Loss of the Interchain Disulfide Peptide and Dissociation of the Tetramer Following Limited Proteolysis of Native Human Serum Cholinesterase*

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Highly purified human serum cholinesterase had a single electrophoretic band, ChE-5, on gels stained for activity or protein. When cholinesterase in its native conformation was treated with trypsin, three new bands were obtained on nondenaturing gel electrophoresis. Each protein band had cholinesterase activity. The products of limited proteolysis were designated ChE-1', ChE-3', ChE-4', and ChE-5' because their mobilities were the same as those of the naturally occurring cholinesterase isozymes present in human serum. The fastest moving ChE-1' had an $M_r = 60,000$ on sodium dodecyl sulfate (SDS) gels, which is the same molecular weight as monomer from untreated cholinesterase. ChE-3', -4', and -5' were dimer, trimer, and tetramer, and their subunit molecular weight was also 90,000. Small peptides containing the interchain disulfide bond and the hydrophobic bond were cut out by trypsin. Since removal of these peptides caused no detectable change in subunit molecular weight on SDS gels, these small peptides must be located near the terminals of the subunit. For quantitation of the interchain disulfide peptide, ChE-5 was selectively reduced and alkylated with $[1,3-^3H]$diisopropylfluorophosphate. Trypsin selectively released the $[^3H]$labeled peptides, and these were isolated by high performance liquid chromatography. ChE-5' was of particular interest. On SDS gels in the absence of reducing agent, ChE-5' showed a much higher proportion of monomer than did ChE-5. This suggested that purified cholinesterase preparations which are homogeneous on non-SDS gels may, nevertheless, contain cuts due to proteolysis. Cholinesterase purified by our method was nearly free of proteolytic cuts. The results were consistent with our previously reported structure of cholinesterase as a tetramer of identical subunits held together by hydrophobic and interchain disulfide bonds.

Human serum cholinesterase (EC 3.1.1.8 acylcholine acylhydrolase; also known as pseudocholinesterase, nonspecific cholinesterase, and butyrylcholinesterase), isolated from human plasma, is a globular tetrameric molecule with a molecular weight of approximately 340,000 (1). It has four identical subunits arranged as a dimer of dimers (2, 3), that is, pairs of subunits are covalently linked through a single disulfide into dimers, and two such dimers are hydrophobically linked into a tetramer. The molecule remains a tetramer after the interchain disulfide bonds have been reduced and alkylated (2).

The tetramer appears to be a prolate ellipsoid (3) and contains four active sites/molecule (4).

This structure is similar to the globular tetrameric form of acetylcholinesterase (SC 3.1.1.7) (5-7). The cholinesterase in human plasma seems to lack a collagen-like tail. Cholinesterases that do have a tail include the butyrylcholinesterase and acetylcholinesterase from chicken muscle (8) and rat tissues (9), as well as acetylcholinesterase from electric eel (10), Torpedo californica (11), bovine brain (7), and human muscle (12). As many as three globular tetramers may be linked via collagen tails to form an asymmetric cluster. Acetylcholinesterase and butyrylcholinesterase have many other points of similarity, though the two can be distinguished on the basis of substrate specificity (13) and sensitivity to inhibition by organophosphates (14).

Not much detail is known about the structure of any of these cholinesterases. The present study on limited proteolysis of native cholinesterase shows that the interchain disulfide peptide and the hydrophobic bond region are both located near a terminal of the subunit polypeptide chain. Both functions reside in small peptides that can be removed without causing a detectable loss of mass.

**EXPERIMENTAL PROCEDURES**

**Materials**—Outdated human plasma was a gift from Dr. Harold Gallick of the Michigan Department of Public Health, Lansing. Trypsin/1,1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington. Iodo-$[^3H]$acetamide (23.0 mCi/mmol in ethanol) and $[^3H]$diisopropylfluorophosphate (0.9 Ci/mmol, in propylene glycol) were from New England Nuclear.

**Cholinesterase Genotype**—The cholinesterase used in this study had the "usual" genotype, as determined from characteristic dibucaine (15) and fluoride (16) numbers of 80 and 60, respectively.

