Location of Disulfide Bonds within the Sequence of Human Serum Cholinesterase*

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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 Cys56, Cys82, Cys152-Cys283, and Cys460-Cys481. A disulfide bond with Cys82 rather than Cys56 was inferred by homology with Torpedo acetylcholinesterase. Cys571 forms a disulfide bridge with Cys571 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 in human cholinesterase have exactly the same location as in Torpedo californica acetylcholinesterase. There is one potential free sulfhydryl in human cholinesterase at Cys56, 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.

The abbreviation used is: HPLC, high pressure liquid chromatography or high pressure liquid chromatograph.
ultiple with 0.1% trifluoroacetic acid and acetonitrile, or the Synchro- 
apk RP-P column eluted with 0.1 M hexafluoroacetone-ammonia, pH 7.3, and acetonitrile. Hexafluoroacetone (Aldrich) is at present the 
only available neutral pH buffer that is volatile and UV transparent 
(15).

Identification of Disulfide-containing Peptides—Thanhauer 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 
Thanhauer's cleavage buffer, incubated for 10 min, and then re- 
acted 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 
detectable during sequencing and amino acid analysis (15).

Amino Acid Sequencing—Peptides were sequenced by the manual 
method of Thayer (16). Thayer's modifications of the Edman degradation 
allowed sequencing 5 to 50 peptides at the same time using 200 pmol 
of each peptide. Phenylthiodyantoins were identified on a Waters 
HPLC using an Ultrasphere ODS 5-µm column (Altex) at 
50 °C. The column was equilibrated with 100 mM ammonium acetate, 
pH 4.5, containing 25% acetonitrile. Phenylthiohydantoins were identified on 
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 
directed cysteine 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 
carboxyl 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 digesting cholinesterase with pepsin 
at pH 1.3. The Thanhauer 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-con- 
taining 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.

HPLC chromatograms of cysteine peptides, before and after 
reduction of disulfide bonds, are shown in Fig. 2. Peptide Ia 
of Fig. 2 is Leu-Gly-Ile-Pro-Tyr-Ala-Gln-Pro-Pro-Leu-Gly-Arg-Leu-Arg-Phe-Lys-Lys-Pro-Gln-Ser-Leu-Thr-Lys-Trp-

**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 di- 
gested 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 disul-
fide 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 
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directed cysteine or nonalkylated cysteine. Finding the expected 
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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 iodoacetamide. In B2, B3, and B4 alkylation was with vinylpyridine. The peptide peaks are IIa and of the same peptides after reduction and alkylation. The peptide peaks are B2, B3, B4 used a phenyl column equilibrated with solvent A (0.1% trifluoroacetic acid) and eluted with a gradient increasing in solvent B (acetoni trile 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 Synchropak 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.

Other disulfide-containing HPLC peaks, in addition to those in Fig. 2, were sequenced. These were subfragments produced by pepsin cleavage at additional sites. For example,
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-Glu-Thr-Glu-Asp-Glu-Asp-Pro 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 Cys66.

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 sulfonic 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 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 were 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 Cys66.

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 sulfonic 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 Cys66-Cys96 (Cys96 rather
than Cys²⁶ being inferred from homology with Torpedo acetylcholinesterase), and Cys²⁸, Cys³⁵, and Cys⁶⁰, Cys¹³. A fourth disulfide bridge involves Cys⁷¹ which appears to be covalently attached to Cys⁷² of an identical subunit. One potential free sulfhydryl was suggested, though the inaccessibility of Cys⁶⁶ to alkylation left open the possibility that it was not free.

**Comparison with 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 Cys⁷¹-Cys⁴, Cys⁸⁴-Cys³⁵, Cys⁴²-Cys⁸⁴, and Cys⁷²-Cys⁷³. Cys⁷³ is the interchain disulfide bond. The free sulfhydryl is Cys²⁹.

### Table 3. Comparison of Cholinesterase Sequences

<table>
<thead>
<tr>
<th>Species</th>
<th>Sequence</th>
<th>% Sequence identity</th>
<th>% Amino acid identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human ChE</td>
<td>++--------------------------------</td>
<td>53.8%</td>
<td>52%</td>
</tr>
<tr>
<td>Torpedo AChE</td>
<td>+---------------------------------</td>
<td>52%</td>
<td>49%</td>
</tr>
<tr>
<td>Drosophila AChE</td>
<td>+---------------------------------</td>
<td>28%</td>
<td>52%</td>
</tr>
</tbody>
</table>

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-glyco-
**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 Cys**—Identification of Cys 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. Cys is 4 residues away from Leu 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 Lys or Lys 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 Cys 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 mercaptoethanol 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 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

**Fig. 4. Hydropathy Index.** The amino acid sequences of human cholinesterase, Torpedo acetylcholinesterase, and Drosophila acetylcholinesterase were evaluated using Kyte and Doolittle’s hydropathy scale (23). On this scale, arginine has the lowest value of −4.5, while isoleucine has the highest value of +4.5. The PRPLOT program, with a window size of six amino acids and scoring index no. 8, was used. The midpoint line at −0.5 is the average hydropathy value. Regions above the midpoint line have a high probability of being in the interior of the protein, while regions below the midpoint line have a high probability of being on the exterior of the protein. The 40 amino acids estimated to be in the Drosophila signal peptide have been omitted. The signal peptide of Torpedo has also been omitted. The active site serine is indicated by an arrow. In Drosophila, the nonhomologous 33-residue peptide at 107–140 is indicated by a bar.
The serine proteases include 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$^{423}$ or His$^{438}$, because these are the only conserved histidines in the three cholinesterases. This location for histidine is very different from the charge relay His$^{61}$ of chymotrypsinogen. The three cholinesterases have a conserved aspartic acid at Asp$^{102}$ near the charge relay Asp$^{32}$ of chymotrypsinogen. This makes Asp$^{102}$ 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.

REFERENCES