Tsai, A., Hudson, B., & Simoni, R. D. (1980) *Methods Enzymol.* (in press).
Warshel, A., & Karplus, M. (1972) *J. Am. Chem. Soc. 94*, 5612.
Watts, A., Harlos, K., Maschke, W., & Marsh, D. (1978) *Biochim. Biophys. Acta 510*, 63.
Wolber, P. K. (1980) Ph.D. Thesis, Stanford University, Stanford, CA.
Yellin, M., & Levin, I. W. (1977a) *Biochim. Biophys. Acta 468*, 490.
Yellin, N., & Levin, I. W. (1977b) *Biochim. Biophys. Acta 489*, 177.

Characterization of Pepsin-Resistant Collagen-like Tail Subunit Fragments of 18S and 14S Acetylcholinesterase from *Electrophorus electricus*†

Carol Mays‡ and Terrone L. Rosenberry*

ABSTRACT: Digestion of 18S and 14S acetylcholinesterase from eel electric organ with pepsin at 15 °C for 6 h results in extensive degradation of the catalytic subunits, but a major portion of the collagen-like tail structure associated with these enzyme forms resists degradation. The pepsin-resistant structures partially aggregate and can be isolated by gel exclusion chromatography on Sepharose CL-6B in buffered 1 M sodium chloride. The largest structure, denoted F_3, has a molecular weight of 72000 according to gel electrophoresis in sodium dodecyl sulfate and is composed of three 24000 molecular weight polypeptides linked by intersubunit disulfide bonds. This structure is largely, but not completely, a collagen-like triple helix as indicated by a circular dichroism spectrum typical of triple-helical collagen and an amino acid composition characterized by 27% glycine, 5% hydroxyproline, and 5% hydroxylysine. Continued pepsin action results in degradation of the disulfide linkage region such that disulfide-linked dimers F_2 and finally F_1 monomers become the predominant forms in sodium dodecyl sulfate. Digested samples in which either F_3 or F_2 predominate have virtually identical circular dichroic spectra and amino acid compositions and generate similar diffuse 24000 molecular weight polypeptides following disulfide reduction. Thus the intersubunit disulfide linkages in F_3 must occur close to the end(s) of the fragment polypeptide chains. Pepsin conversion of F_3 to F_2 is particularly accelerated between 25 and 30 °C, suggesting that the triple-helical structure in the disulfide linkage region undergoes thermal destabilization in this temperature range. Digestion at 40 °C yields presumably triple-helical F_1 structures devoid of disulfide linkages, although their degradation to small fragments can be detected at this temperature. The question of whether the three tail subunits that give rise to F_1 polypeptides are identical remains open.

Acetylcholinesterase (EC 3.1.1.7) from the electric organs of the eel *Electrophorus electricus* is extracted at high ionic strength as a mixture of asymmetric forms (Massoulié & Rieger, 1969). The predominant form, an 18S species, can be categorized[1] as an A_{12} molecule and is composed of twelve catalytic subunits (Rieger et al., 1976) linked asymmetrically by disulfide bonds to three collagen-like tail subunit polypeptides (Rosenberry & Richardson, 1977; Anglister & Silman, 1978; Rosenberry et al., 1980). A similar tail structure has been identified in the 14S (A_8) and 8S (A_4) forms that are also present in these extracts (McCann & Rosenberry, 1977). The A_{12} nature of the principal asymmetric form from several rat, chicken, and human muscles and from bovine superior cervical ganglion also has been confirmed (Bon et al., 1979), but, in contrast to eel electric organ, these tissues also contain considerable amounts of globular G_4, G_2, and G_1 forms (Massoulié, 1980). The A_{12} form in rat diaphragm is localized primarily in the end-plate region and virtually disappears on denervation of the muscle (Hall, 1973), while the globular forms are distributed nearly uniformly in this muscle and show a much smaller decrease on denervation. A basement membrane localization of a significant fraction of end-plate acetylcholinesterase by means of its collagen-like tail was suggested by observations that collagenase degrades end-plate basement membrane and releases this enzyme from the end plate (Hall & Kelly, 1971; Betz & Sakmann, 1973), and this suggestion was supported by direct observation of acetylcholinesterase activity in the end-plate basement membrane matrix that survived selective destruction of nerve and muscle cell elements (McMahan et al., 1978). Although end-plate A_{12} forms have not been isolated and subjected to subunit analysis, the similarities of end-plate and electric organ A_{12} forms in their native molecular weights (Bon et al., 1979) and their solubility, aggregation, and collagenase digestion patterns (Bon et al., 1978, 1979; Johnson et al., 1977; Watkins et al., 1977; Webb, 1978; Anglister & Silman, 1978; Rotundo & Fambrough, 1979) indicate that their collagen-like tail structures must be quite comparable.

† From the Departments of Biochemistry and Neurology, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received August 21, 1980; revised manuscript received January 6, 1981.* This investigation was supported, in part, by National Institutes of Health Grants NS-03304 and NS-11766, by National Science Foundation Grant PCM77-09383, by the Muscular Dystrophy Association, and by the New York Heart Association.

* Correspondence should be addressed to this author at the Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106.

‡ Present address: The Division of Arteriosclerosis and Metabolism, Mount Sinai School of Medicine, New York, NY 10029.

[1] This nomenclature (Bon et al., 1979) distinguishes two classes of acetylcholinesterase: "asymmetric" forms A_n that are assemblies of n catalytic subunits associated with a collagen-like tail structure and "globular" forms G_n in which the assemblies of n catalytic subunits are devoid of a collagen-like tail.