**Activity Assays**—Activity was routinely measured according to the method of Kalow and Lindsay (17) by recording the rate of hydrolysis of 50 $\mu$M benzoylcholine in 0.067 M sodium/potassium phosphate buffer, pH 7.4, 25 $^\circ$C, as the decrease in absorbance at 240 nm $(\Delta E_{240}) = 6700$ $M^{-1} cm^{-1})$. One unit of enzyme hydrolyzed one $\mu$mol of benzoylcholine/min. For comparison with results of other workers, activity was also measured with butyrylthiocholine (1.0 $\mu$mol butyrylthiocholine in 0.1 M sodium phosphate, pH 8.0, 25 $^\circ$C by the method of Ellman et al. (18).

**Protein Concentration**—This was calculated from absorbance at 280 nm using $E_{280} = 18.0$. There is no generally accepted value for serum cholinesterase. Other laboratories use values of 25 (19), 20 (20), 15.2 (21), 14.5 (1), 13.6 (22), and 11.3 (23), while for eel acetylcholinesterase a value of 18.0 (24) has gained wide use.

**Purification of Cholinesterase**—Our previously described purification method (4) was modified to obtain larger quantities of highly purified enzyme. 30 bags of outdated human plasma, containing about 10 liters, were chromatographed on DE52 (Whatman) at pH 4.0, followed by a second ion exchange chromatography step at pH 7.0, and finally by affinity chromatography on procainamide-Sepharose 4B. The yield of pure cholinesterase was approximately 12 mg. Freshly prepared cholinesterase had a single activity band on polyacrylamide gels stained for activity with either a-naphthyl acetate (25) or butyry-
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| 150 | 458 | with trypsin, three additional bands appeared. All four bands had activity with α-naphthyl acetate and butyrylcholinesterase. The bands were designated ChE-1', ChE-3', ChE-4', and ChE-5' because their mobilities were similar to those of the naturally occurring isozymes in human serum, ChE-1, ChE-3, ChE-4, and ChE-5. No band corresponding to mobility to the natural isozyme ChE-2 was observed. Saeed et al. (31) first reported that trypsin as well as pepsin, chymotrypsin, ficin, and plasmin degraded the major cholinesterase component, ChE-5, into these bands. Our results with pure cholinesterase confirm the results of Saeed et al. for plasma and for partially purified cholinesterase. The protein-stained bands corresponded exactly to the activity-stained bands. There were no protein-stained bands that did not also have activity, although peptides smaller than 20,000 in molecular weight would not have been detected on these gels.

The 14C-cholinesterase, which had been selectively reduced and alkylated at the interchain disulfides, gave the same elctrophoretic bands following trypsin treatment as did unlabeled cholinesterase. It is not surprising, therefore, that addition of 100 μM dithiothreitol to trypsin-digested cholinesterase did not affect the patterns of activity and protein-staining visualized on non-SDS gels (Fig. 2).

**Fig. 1. Trypsin digestion of highly purified, native cholinesterase at pH 8.1, 37 °C.** 0.5-ml aliquots of cholinesterase at a protein concentration of 0.11 mg/ml in 0.1 M Tris-Cl, pH 8.1, buffer were digested with various amounts of trypsin/1-1-tosylamido-2-phenylethyl chloromethyl ketone. Freshly prepared solutions of trypsin/t-1-tosylamido-2-phenylethyl chloromethyl ketone in 1 mM HCl were added in a volume of 25 μl to give final trypsin concentrations of 0.001, 0.01, and 0.1 mg/ml. Solid trypsin/t-1-tosylamido-2-phenylethyl chloromethyl ketone was added to give final trypsin concentrations of 1.0 and 10 mg/ml. The pH of the digestion mixtures receiving solid trypsin had to be readjusted to 8.1 with 1 M unneutralized Tris at zero time. After 1, 2, 3, 5, 7, 11, 22, and 24 h of incubation at 37 °C, 5-μl aliquots were assayed in quadruplicate for activity with benzoylcholine. 100% activity was 22 units/ml. ---, zero trypsin; O--O, 0.001 mg/ml of trypsin; △-△, 0.01 mg/ml; ■-■, 0.10 mg/ml; △-△, 1 mg/ml; O--O, 10 mg/ml.

