cys-Arg-Phe-Trp-Thr-Ser-Phe linked via a disulfide bond. The sequence of both peptides was obtained simultaneously when the purified disulfide peptide IIIa was subjected to amino acid sequencing. After reduction and alkylation of IVa, the peptide separated into IIb and IIIc as shown in panel B of Fig. 2. Both fragments were sequenced and subjected to amino acid composition analysis. Results agreed with the sequence shown and led to the conclusion that the disulfide bond is between Cys61 and Cys62.

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the disulfide peptide in panel A2 was isolated not only as the intact 66-residue peptide but also as the 60-residue peptide covalently attached to Ser-Glu-Asp-Cys-Leu. The peptide in panel A2 was also found as the subfragment Ala-Lys-Leu-Thr-Gly-Cys-Ser-Arg-Glu-Asn-Glu-Thr-Glu-Ile-Ile-Iys-Cys-Leu. The peptide pair in panel A3 was also found as the subfragment Arg-Ala-Glu-Gln-Cys-Arg-Phe-disulfide-bonded to Phe-Ile-Cys-Pro-Ala-Leu. The peptide in panel A4 was found not only as the intact 49-residue peptide but also as a disulfide-linked mixture whose amino acid sequence started with Phe-Pro-Lys-Val-Leu-Glu-Met and Met-Thr-Gly-Asn-Ile-Asp-Glu and Thr-Gly-Asn-Ile-Asp-Glu-Ala.

Residue 66 may be a free sulfhydryl. However, we were unable to specifically alkylate it with [3H]iodoacetic acid. Cys66 was successfully alkylated only under conditions which alkylated all 8 cysteines, that is following reduction with dithiothreitol in the presence of 6 mM guanidine chloride, 1 mM EDTA, pH 8.0.

Steric hindrance from the adjacent disulfide bond at position 65 could be a reason for lack of reactivity of the free sulfhydryl. To lessen this effect and make residue 66 more accessible, the protein was digested with various proteases before alkylation was attempted with radioactive iodoacetic acid. To test for incorporation of iodoacetic acid, the digests were chromatographed on HPLC and the HPLC fractions counted for radioactivity. No peptide was found to contain significant radioactivity. It is possible that the adjacent disulfide bridge blocked access to the sulfhydryl even in relatively short peptides.

Another explanation for the lack of reactivity of a free sulfhydryl could be that the free sulfhydryl is unstable once the protein is digested and that it becomes oxidized during the digestion period. With this possibility in mind, the protein was digested under conditions that stabilized free sulfhydryls, that is under a blanket of nitrogen, in the presence of 4 mM EDTA, for the short time of 4 h. [3H]iodoacetic acid was added at the end of the 4-h digestion period. The result was that even with these precautions, no alkylation was detected. Another possibility is that the free sulfhydryl became oxidized during protein purification or storage.

The possibility was considered that the free sulfhydryl was not free but was disulfide bonded to a collagen tail fragment or to some other peptide. NH-terminal sequencing of the intact protein showed only one sequence, whereas two sequences would have been expected if a peptide was disulfide-linked to a collagen tail fragment or to any other peptide. NH-terminal sequencing of the intact protein showed only one sequence, whereas two sequences would have been expected if a peptide was disulfide-linked to Cys66. Amino acid composition analysis of peptide Ia revealed no unknown peptide that might have been covalently attached to Cys66. Chymotryptic as well as tryptic subfragments of this peptide were sequenced (7), and all subfragments were found to fit the known 574-residue sequence. In conclusion there was no evidence to suggest that Cys66 is disulfide-linked to a collagen tail fragment or to any other peptide. Similarly, MacPhee-Quigley et al. (9) found no evidence for linkage to a collagen tail via Cys231, the free sulfhydryl of Torpedo acetylcholinesterase.

Other possible explanations for the lack of reactivity of Cys66 are that Cys66 is disulfide-bonded to a low molecular weight sulfhydryl compound such as glutathione, or that it is in a thioester linkage, or that Cys66 is a sulfenic or sulfinic acid (19). The possibility that Cys66 is protected by ligation to metal is considered unlikely because EDTA was present in all alkylation experiments.