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The digestion of tissue or tissue extracts with pepsin is frequently employed to solubilize collagen-like proteins or to selectively degrade noncollagen-like regions in these proteins. Pepsin digestion of acid-soluble calf skin collagen at low pH releases small peptides to the dialyzate with amino acid compositions not typical of triple-helical collagen while retaining most of the protein in native triple-helical form (Rubin et al., 1963). Collagen-like regions that resist degradation by pepsin can be fractionated into various collagen types by differential salt precipitation (Trelstad et al., 1972; Sage & Bornstein, 1979). In this paper we describe an isolation procedure for the collagen-like domain of the A_{12} tail structure that resists pepsin degradation and demonstrate the triple-helical nature of this fragment domain. Our results appear consistent with those recently reported in abstract form by Anglister et al. (1979).

Materials and Methods

Proteins. 18S and 14S acetylcholinesterase was purified from buffered 1.0 M sodium chloride extracts of fresh electric organ tissue by affinity chromatography as described in Rosenberry & Richardson (1977). In this procedure, the purified enzyme is obtained in buffered 1.0 M sodium chloride containing 5 mM decamethonium bromide and consists of about 75% 18S and 25% 14S forms. The purified enzyme was concentrated by dialysis against 10 mM sodium phosphate, pH 7, (4–16 h, 4 °C) followed by centrifugation at 75000*g* for 7–16 h in an SW 25.1 rotor. Under these low ionic strength conditions, 18S and 14S acetylcholinesterase aggregates (Massoulié & Rieger, 1969; Dudai et al., 1972) and is recovered by suspension of the centrifugation pellet. Pellet recoveries corresponded to 80% of the enzyme activity (average of 10 runs) at concentrations up to 8 mg/mL with specific activities comparable to those in the peak fractions from affinity chromatography (Rosenberry & Richardson, 1977).

11S eel acetylcholinesterase was purified from a crude lyophilysate (Rosenberry et al., 1972) by the same procedure used for the 18S and 14S enzyme stock except that 1.0 M sodium chloride was deleted from all column solvents. Acid-soluble calf skin collagen and soybean trypsin inhibitor (type I-S) were obtained from Sigma Chemical Co.

Pepsin Digestion. To suspensions of acetylcholinesterase (2–6 mg/mL in 10 mM sodium phosphate, pH 7) were added glacial acetic acid and stock porcine pepsin (Worthington Biochemical Corp., 2× recrystallized, 1–10 mg/mL in 50 mM acetic acid). Final concentrations were 0.5 M acetic acid and 0.02–0.1 mg/mL pepsin. The active pepsin concentrations were estimates because a single pepsin stock that was used throughout (stored as the commercial lyophilysate at −20 °C) lost ~80% of its activity over a 1-year period. The digestion mixture was maintained at 15 °C for 6 h and then terminated by the addition of stock Tris-HCl[2] (2.9 M, pH 8.5) to a final concentration of 1.0 M, pH 8.0.

Gel Exclusion Chromatography. The pH 8 digestion mixture (2–8 mL) was applied to a 145-mL Sepharose CL-6B column (1.5 × 82 cm) equilibrated in buffered 1.0 M sodium chloride (50 mM Tris-HCl or 20 mM sodium chloride, pH 7) at 4 °C. Inclusion of 1 M sodium chloride in the elution solvent was necessary in order to prevent the loss by adsorption of considerable amounts of the tail subunit fragments. A flow rate of 10 mL/h was maintained by a hydrostatic pressure differential. Equal fractions of 2–3 mL were collected, and the absorbances of each fraction at 280, 250, 225, and 215 nm were measured. The absorbance recovery at 280 nm (A_{280} recovery) for a given pool of column fractions is defined as the sum of the products of A_{280} times the volume for the fractions in that pool. Stokes radii R_e were estimated from a calibration curve of $(-\log K_D)^{0.5}$ vs. R_e with assumed R_e values of 15.6 nm for 18S acetylcholinesterase, 8.75 nm for 11S acetylcholinesterase, and 2.4 nm [from Birk et al. (1963)] for soybean trypsin inhibitor (Bon et al., 1976).

Protein Determinations. Concentrations of 18S and 14S acetylcholinesterase were estimated by absorbance measurements at 280 nm in buffered 1 M sodium chloride, assuming $\epsilon_{280}^{1\%} = 18.0$ (Rosenberry & Richardson, 1977). The protein content of pepsin-resistant fragments was determined by direct amino acid analysis as indicated below. From these determinations, values of $\epsilon_{280}^{1\%} = 50$ and of $(\epsilon_{215} - \epsilon_{225})^{1\%} = 150$ for fragments in column pool I were estimated. In some experiments, fragments containing the enzyme active-site serine were monitored by prelabeling the enzyme stock (40 μN) with 25 μM [^{3}H]iPr_2PF[2] (0.9 Ci/mmol, New England Nuclear) in 10 mM sodium phosphate buffer for 1 h prior to initiation of the pepsin digestion and subsequent application to the Sepharose CL-6B column. Dialysis of parallel labeled samples without pepsin digestion showed that about one-half of the total label is incorporated into the protein under these conditions, presumably at the active-site serine [see Barnett and Rosenberry (1979) for the specificity of this labeling procedure].