**Fig. 2. SDS-Gel Electrophoresis of Cholinesterase Digested with Trypsin.** Aliquots of the same samples shown in Fig. 2 were also applied to SDS gels. Fig. 3 shows that on SDS gels, both in the presence and in the absence of reducing agent, the trypsin-digested cholinesterase had only two bands. These bands had M, = 90,000 and 180,000. No fragments smaller in size than 90,000 were observed. Untreated cholinesterase also had bands of M, = 90,000 and 180,000, corresponding to monomer and dimer (2). Thus, the trypsin-generated monomers appeared to have the same apparent molecular weight as monomers from control cholinesterase, despite the observation in Fig. 2 that trypsin had cut cholinesterase. This suggested that trypsin had removed only small peptides from cholinesterase. In the absence of reducing agent, a difference

\[ \text{Activity} = \frac{100}{\text{Time, hours}} \]

\[ \text{Time, hours} = \begin{cases} 5 & \text{Activity} = 50 \\ 10 & \text{Activity} = 25 \\ 20 & \text{Activity} = 10 \end{cases} \]

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Fig. 2. Polyacrylamide gel electrophoresis of cholinesterase digested with various amounts of trypsin. Highly purified cholinesterase at a concentration of 0.25 mg/ml was digested in 0.1 M Tris-Cl, pH 8.1, buffer, at 37 °C, with various amounts of trypsin/7.1-thesylamido-2-phenylethyl chloromethyl ketone for 24 h. At the end of that time, each sample was tested for activity and applied to a 7.5% polyacrylamide slab gel. Direction of migration was from top to bottom. The percentage of activity remaining was 1, 38, 39, 62, 92, and 100% in digests containing 10, 1, 0.1, 0.01, and 0.001 mg/ml and 0 trypsin. The unmarked lane adjacent to the 100% activity sample received trypsin alone, 10 mg/ml, which had been incubated for 24 h at 37 °C at pH 8.1. The unmarked lane at the right edge of the gel received human serum alone. ChE-1, ChE-2, ChE-3, ChE-4, and ChE-5 designate the cholinesterase isozymes present in serum. The serum sample has an additional band of activity near the bottom of the gel due to albumin. The gel was stained for activity with o-naphthyl acetate. Staining for activity with butyrylthiocholine or for protein with Coomassie blue showed the same bands. Addition of 100 mM dithiothreitol to each trypsin-treated cholinesterase sample before electrophoresis did not alter the banding pattern in either the activity or protein-stained gels.

between trypsin-digested and control cholinesterase was seen on SDS gels (Fig. 3, top gel). Trypsin-digested cholinesterase had a much higher proportion of monomeric (90,000) protein. Thus the effect of trypsin was similar to the effect of reducing agent, in that both reagents converted dimer to monomer as seen on SDS gels. These results suggested that the small peptides which were removed by trypsin contained the interchain disulfide bond.

To determine the size composition of the individual bands, ChE-1', -3', -4', and -5' were isolated and subjected individually to SDS-gel electrophoresis. Fig. 4 shows the results of SDS-gel electrophoresis in the absence of reducing agent. All four species contained \(M_\text{r} = 90,000\) monomer. In addition they contained variable proportions of \(M_\text{r} = 180,000\) dimer. ChE-1' contained only the monomer. When the same experiment was performed in the presence of reducing agent, no difference could be observed between them as all the protein migrated in the monomer band.

A comparison of Fig. 4 (lane ChE-5') and Fig. 3 (top gel, lane 0) shows that the isolated ChE-5' was not identical with control cholinesterase, that is to ChE-5. The proportion of monomeric protein was much higher in ChE-5' than in ChE-5. This indicates that cholinesterase, which by the criterion of mobility on non-SDS gel, appears to be intact tetrameric protein of \(M_\text{r} = 340,000\), can, nevertheless, contain nicks and cleavages in its structure. These structural alterations are revealed on SDS-gel electrophoresis by the presence of monomeric protein on gels run without reducing agent. Thus, ChE-5' appears to have lost a portion of its interchain disulfide linkages.