**DISCUSSION**

There are 8 cysteines/subunit in the complete amino acid sequence of human serum cholinesterase (7). The present results suggest that six of these cysteines are involved in forming three internal disulfide bridges within one subunit. The intrachain disulfide bonds are at Cys56-Cys66 (Cys66 rather...
### Comparison of Cholinesterase Sequences

Human cholinesterase (CHE) was sequenced by Edman degradation of the serum protein (7). *T. californica* and *Drosophila melanogaster* acetylcholinesterase (ACHE) sequences were deduced from the nucleotide sequences of cDNA clones (8, 10). Sequences start at the amino terminus for the human and *Torpedo* enzymes but include the signal peptide for the *Drosophila* enzyme. The residue numbers correspond to the sequence of human cholinesterase. The disulfide bonds are known for the human and Torpedo enzymes but are speculative for *Drosophila* acetylcholinesterase. The disulfide bridge at Cys571 is the interchain disulfide which covalently links two identical subunits. Human cholinesterase has nine glycosylated (CHO) asparagines. The number of glycosylated asparagines in *Torpedo* and *Drosophila* acetylcholinesterase could be four and five, respectively. Two histidines, His180 and His186, are conserved in all three cholinesterases. The active site serine is Ser194. The sequences have been lined up to maximize homology with human cholinesterase. *Torpedo* acetylcholinesterase has 309 identical residues, and *Drosophila* has 317 identical residues. *Torpedo* acetylcholinesterase has 216 identical residues when compared to human cholinesterase.

### Comparison of Disulfide Bonds of Torpedo Acetylcholinesterase

Acetylcholinesterase from the electric organ of *T. californica* has been cloned and sequenced (8). It has 575 amino acids. There are 5 cysteines, and 8 cysteines. A fourth disulfide bridge involves Cys651 which appears to be covalently attached to Cys571 of an identical subunit. One potential free sulfhydryl was suggested, though the inaccessibility of Cys571 to alkylation left open the possibility that it was not free.

Comparison of Disulfide Bonds of Torpedo Acetylcholinesterase—Acetylcholinesterase from the electric organ of *T. californica* has been cloned and sequenced (8), and its disulfide bonds have been established (9). Acetylcholinesterase has a chain length of 575 amino acids. It has 8 cysteines. Its disulfide bonds are at Cys57-Cys64, Cys66-Cys86, Cys91-Cys115, Cys118-Cys123, Cys118-Cys123, Cys118-Cys123, the interchain disulfide bond. The free sulfhydryl is Cys253. Comparison of the disulfide bonding in acetylcholinesterase and cholinesterase shows that the disulfide bonds are in identical positions and involve the same cysteines (Fig. 3). Both proteins contain three internal disulfide loops of exactly the same length, and both are linked to an identical subunit via a cysteine that is 4 residues from the carboxyl terminus. The amino acid sequences are 53.8% identical. It is expected that the secondary and tertiary structures will have much in common.

One significant difference in the structures of these two proteins is in the number of carbohydrate chains. Human serum cholinesterase has nine asparagine-linked carbohydrate chains, at Asn57, Asn61, Asn78, Asn91, Asn200, Asn227, Asn451, Asn455, and Asn533 (7). *Torpedo* acetylcholinesterase has the possibility of four asparagine-linked carbohydrate chains, at Asn68, Asn174, Asn179, and Asn533 (9), where the sequence Asn-X-Thr/Ser occurs. This sequence is common to all N-glycosidically linked carbohydrate chains, though not every asparagine in such a sequence needs to be glycosylated (20). Cholinesterase and acetylcholinesterase have only two common glycosylation sites. Carbohydrate chains are known to be directed toward the surface of the molecule (21). The differences in number and location of carbohydrate chains may account for some structural differences near the surface and may partly explain why antibodies to one enzyme do not recognize the other enzyme.

The comparison can be extended to bovine thyroglobulin because 544 amino acids at the carboxyl terminus of this protein have 28% sequence identity with both *Torpedo* acetylcholinesterase and human cholinesterase (7, 8). The carboxyl-terminal portion of thyroglobulin has 6 cysteines located at positions homologous to the 6 cysteines which form intrachain disulfide bonds in cholinesterase and acetylcholinesterase. These 6 cysteines are expected to form the same three disulfide pairs in thyroglobulin as in cholinesterase and acetylcholinesterase. Thyroglobulin has two potential N-gly-
Cosylation sites in this region, but neither site is present in the cholinesterases.