Amino Acid Compositions. Analyses were conducted on a Beckman 119CL amino acid analyzer with a Model 126 Data System. Analyzer buffers A, B, and C corresponded respectively to 0.2 M sodium citrate, pH 3.10; 0.2 M sodium citrate and 0.2 M sodium chloride, pH 3.95; and 0.2 M sodium citrate, 0.8 M sodium chloride, pH 5.10. Protein hydrolysis of dried samples dissolved in 6 N hydrochloric acid (Baker, Ultrapure) containing 80 mM mercaptoethanol was conducted in tubes sealed under argon for 16–20 h at either 110 or 115 °C. Hydrolyzed samples were dried, dissolved in buffer A, pH 2.2, and applied to the analyzer in 0.1–1.0-mL aliquots. Analyses involving quantitative protein determinations used nondialyzed dried samples (see footnote 4 below) containing up to 1.4 mmol of sodium chloride and 0.3 mmol of sodium phosphate in 2.0 mL of acid solution, and application aliquots contained no more than one-half of each sample in 1.0 mL. Amino acid standards for these determinations contained a similar quantity of salt and were carried through an identical hydrolysis procedure.

Estimates of the hydroxylysine content in polyacrylamide gel bands were made from dried samples containing 10–12 1-mm gel slices (Rosenberry et al., 1974) to which norleucine was added as an internal standard. Hydrolysis was conducted as described above in 1.0 mL of acid solution, and the subsequent sample workup followed Houston (1971). Norleucine recoveries from each sample were 50–75%, and hydroxylysine contents were normalized to correspond to complete recovery of norleucine. Hydroxylysine quantities down to the level of the gel blanks (0.04 nmol) could be determined.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Samples were prepared, electrophoresed on 5.8% gels at 3 mA/tube, and stained with Coomassie Brilliant Blue R with only slight modifications (Rosenberry & Richardson, 1977) of the procedure of Fairbanks et al. (1971). Calibration standards for polypeptide molecular weights were cyanogen bromide fragments of α1(I) and α2(I) acid-soluble calf skin collagen prepared according to Miller et al. (1969). The

[2] Abbreviations used: iPr_2PF, diisopropyl fluorophosphate; Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; CD, circular dichroism.

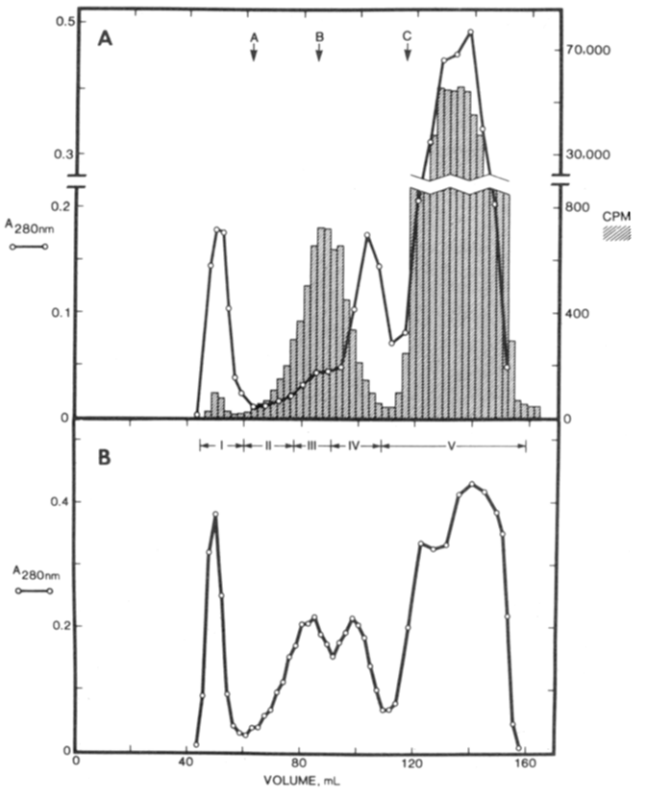

FIGURE 1: Gel exclusion chromatography following pepsin digestion of low ionic strength aggregates of 18S and 14S acetylcholinesterase. (A) Acetylcholinesterase (10 mg), prelabeled with [^{3}H]iPr_2PF to indicate peptides containing the enzyme catalytic site, was digested with 0.1 mg/mL pepsin. The neutralized sample (8.0 mL) was chromatographed on Sepharose CL-6B as described under Materials and Methods. The absorbance (A_{280nm}) and cpm (10-μL aliquot) of each column fraction (2.3 mL) are shown. Fractions were combined in the five pools indicated by Roman numerals for further characterization. (B) Digestion conditions are similar to those in (A) with the exception that the pepsin concentration was ~0.03 mg/mL. Column conditions and pools are the same as those in (A). The column onput contained a neutralized digest of 10 mg of 18S and 14S acetylcholinesterase in 6.4 mL. Elution volumes of the following standard proteins were determined from parallel chromatographic runs in the same buffer: (A) 18S eel acetylcholinesterase (M_r 1 100 000); (B) 11S eel acetylcholinesterase (M_r 320 000); (C) soybean trypsin inhibitor (M_r 22 000). These elution volumes are denoted by arrows and correspond to respective K_D values of 0.139, 0.406, and 0.777.

following molecular weights for these fragments were calculated from the data of Fietzek & Kuhn (1976) by assuming mean amino acid residue weights of 91.0: α1 and α2, 94 000; α2CB3,5, 60 000; α2CB4, 29 000; α1CB8, 25 000; α1CB6, 18 000.

Circular Dichroism Spectra. Measurements were made on a Cary Model 60 spectropolarimeter with a standard CD attachment in 1 mm pathlength cells at 25 °C. The mean residue ellipticity, $[\theta]_{MR}$, was expressed in units of deg·cm^2/$dmol_{MR}$ with mean residue weights of 112 g/mol for 11S acetylcholinesterase, of 91 g/mol for collagen, and of 100 g/mol for pepsin-resistant fragments.