Size Exclusion Chromatography—Molecular weights estimated from filtration on Sephacryl were 340,000 for ChE-5', 245-281,000 for ChE-4', 177-198,000 for ChE-3', and 92-100,000 for ChE-1'. These results, together with the results of

Fig. 3. SDS-gel electrophoresis of cholinesterase digested with various amounts of trypsin. The same cholinesterase samples described in the legend to Fig. 2 were used. After 24 h of digestion with trypsin, each sample was incubated with 16 mM DFP for 1 h before the addition of SDS. The purpose of the DFP was to inactivate trypsin. This precaution was necessary in order to prevent additional cleavages of SDS-denatured cholinesterase by trypsin. Two slab gels containing 10% acrylamide and 0.1% SDS (27) are shown. In the top gel no reducing agent was added. In the bottom gel, each sample was incubated with 50 mM dithiothreitol before electrophoresis. The unmarked lane in each gel received 25 \(\mu\)l of 10 mg/ml of trypsin which had been incubated in pH 8.1 buffer at 37 °C for 24 h. The other lanes received 6.3 \(\mu\)g of cholinesterase treated with 10, 1, 0.1, 0.01, and 0.001 mg/ml and 0 trypsin. The top lane of each gel is the trypsin concentration. The apparent molecular weights of the protein bands were 90,000 and 180,000. The gel was stained for protein with Coomassie brilliant blue R.
Digestion with Trypsin—The peptide following digestion with trypsin was quantitated using cholinesterase which had been selectively reduced at the interchain disulfide bond and alkylated with iodo\[%\]acetamide. This %-cholinesterase preparation, at a concentration of 0.25 mg/ml in 0.1 M Tris-Cl, pH 8.1, was digested with 0.0125 mg/ml of trypsin at 37 °C in a total volume of 3.60 ml. for 5 different analyses: 1) aliquots (3 x 80 ~1) were placed for HPLC analysis of peptides; 4) aliquots (4 x 5 ~1) were used for protein-stained polyacrylamide gels; 2) 80 ~1 (containing 20 pg of protein) were placed into tubes containing 11~1 of 0.222 mM \[^{3}H\]DFP, and used for protein-stained polyacrylamide gels; 3) 200 ~1 were used for HPLC analysis of peptides; 4) aliquots (4 x 5 ~1) were assayed for activity with benzoylcholine; 5) aliquots (2 ~1) were mixed with 20 ~1 of 1 mg/ml of soybean trypsin inhibitor and used for activity-stained gels. Results are given in Figs. 5–8.

The stoichiometry of labeling was 222 pmol of \[^{3}H\]DFP/20 ~1 of cholinesterase, which is equivalent to 1 mol of \[^{3}H\]DFP/90,000 g. This stoichiometry is in agreement with our previous report of 4 active sites/molecule in the tetramer (4).

Progress of the digestion was quantitated in Fig. 5 by measuring the amount of \[^{3}H\]DFP label in the isolated electrophoretic bands. The major component during all stages of digestion was ChE-5'. We were unable to produce a majority of the small active fragments, indicating that ChE-1', -3', and -4' were equally or more susceptible to digestion than was ChE-5'. Bands were formed and degraded at different rates so their relative concentrations varied during the course of digestion.

The total amount of material recovered on gels decreased as the digestion progressed. This decrease correlated with the activity loss measured in each sample prior to electrophoresis. For example, at zero time 20 ~1 of sample bound a total of 222 pmol of \[^{3}H\]DFP label and had an activity of 44.2 units/ml. After 22 h of digestion, the sample bound a total of 125 pmol of \[^{3}H\]DFP label and had an activity of 27.3 units/ml. The lost material was not detected on SDS gels (see Fig. 7), and was, therefore, smaller than 20,000 in molecular weight. This stoichiometry of labeling was 222 pmol of \[^{3}H\]DFP/20 ~1 of cholinesterase, which is equivalent to 1 mol of \[^{3}H\]DFP/90,000 g. This stoichiometry is in agreement with our previous report of 4 active sites/molecule in the tetramer (4).

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The earlier time points in the digestion yielded similar amounts of interchain disulfide peptide (represented by $^{14}$C) and active sites (represented by $^{3}$H). Each electrophoretic band, including ChE-5', contained less $^{14}$C label per active site than the untreated control cholinesterase. This result confirmed the conclusion that the effect of trypsin on native cholinesterase was to cut out the interchain disulfide bond.

For most of the digestion period the ratio of picomoles of $^{14}$C/$^{3}$H was highest in ChE-5' and progressively less in ChE-4', ChE-3', and ChE-1'. However, Fig. 6 also shows that this ratio was not a fixed number for any individual band, but varied during the course of digestion. This indicated that each band represented a mixture of nonidentical polypeptides.