Comparison to Drosophila Acetylcholinesterase—The cDNA of Drosophila acetylcholinesterase, including the signal peptide, indicates a total of 11 cysteines (10). Six cysteines are located in positions which would allow the same three internal disulfide bridges in Drosophila that are present in human cholinesterase and in Torpedo acetylcholinesterase (Fig. 3). The carboxyl-terminal portion of Drosophila acetylcholinesterase has very little homology with the Torpedo and human enzymes, and, therefore, it is unclear whether either of the two cysteines in that region is likely to have the function of linking two subunits. Drosophila acetylcholinesterase has five potential N-glycosylation sites, none of which is in exactly the same position as in the Torpedo or the human enzymes.

Disulfide at Cys571—Identification of Cys571 as the interchain disulfide is supported by earlier work where we had shown that there was one disulfide bond between two subunits (3) and that this interchain disulfide is located very near the subunit terminus (12). At that time we did not know whether this disulfide is near the amino or the carboxyl terminus. Cys571 is 4 residues away from Leu574 at the carboxyl terminus. Our earlier work showed that a peptide containing the interchain disulfide was easily removed by trypsin digestion. There are two lysines near the carboxyl terminus. Cleavage by trypsin at either Lys567 or Lys568 would remove 800–900 daltons and would explain why the cleaved subunit has the same apparent subunit weight of 85,000–90,000 as the intact subunit. Cholinesterase has a broad band on sodium dodecyl sulfate gels, probably because of its nine carbohydrate chains. The broadness of the band does not allow discrimination of differences less than 5,000 daltons.

Anglister et al. (22) also concluded that the interchain disulfide bridge for eel acetylcholinesterase was at one end of the catalytic subunit. For both eel acetylcholinesterase and human cholinesterase it has been observed that the interchain disulfide bridges are not essential for maintenance of quaternary structure under nondenaturing conditions.

The finding that the interchain disulfide is at Cys571 supports our earlier interpretation regarding the sodium dodecyl sulfate gel electrophoresis pattern of purified cholinesterase preparations. A preparation that has both monomer and dimer bands in the absence of reducing agent can be concluded to have experienced some proteolysis during the purification procedure. A preparation that has only a dimer-sized band of approximately 170,000–180,000 daltons in the absence of reducing agent can be concluded to be free of proteolytic cleavage. The effect of proteolysis is similar to the effect of mercaptethanol insofar as both yield a monomer-sized band on sodium dodecyl sulfate gel.

Hydropathy Index—The hydropathy (23) profiles for human cholinesterase and Torpedo acetylcholinesterase are similar in Fig. 4, suggesting that folding in the two proteins is similar. The region from residue 538 to 569 falls below the midpoint line and is, therefore, predicted to be on the exterior in both human cholinesterase and Torpedo acetylcholinesterase. This prediction agrees with the results of limited proteolysis discussed above. The evidence strongly supports the conclusion that the subunits in the globular tetrameric serum cholinesterase protein are arranged in such a way that the carboxyl ends are near the surface of the molecule.

The hydropathy profile of Drosophila acetylcholinesterase has less resemblance to those of the other two cholinesterases. Two regions stand out as different: the region from residue 33 to 53 and when it is omitted from the hydropathy figure the profile becomes more similar to the others. The 33-residue peptide starting at 107 has less resemblance to those of the other two cholinesterases. Two regions stand out as different: the region from residue 107 to 140, and the region at the carboxyl terminus. The 33-residue peptide starting at 107 is a nonhomologous extra peptide (see Fig. 3), and when it is omitted from the hydropathy figure the profile becomes more similar to the others. This peptide is below the midpoint line in Fig. 4 and, therefore, is likely to be near the enzyme surface. The carboxyl terminus of Drosophila acetylcholinesterase has a prominent peak above the line which contrasts strongly with the carboxyl-terminal profiles of the other two cholinesterases. The hydropathy profile suggests that the carboxyl terminus of the Drosophila enzyme is buried.