Results

Fractionation of Pepsin-Resistant Fragments of 18S and 14S Acetylcholinesterase by Gel Exclusion Chromatography on Sepharose CL-6B. Pepsin digestion generated several comparatively resistant polypeptide fragments, and the relative amounts of these fragments depended on the extent of pepsin degradation. Gel exclusion chromatography profiles of two samples that were digested with different concentrations of pepsin are compared in Figure 1. Fractions from both profiles

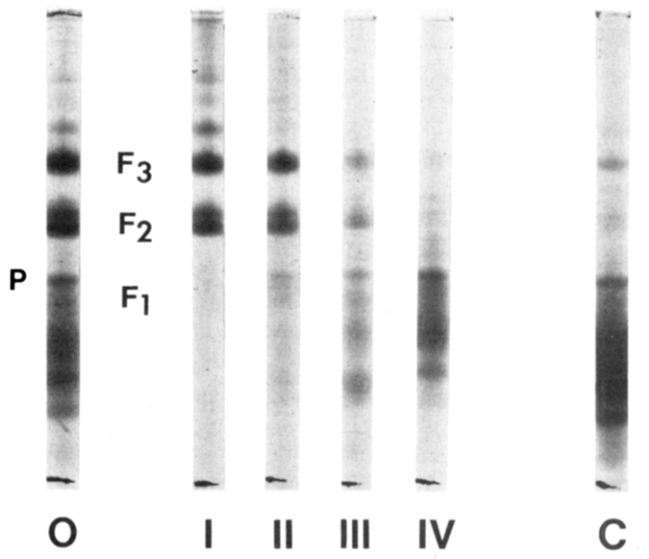

FIGURE 2: Polyacrylamide gel electrophoresis of pepsin digests and of selected fractions shown in Figure 1A in 1% sodium dodecyl sulfate. Samples were dialyzed overnight against water, lyophilized, dissolved in 25 μL of 1% sodium dodecyl sulfate sample buffer in the absence of disulfide reducing agents, electrophoresed, and stained with Coomassie Brilliant Blue R as outlined under Materials and Methods. (O) 0.16 mL of the column onput described in Figure 1A. (I–IV) Representative samples of pools I–IV: (I) pool I, 0.7 mL of fraction at 52 mL; (II) pool II, 0.9 mL of fraction at 77 mL; (III) pool III, 0.8 mL of fraction at 91 mL; (IV) pool IV, 0.8 mL of fraction at 100 mL. (C) 0.2-mL pepsin digest of 0.2 mg of [^{3}H]iPr_2PF-labeled 11S acetylcholinesterase prepared as described in Figure 1A.

can be grouped somewhat arbitrarily into the five pools indicated in the figure. Pool I eluted between 44 and 60 mL and corresponded to the column void volume. Protein in this pool was aggregated, as suggested by the low absorbance ratio A_{280}/A_{250} of ~0.8 for the fractions in this pool. In contrast, fractions that eluted after pool I were characterized by A_{280}/A_{250} values of >2 that are typical of undigested acetylcholinesterase. The lower pepsin activity in the digestion associated with Figure 1B resulted in A_{280} recoveries that were about twice, in pool I, and four times, in pool II, the corresponding recoveries in Figure 1A.

Gel electrophoresis in sodium dodecyl sulfate of samples representative of pools I–IV in Figure 1A gave banding patterns shown in Figure 2. Pepsin-digested fragments large enough to be retained by dialysis tubing are shown in the gel corresponding to the column onput (O). The largest discrete polypeptide fragment bands are denoted F_3 and F_2 and were the only major polypeptides in the digest larger than pepsin (P). F_3 is a single band while F_2 is a closely spaced doublet. After the column fractionation in Figure 1A, F_3 and F_2 were the only major polypeptide bands readily observed in pools I and II. A faint diffuse band just below pepsin at the position labeled F_1 was observed in pools I and II in some pepsin digests and is characterized further below. Pool III was a mixture representative of most of the polypeptides in the column onput, and pool IV included only pepsin and smaller polypeptides. The A_{280} recovery in pool V corresponded to nearly 70% of the total A_{280} onput, but virtually everything was dialyzable; thus little gel staining was observed under the conditions in Figure 2. Gel electrophoresis of fractions from Figure 1B resulted in banding patterns that differed from those in Figure 2 in two respects (data not shown): (1) the amount of F_3 relative to F_2 appeared to be about twice that shown in Figure 2 for all fractions; (2) pool II gels contained polypeptide bands below pepsin similar to those in the pool III gel in Figure 2, although, as in Figure 2, pool I gels remained free of any

polypeptide bands below pepsin.

While pool I contained aggregated material in the column void volume, pool II was comprised of apparently soluble protein in the included volume of the column with a separate peak of F_2 and F_3 distinct from that in pool I. Thus F_3 and F_2 were obtained both in large aggregates and as discrete "dissociated" complexes prior to denaturation and separation into individual polypeptides in sodium dodecyl sulfate. The elution volume of the dissociated complex in pool II could be estimated from the staining intensities of F_3 and F_2 on sodium dodecyl sulfate gels that were routinely used to characterize fractions from column fractionations similar to those in Figure 1. For twelve such fractionations, this estimate was 78 ● 1 mL and thus corresponded to a Stokes radius of 10.8 nm, between that of 18S and 11S acetylcholinesterase (Figure 1). It is also noteworthy that the aggregation of F_3 and F_2 that occurs in pool I was not dependent on low ionic strength aggregation of 18S and 14S acetylcholinesterase prior to pepsin digestion. Maintenance of 1 M sodium chloride throughout the digestion did not significantly affect the distribution of F_3 and F_2 between pool I and pool II.