The observation that ChE-5' contained a lower ratio of picomoles of $^{14}$C/$^{3}$H than control cholinesterase, ChE-5, showed again that ChE-5' had been partially degraded by trypsin. Since ChE-5' and ChE-5 had the same mobilities on polyacrylamide gel (Fig. 2) and on size exclusion chromatography, it follows that mobility on these systems cannot be the criterion for deciding whether cholinesterase is completely intact. As already pointed out, partial proteolysis can be detected by the presence of monomeric protein on SDS gels run in the absence of reducing agent.

In Fig. 7 the double-labeled cholinesterase digest was subjected to SDS-gel electrophoresis in the absence of reducing agent. At zero time, we had expected cholinesterase to be completely in the monomeric form because its interchain disulfides had been reduced and alkylated. However, Fig. 7 shows that 25% of the $[^{3}$H]DFP label was in the dimer. During the first 3 h of digestion, $^{14}$C-peptide was rapidly lost from the monomer, while the total amount of monomer, as represented by $^{3}$H, remained virtually constant. For the interval 0 to 1 h, the unchanged level of monomer $^{3}$H may be attributed to replenishment from dimer $^{3}$H. For the interval 1 to 3 h, the ratio of picomoles of $^{14}$C/$^{3}$H in the monomer decreased from 0.76 to 0.64, while after 22 h of digestion, this ratio was 0.56. Thus, $^{14}$C-peptide was lost from the monomer even though the monomer did not change in apparent molecular weight. This again indicated that the peptide or peptides cut out were small in size, probably significantly less than $M_t$ = 5000, and were located at the terminal, either the COOH- or NH$_2$- terminal end of the cholinesterase subunit.

The total amount of material recovered on SDS gel (Fig. 7) decreased during the course of digestion. A similar observation had been apparent from the non-SDS gel in Fig. 5. Since there were no protein-stained bands on the SDS gel smaller in molecular weight than 90,000, it follows that the lost material was digested to small peptides that could not be fixed on the gel. The disappearance of total $^{14}$C was faster than the disappearance of total $^{3}$H, suggesting that trypsin selectively cut out $^{14}$C-peptide more rapidly than it digested the remainder of the molecule.

The same digests that were subjected to gel electrophoresis in Figs. 5 to 7 were also injected into a reverse phase column on HPLC. Only peptides could be eluted from the HPLC column. The $M_t$ = 340,000 tetrameric cholinesterase and its $M_t$ = 90,000 subunit were permanently retained on the column. In Fig. 8, two $^{14}$C-containing peaks eluted early in the chromatogram, ahead of most of the nonlabeled peptides, and, therefore, the radioactive peaks were probably small peptides. These $^{14}$C-peptides originated from the interchain disulfide peptide. The $^{14}$C counts recovered on HPLC (59% after 22 h of digestion) correlated with the $^{14}$C counts lost on gels from the same sample (63% on non-SDS gel, and 66% on SDS gel). This same sample had lost 38% activity; it is likely that the nonlabeled peptides in Fig. 8 originated from the 38% of cholinesterase protein that had been digested to completion.

Material was recovered as peptides on HPLC as illustrated in Fig. 8.

Loss of the interchain disulfide peptide from ChE-1', -3', -4', and -5' was quantitated in Fig. 6 by measuring the relative picomoles of $^{14}$C/pmol of $^{3}$H. The ratio of picomoles of $^{14}$C/pmol of $^{3}$H in the whole sample was 0.87 at zero time and 0.51 after 22 h of digestion.