Comparison to Serine Proteases—The trypsin family of...
serine proteases includes trypsin, chymotrypsin, the blood coagulation factors, and other hydrolytic enzymes (24). The serine proteases are irreversibly inhibited by organophosphate esters such as diisopropyl fluorophosphate, due to acylation of the active site serine (25). Another common feature is the charge relay system serine, aspartic acid, histidine (26). The blood coagulation factors share characteristic domains called the catalytic domain, kringle domain, growth factor domain, finger domain, and vitamin K-dependent domain (27).

Subtilisin is a bacterial serine protease with a different three-dimensional structure than the trypsin family of serine proteases (28). Though subtilisin has serine at the active site and has a charge relay system, the aspartic acid of the charge relay system is at a different location in the sequence. Subtilisin has no disulfide bonds.

The cholinesterases are similar to the serine proteases. They have serine at the active site, and this serine is irreversibly labeled by diisopropyl fluorophosphate. A charge relay system may exist (29), since histidine appears to be essential for catalysis (30, 31). However, neither aspartic acid nor histidine of such a possible charge relay system has been unequivocally labeled or identified.

Unlike the serine proteases, the cholinesterases are primarily esterases. There is controversy regarding the possibility that cholinesterases may also be peptidases (32-39). If cholinesterases hydrolyze esters exclusively, then this would clearly distinguish them from the serine proteases, because the latter hydrolyze esters, peptides, and proteins. Cholinesterases can also be distinguished from the serine proteases by the chemical nature of the stable serine derivative obtained after reaction with diisopropyl fluorophosphate (40). The cholinesterases initially form diisopropyl phosphorylserine, but this quickly ages to yield the monoisopropyl phosphorylserine (41). In contrast, the serine proteases form only the diisopropyl phosphorylserine derivative.

The serine protease family and the cholinesterase family also show structural differences. Fig. 5 compares the locations of the active site serine, the charge relay aspartic acid and histidine, and the disulfide bonds. When these proteins are lined up so that the active site serines are on the same line, it becomes apparent that the cholinesterases differ from the serine proteases. The active site serine in the cholinesterases is closer to the amino terminus than the carboxyl terminus. A charge relay histidine in cholinesterase is likely to involve His<sup>423</sup> or His<sup>438</sup>, because these are the only conserved histidines in the three cholinesterases. This location for histidine is very different from the charge relay His<sup>61</sup> of chymotrypsinogen. The three cholinesterases have a conserved aspartic acid at Asp<sup>129</sup> near the charge relay Asp<sup>92</sup> of chymotrypsinogen. This makes Asp<sup>129</sup> a candidate for the charge relay aspartic acid, but there are five other conserved aspartic acids that need also be considered for this function.

FIG. 5. Comparison of serine proteases and cholinesterases. The amino acid sequences of bovine chymotrypsin (24), bovine prothrombin (24), human cholinesterase (7), Torpedo acetylcholinesterase (8), Drosophila acetylcholinesterase (10), and subtilisin BPN' (28) are represented by vertical lines. The active site serine and the charge relay aspartic acid and histidine are shown. For the cholinesterases the locations of the charge relay aspartic acid and histidine are speculative. The histidines shown in the cholinesterases are the only histidines conserved in all three cholinesterases. The aspartic acid shown is conserved in all three cholinesterases, but there are five other conserved aspartic acids. Disulfide bonds are indicated by boxes. The interchain disulfide bond is indicated by -S-S-next subunit. The disulfide bonds in the Drosophila enzyme are suggested by homology rather than by direct experimental evidence. Asparagine-linked carbohydrates are indicated by CHO.
At the domain level there are also significant structural differences. The amino acid sequence of human serum cholinesterase was examined for the presence of kringle, finger, growth factor, and vitamin K-dependent domains. Tissue plasminogen activator, prothrombin, and urokinase were used for comparison. Cholinesterase has no significant sequence homology with any of these domains nor is any significant sequence homology found around the active site serine. Furthermore, the pattern of disulfide bonding in cholinesterase does not resemble the pattern in these serine proteases.

These findings lead to the conclusion that the cholinesterases constitute a separate family of serine esterases that differs from the trypsin family of serine proteases and from subtilisin.

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