Identification of Pepsin-Resistant Fragments Not Derived from Catalytic Subunits. The chromatographic procedure in Figure 1 is intended to isolate collagen-like regions of the acetylcholinesterase tail subunits that are resistant to pepsin, and several criteria were examined to demonstrate that F_3 and F_2 were collagen-like fragments that could be isolated essentially free of other polypeptide contaminants. Contamination could arise either from small amounts of residual undigested acetylcholinesterase or from noncollagen-like fragments. As an initial test of such contamination, 18S and 14S acetylcholinesterase labeled at the catalytic sites with [^{3}H]iPr$_2$PF was digested with pepsin. The ^{3}H cpm profile in Figure 1A indicates that very little 18S and 14S acetylcholinesterase survived the digestion intact. Less than 1.5% of the total ^{3}H cpm eluted in pools I–IV, virtually all at or near the elution volume of 11S acetylcholinesterase [the G_4 form of the enzyme generated from the eel 18S and 14S forms by several proteases (Massoulié, 1980)]. A negligible amount appeared at the earlier elution volumes that correspond to 14S and 18S acetylcholinesterase. These percentages are considerably less than the corresponding A_{280} recoveries, but it is reasonable that pepsin should preferentially degrade the relatively exposed region of the enzyme catalytic site. A more rigorous second criterion was provided by a control pepsin digest of a protein free of collagen-like sequences. Since collagen-like tail subunits are associated with 14S and 18S acetylcholinesterase but not with 11S acetylcholinesterase, pepsin digestion of the 11S enzyme provided an excellent control for fragments arising from the catalytic subunits. A gel profile (C) corresponding to this control is included in Figure 2, and it is apparent that polypeptide bands in the region of F_3 and F_2 above pepsin are virtually nonexistent, while bands below pepsin roughly correspond to the 14S and 18S enzyme digest. Thus, F_3 and F_2 appear to be excellent candidates for pepsin-resistant tail fragments.

To ensure that ^{3}H-labeled noncollagen fragments do not coincidentally electrophorese with F_3 and F_2, we sliced and counted four of the gels in Figure 2. Results for the onput sample gel (O) are shown in Figure 3. Four distinct ^{3}H-labeled peaks are apparent between 4.2 and 7.5 cm. The same four bands characterized gel slice profiles for gels I, III, and C with only minor variation in the relative amounts of the 7.5-cm band. Trace amounts of ^{3}H at 0.1 and 2.5 cm in Figure 3 could represent residual intact oligomers from the 18S and

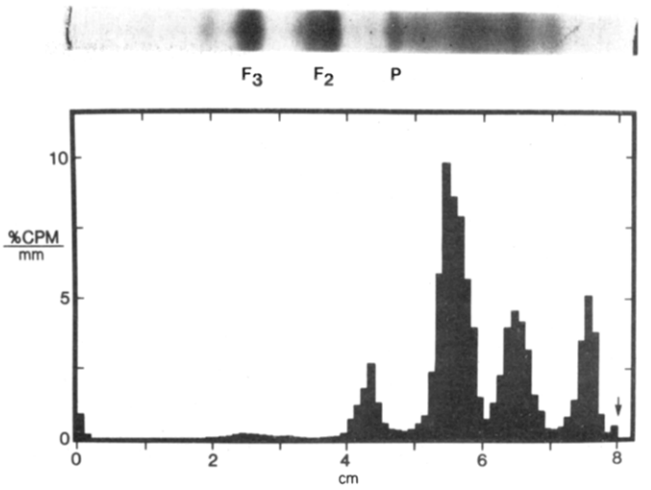

FIGURE 3: ^{3}H-Labeled polypeptide components from pepsin digestion of [^{3}H]iPr$_2$PF-labeled 18S and 14S acetylcholinesterase following gel electrophoresis. Gel O in Figure 2 was sliced in 1-mm segments for scintillation counting according to Barnett & Rosenberry (1979). The percentage of the total cpm recovered is indicated for each slice. The total recovery was 1.1% of the cpm in the gel onput, and this corresponded to ~75% of the column recovery in pools I–IV of Figure 1A. The gel photograph at the top (from Figure 2) was taken prior to slicing and corresponds to the histogram scale. The histogram terminates at the dye front.

14S enzyme [see Rosenberry & Richardson (1977); these oligomers remain at the top of 5.8% gels] and catalytic subunit monomers, respectively. These relative trace amounts were even smaller in gel I, and it was calculated that pool I had <0.0005% of the intact catalytic subunits that were in the sample prior to pepsin digestion. This observation is also important because it indicates that the light bands above F_3 in gel I (Figure 2) are probably oligomers of F_3 and/or F_2 and are not undigested acetylcholinesterase. No significant ^{3}H label coelectrophoreses with F_3 and F_2. Similar results are obtained with digests like that in Figure 1B in which pepsin degradation was less extensive. For example, termination of a digestion of [^{3}H]iPr$_2$PF-labeled enzyme with 0.1 mg/mL pepsin after only 1 h resulted in a 3-fold increase in ^{3}H label in pools I–IV and a 4-fold increase in ^{3}H label in pool I alone relative to Figure 1A. Even in this case, however, gel slicing analyses revealed that pool I had <0.005% of the intact catalytic subunits that were in the sample prior to pepsin digestion.