FIG. 7. Rate of disappearance of $^{14}$C (interchain disulfide peptide) from cholinesterase isolated by SDS-gel electrophoresis. Aliquots of the same double-labeled cholinesterase digests which were used for polyacrylamide gel electrophoresis in Figs. 5 and 6 were also applied to an SDS gel. No reducing agent was added. Bands with molecular weights corresponding to monomer and dimer were the only Coomassie-staining bands present. Their apparent molecular weights did not alter during the course of trypsin digestion, though their intensities decreased. The graph shows picomoles of radioactive isotope present in the monomer and dimer after various times of digestion by trypsin. $^{14}$C represents Cys of the interchain disulfide bond alkylated with iodo[2$^{14}$C]acetamide and not with $[^{3}$H]DFP. 200 µl of the digestion mixture containing 50 pg of cholinesterase protein were digested with trypsin as in Fig. 5. The HPLC chromatogram shows the 22-h time of digestion by trypsin. $^{14}$C represents Cys of the interchain disulfide bond alkylated with iodo[2$^{14}$C]acetamide and not with $[^{3}$H]DFP. At zero time, the sample contained 156 pmol of monomer $^{3}$H, 92 pmol of dimer $^{3}$H, 170 pmol of $^{14}$C in the monomer, and 7 pmol of $^{14}$C in the dimer. The ratio of picomoles of $^{14}$C/pmol of $^{3}$H in the whole sample was 0.87 at zero time and 0.51 after 22 h of digestion.

FIG. 8. Isolation of interchain disulfide peptides by HPLC. Cholinesterase whose interchain disulfide bonds had been selectively reduced and alkylated with iodo[iodo[2$^{14}$C]acetamide was digested with trypsin as in Fig. 5. The HPLC chromatogram shows the 22-h time point from the same digestion. In contrast to the protocol in experiments for Figs. 5 to 7, the sample injected into the HPLC was labeled only with iodo[iodo[2$^{14}$C]acetamide and not with $[^{3}$H]DFP. 200 µl of the digestion mixture containing 50 µg of cholinesterase protein were injected into a reverse phase column (Varian Associates, MCH-10, which is C$_3$-bonded to 10-µm silica) on a Varian model 5060 HPLC. Peptides were eluted with a gradient at a flow rate of 1.0 ml/min at ambient temperature. The gradient was formed between solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile containing 0.1% trifluoroacetic acid) increasing in solvent B at 1%/min. Absorbance was monitored at 220 nm (solid line). 1-ml fractions were collected and their radioactivity counted. The two radioactive peaks eluting at 15 and 26 min contained a total of 59% of the $^{14}$C counts present in 50 µg of cholinesterase. Cholinesterase that had not been treated with trypsin was completely retained on this column with zero counts recovered. The large peaks at 2-4 min are solvent injection peaks.
results. That is, the $^{14}C$ counts recovered on HPLC correlated with $^{14}C$ counts lost from gels. $^{14}C$-peptide was recovered on HPLC in quantities far greater than activity loss. This confirmed the conclusion that $^{14}C$-peptide (representing interchain disulfide peptide) was selectively cleaved from cholinesterase leaving a molecule which, though depleted of $^{14}C$-peptide, had activity.

Only one $^{14}C$-peptide had been expected. To understand the significance of isolating two $^{14}C$ peaks on HPLC, we will need to analyze each peak for amino acid composition and sequence. One possible explanation is that the amino acid sequence of one peptide may be wholly contained within the second peptide.

**Effect of Trypsin on Hydrophobic Bonding between Subunits**—In our model of cholinesterase, the 4 subunits are linked by hydrophobic bonds as well as by disulfide bonds (2). Trypsin had two effects. It selectively cut out interchain disulfide bonds, and it also dissociated the subunits (Fig. 2), producing cholinesterase trimers, dimers, and monomers. It follows that trypsin cleaved off a peptide containing the function of linking subunits via hydrophobic bonds. This peptide had a low molecular weight and was located near the end of the polypeptide chain, as shown by the observation that ChE-1', though depleted of hydrophobic attachment to other subunits, had no significant loss of mass. We did not determine whether the interchain disulfide function and the hydrophobic bond function were located in a single tryptic peptide or in several peptides.

**DISCUSSION**

In these experiments we have focused on the products resulting from limited proteolysis of native serum cholinesterase. When native cholinesterase, ChE-5, was treated with trypsin, four products were observed on non-SDS gels. These were called ChE-1', ChE-3', ChE-4', and ChE-5' because they had the same mobilities on gels as the naturally occurring isozymes of cholinesterase ChE-1, ChE-3, ChE-4, and ChE-5. ChE-5 is the major enzymic component. The naturally occurring serum isozyme ChE-2 was not produced by trypsin. Like the native isozymes, the trypsin-generated products had activity. Saeed *et al.* (31), using partially purified human cholinesterase, had previously reported that the major component of cholinesterase was converted to isozyme-like products by proteases. Whittaker and Charlier (32) found that papain, carboxypeptidase A, leucine aminopeptidase, and chymotrypsin modified the cholinesterase isozymes in human plasma.

Because we used highly purified cholinesterase we were able to stain gels for protein. We found that proteolysis resulted in a maximum of four protein-staining bands, and these bands all had activity. It was surprising to find that no protein-staining bands were produced that were not associated with activity.

The quaternary structure of tetrameric serum cholinesterase is stabilized by covalent (disulfide linkage) and noncovalent interactions. The disulfide linkage does not appear to be essential for the maintenance of the tetramer (2). Noncovalent interactions in the form of hydrophobic bonds are essential for maintenance of the tetramer. Digestion of the tetramer with trypsin resulted in the formation of disulfide-depleted tetramer, trimer, dimer, and monomer.

The molecular weight of the smallest product, ChE-1', was 90,000. ChE-1' was depleted of both an interchain disulfide linkage and a hydrophobic bond region. Despite the fact that trypsin treatment had resulted in the loss of these subunit association functions, the molecular weight of ChE-1' was not detectably lower than the subunit molecular weight of the native cholinesterase. This led to the conclusion that the interchain disulfide and hydrophobic bond functions were both contained in peptides of molecular weight, significantly less than 5,000. Furthermore, it followed that these peptides were located near the terminal of the polypeptide chain; had they been cut out of the monomer's interior, the molecular weight of ChE-1', as determined by SDS-gel electrophoresis, would have been much less than 90,000. Small peptides carrying the interchain disulfide linkage were identified and isolated by HPLC. Whether the interchain disulfide and hydrophobic bond functions resided in a single tryptic peptide or in separate peptides was not determined.

Like ChE-1', the other products, ChE-3', -4', and -5', also had subunit molecular weights of 90,000. ChE-1' was a monomer, ChE-3' a dimer, ChE-4' a trimer, and ChE-5' a tetramer. Each of these trypsin-generated products was depleted in interchain disulfide linkage. Thus, trypsin cut out the interchain disulfide peptide and the hydrophobic bond peptide, resulting in the dissociation of the tetrameric molecule to give a mixture of monomer, dimer, trimer, and tetramer.

The tetrameric molecule appeared to be equally or more stable to further trypsin digestion than the smaller products, as concluded from our inability to accumulate ChE-1', -3', and -4' at the expense of ChE-5'. The majority of protein was always in the ChE-5' band. This conclusion agrees with Chiou *et al.* (33), who digested horse serum cholinesterase with trypsin and reported that it was resistant to dissociation into smaller active forms by treatment with chemical agents (36-38). While our results support the possibility that the isozymes may have arisen by proteolysis, they do not prove this. There are a number of similarities between the natural isoforms and the trypsin-generated isozymes. These similarities include mobility on gel electrophoresis, activity in each of the bands, and molecular weights (35, 36, 39-41). Harris *et al.* (25), Masson (3), and Gaffney (42) concluded that the isozymes ChE-5, -3, and -1 varied in size but not in charge, while ChE-2 was a dimer with increased charge density. Further structural work needs to be done before a conclusion can be reached regarding the origin of the isozymes in serum.

**Limited Proteolysis Effects on Cholinesterase from Other Sources**—Vigny *et al.* (7) isolated the globular tetrameric form of partially purified bovine brain acetylcholinesterase by sucrose density centrifugation. Trypsin treatment yielded an active monomer that had no detectable difference in sedimentation coefficient from naturally occurring monomer. The monomer could not be dissociated into smaller active entities by either reduction or proteolysis. Allemand *et al.* (8), working with chicken muscle butyrylcholinesterase, similarly found that trypsin converted the tetrameric globular form into an active monomer form. These results agree with our results for human serum cholinesterase.

Highly purified acetylcholinesterase from electric eel contains a DFP-labeled fragment of $M = 50,000$ to 59,000 (5, 6, 43) as a result of proteolysis by either trypsin or autolysis. We did not observe a fragment of similar size in human serum cholinesterase.

In conclusion, we have shown that the function of linking subunits via disulfide bonds, and the function of linking sub-
units via noncovalent bonds, are contained in small peptides located near the end of the cholinesterase polypeptide chain.

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