Quantitation of Column Fractionation Recoveries by Amino Acid Analysis. To further support the collagen-like nature of F_3 and F_2 as well as to quantitate the recovery of total hydroxyproline and hydrolysine from the column chromatography procedure, we hydrolyzed samples from the five pools in Figure 1B and subjected them to amino acid analysis. A summary of the quantitative recoveries of amino acids in these pools is presented in Table I. Pool I and thus its major components F_3 and F_2, with nearly 28% glycine and over 5% each of hydroxyproline and hydroxylysine, were predominantly collagen-like in composition. Pool II had only one-half the mole percentages of hydroxyproline and hydroxylysine found in pool I and thus appeared to consist of about equal amounts of the F_3–F_2 mixture and of noncollagen-like fragments. As noted above, the polypeptide content of pool II is quite sensitive to the extent of pepsin digestion, and more extensive digestion may increase the percentage of its collagen-like components. Pool III seemed to be at most 20% collagen-like, and pools IV and V were devoid of any fragments derived from collagen-like domains. The overall amino acid recovery was quantitative, although the total recoveries of hydroxyproline and hydroxylysine were 81% and 79%, respectively. Of the

Table I: Recovery of Total Amino Acids and of Three Amino Acids Characteristic of Collagen-like Polypeptides from Column Chromatographic Fractionation of the Pepsin Digest

fraction	total amino acids[a] (mg)	mol % Hyp	mol % Gly	mol % Hyl
column onput	10.53	0.6	10.9	0.7
pool I	0.59	5.2	27.9	5.6
pool II	0.41	2.3	18.2	2.5
pool III	0.76	0.9	12.8	1.0
pool IV	0.73	0.0	6.1	0.0
pool V	8.37	0.0	8.9	0.0
total recovery	10.86	0.5	10.3	0.5

[a] Pool samples were not dialyzed to avoid the loss of small peptide fragments, and analyses were conducted in duplicate as outlined under Materials and Methods. Total amino acids are expressed in milligram mean residue weights.

Table II: Amino Acid Composition of Pepsin-Resistant Fragments of 14S and 18S Acetylcholinesterase in Pool I[a]

amino acid	mol %	amino acid	mol %
Hyp	5.0	Met	2.8
Asp	5.0	Ile	2.2
Thr	2.3	Leu	5.2
Ser	5.9	Tyr	1.7
Glu	9.1	Phe	1.2
Pro	8.7	Hyl	5.3
Gly	27.2	Lys	2.3
Ala	3.4	His	2.2
Val	4.3	Arg	4.1

[a] The percentages are the means of nine determinations of pool I from six column fractionations. The standard errors averaged 5% of the means and exceeded 6% only for Asp, Met, and Phe. Cys and Try were not determined in this analysis but were assumed to contribute 2.0 mol %. Cys is present only as cystine, and a maximal estimate of 1.5 for its mole percentage in these fragments can be obtained from the half-cystine content of the intact tail subunits (Rosenberry et al., 1980; P. Barnett and T. L. Rosenberry, unpublished experiments).

recovered hydroxyproline and hydroxylysine, 66% was obtained in pool I. This percentage also appeared sensitive to the extent of pepsin degradation and was probably somewhat lower in Figure 1A. Of primary importance here, however, is the fact that pool I appears to represent a relatively contaminant-free isolation of over one-half of the collagen-like regions of the 14S and 18S acetylcholinesterase tail subunits. This pool was selected for further characterization of the tail subunits in subsequent experiments that follow. The total amino acid composition of pool I is given in Table II. It is noteworthy that the gel chromatographic procedure in Figure 1 resulted in more efficient fractionation than the differential salt precipitation and sedimentation procedures more typically employed for the isolation of other collagen polypeptides.[3]

Molecular Weight Estimates of Fragments. The isolated F_3 and F_2 fragments retain endogenous interpolypeptide disulfide linkages, and exposure to disulfide reducing agents converts them to smaller species on sodium dodecyl sulfate gels. A pepsin digest that had been fractionated as described in Figure 1 was selected for analysis in Figure 4 because pool

[3] For example, centrifugation of the initial 6-h digest (30000*g* for 20 min) before neutralization gave variable distributions of F_3 and F_2 between residue and supernatant. Digestion in 1 M sodium chloride or at lower effective pepsin concentrations favored partitioning of both forms, but particularly of F_3, into the residue. The pool I aggregate seemed particularly susceptible to sedimentation under these conditions. However, the use of this procedure as a fractionation technique was precluded because considerable amounts of small catalytic subunit fragments and some pepsin were also sedimented.

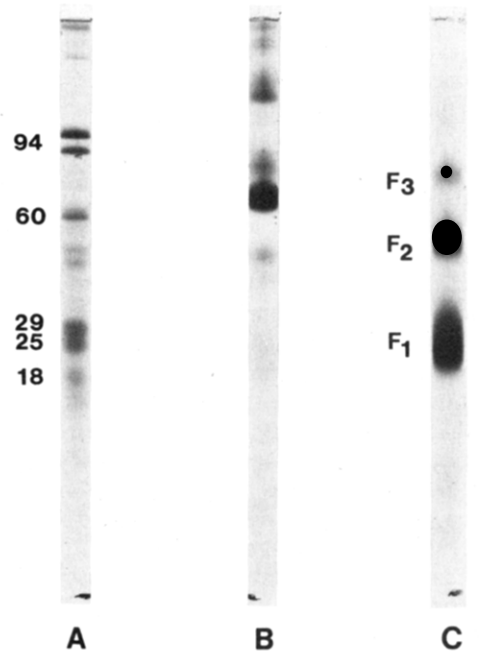

FIGURE 4: Molecular weight determinations of polypeptides arising from pool I by gel electrophoresis in 1% sodium dodecyl sulfate. Electrophoresis conditions are similar to those described in Figure 2. (B and C) Samples correspond to the peak fraction in pool I; (B) 14 μg of protein prepared without disulfide reduction; (C) 20 μg of protein subjected to reduction with 100 mM dithiothreitol at 50 °C for 30 min in the 1% sodium dodecyl sulfate sample solution prior to electrophoresis. (A) Cyanogen bromide fragments of α1(I) and α2(I) acid-soluble calf skin collagen run in parallel with samples B and C. Molecular weights in thousands for these standards are indicated as listed under Materials and Methods.

I was composed almost exclusively of F_3. Exposure to dithiothreitol in the sodium dodecyl sulfate sample buffer resulted in dissociation to a rather broad primary band F_1. Molecular weights of the pepsin-resistant fragments were estimated from electrophoresis standards composed of the well-characterized cyanogen bromide fragments of the α1(I) and α2(I) chains of acid-soluble type I collagen. F_1 corresponds to ~24000 molecular weight. The reduced fragments still associate rather strongly even in 1% sodium dodecyl sulfate, perhaps because a few lysine- or hydroxylysine-derived interpolypeptide cross-linkages may be present. Additional bands with decreasing intensities correspond to 47000, 74000, and 98000 molecular weights. These relative molecular weights are consistent with oligomers of F_1, and thus the designations F_2 and F_3 in Figure 4 indicate dimers and trimers of F_1. Although the electrophoretic mobilities of F_2 and F_3 are somewhat greater prior to disulfide reduction, use of these designations interchangeably between the corresponding nonreduced and reduced gel bands seems a relatively safe assumption. Pool I samples that were predominantly F_3 and those that were largely F_2 gave virtually identical polypeptide banding patterns when run under the completely reduced conditions corresponding to gel C in Figure 4. It should be noted, however, that F_1 in this gel may be quite heterogeneous, as suggested by its broad band. More than one class of dimers and trimers thus may occur, probably due to differing extents of degradation, and could account for the doublet nature of the F_2 band prior to reduction. The question of whether the tail subunits are identical prior to pepsin degradation remains open.

Pepsin Digestion of 18S and 14S Acetylcholinesterase at Various Temperatures. Pool I, prior to disulfide reduction, contains predominantly F_3 and F_2. Several observations

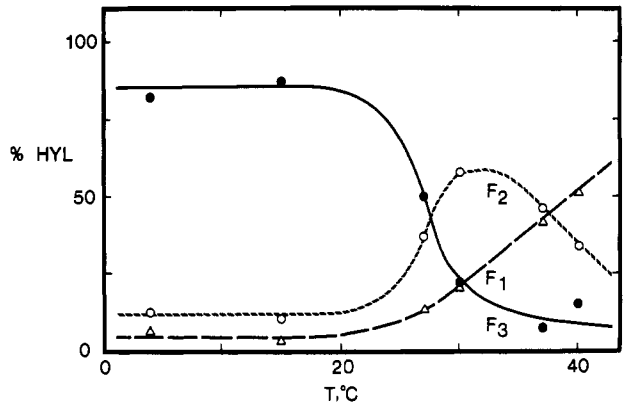

FIGURE 5: Temperature dependence of pepsin digestion of 18S and 14S acetylcholinesterase. Digestion of 1.0 mg of acetylcholinesterase with 38 μg of pepsin (fresh commercial stock) in 2.1 mL was conducted as outlined under Materials and Methods. After the initial 6-h digestion, the sample was divided into equal portions containing 130 μg of digested acetylcholinesterase. Each portion was then adjusted to one of the fixed temperatures indicated, and digestion was continued for an additional 2 h. Gel electrophoresis in sodium dodecyl sulfate of each sample was carried out as described in Figure 2, and the hydroxylysine contents of 1.0–1.2-cm gel segments corresponding to the F_3, F_2, and F_1 regions of the stained gels were determined as outlined under Materials and Methods. The distribution of Hyl among these three regions in each gel is expressed here as the percent Hyl in each region: F_3 (—), F_2 (---), and F_1 (–––). The total Hyl content in these three regions ranged from 1.3 to 2.9 nmol/gel as noted in the text.

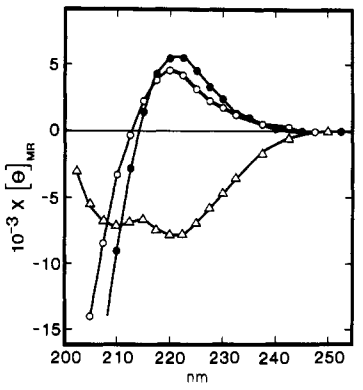

FIGURE 6: CD spectra of 11S acetylcholinesterase, collagen, and pepsin-resistant fragments of 14S and 18S acetylcholinesterase at 25 °C. (△) 11S acetylcholinesterase in 20 mM phosphate buffer. (○) Acid-soluble calf skin collagen in either 10 mM acetic acid or 100 mM sodium chloride and 10 mM acetic acid. (●) Pepsin-resistant fragments of 14S and 18S acetylcholinesterase in phosphate-buffered 1.0 or 2.0 M sodium chloride. The standard error for duplicate determinations of $[\theta]_{MR}$ was 10%.

suggest that F_2 is derived from F_3 by further pepsin degradation. The increased ratio of F_3 to F_2 under conditions of lower pepsin activity was noted above, and we have confirmed that this ratio is also increased when the time of exposure to pepsin is decreased. If F_3 and F_2 represent collagen-like triple-helical domains, as anticipated for the enzyme tail structure, then increasing the pepsin digestion temperature above the helix melting point should result in degradation of F_3 and F_2. To test this expectation, we conducted pepsin digestion of 1 mg of 14S and 18S acetylcholinesterase in two stages. In the first stage, digestion was conducted at 15 °C for 6 h to give a fragment population in which the ratio of F_3 to F_2 was ~8; immediately following the first stage, the sample was divided into six equal portions, each of which was incubated an addition 2 h at a fixed temperature that ranged from 4 to 40 °C. After gel electrophoresis of the samples in sodium dodecyl sulfate, the hydroxylysine content of the collagen-like polypeptides was analyzed as shown in Figure 5 to clarify the relationships among F_3, F_2, and F_1. Samples incubated at 4 and 15 °C showed no further degradation and contained about 85% F_3, 10% F_2, and 5% F_1. A first transition appeared between 15 and 30 °C in which F_3 fell sharply and F_2 and F_1 rose in an ~2:1 ratio. Between 30 and 40 °C a second transition appeared in which F_2 fell and F_1 became predominant. Concomitant with these redistributions was a gradual decrease in the overall recovery of Hyl from ~2.9 nmol (37%)[4] at 4 and 15 °C to ~1.3 nmol (17%) at 35–40 °C. These changes strikingly indicated the degradation of F_3 to F_2 and F_1 and of F_2 to F_1 together with the overall degradation and loss of the triple-helical structures that these polypeptide bands represent. The sharpness of the first transition was further revealed by a second experiment similar to that shown in Figure 5 in which the second-stage digestions were continued for only 1 h. In this case the F_3/F_2 ratio dropped only to 3 at 25 °C but completely inverted to 0.4 at 35 °C. Thus the first transition occurs most readily between 25 and 30 °C.

Circular Dichroism Spectra. To confirm that the pepsin-resistant fragments obtained in pool I indeed have a collagen-like triple-helical secondary structure, we compared CD spectra of this pool with those of acid-soluble calf skin collagen and with 11S acetylcholinesterase shown in Figure 6. Triple-helical collagen is characterized by $[\theta]_{MR}$ values of 5000–6000 at 220–223 nm and −50 000 at 198 nm (Kefalides, 1968; Brodsky-Doyle et al., 1976). The CD spectra for collagen and for the pepsin-resistant fragments in several solvents were virtually identical and consistent with these literature values. In contrast, the CD spectrum of 11S acetylcholinesterase appeared qualitatively similar to that for an α-helical conformation [see Brodsky-Doyle et al. (1976)], although the $[\theta]_{MR}$ values for the 11S form were only ~20% of those for a homogeneous α helix and suggested the presence of considerable amounts of alternative secondary structures. The CD spectrum of 14S and 18S acetylcholinesterase was very similar to that of the 11S form shown in Figure 6. A difference spectrum for these two acetylcholinesterase samples could not be obtained with sufficient accuracy to clearly demonstrate the presence of the triple-helical structure in the 14S and 18S sample.

Discussion

The collagen-like subunits present in native 14S and 18S acetylcholinesterase from eel electric organ form a "tail" structure of ~100 000 molecular weight (Bon et al., 1976) and are linked both to catalytic subunits and to each other by intersubunit disulfide bonds as shown in Figure 7 (Rosenberry & Richardson, 1977; McCann & Rosenberry, 1977; Anglister & Silman, 1978; Bon & Massoulie, 1976; P. Barnett and T. L. Rosenberry, unpublished experiments). The isolated tail subunits have estimated molecular weights of 30 000–40 000 in denaturants following disulfide reduction and thus appear to form a trimeric structure in the native enzyme. However, these isolated subunits contain only 23% glycine, an indication that only certain domain(s) of this trimeric structure can exist in a collagen-like triple-helical conformation with glycine in every third position (Rosenberry et al., 1980; P. Barnett and T. L. Rosenberry, unpublished experiments). The largest polypeptide fragment that resists pepsin digestion of the 18S

[4] A 37% recovery of pepsin-resistant fragments following dialysis and lyophilization was a typical value for small samples. These fragments readily adsorb to a variety of surfaces, and we took care to minimize serial transfers and to limit dialysis time during salt removal (4 h) to avoid even greater losses.

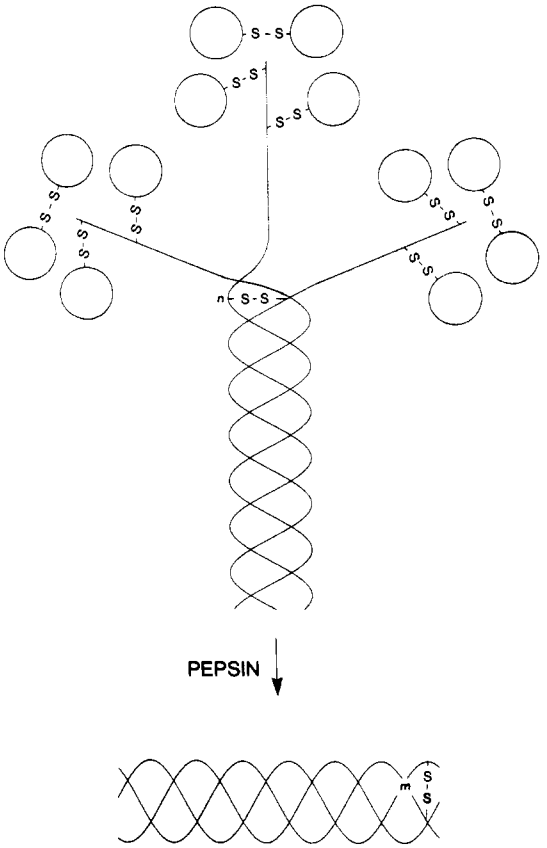

FIGURE 7: Schematic model of digestion of 18S acetylcholinesterase by pepsin. The upper structure represents an 18S molecule, in which each catalytic subunit (circle) is linked by a single disulfide bond either to another catalytic subunit or to a tail subunit. A total of n disulfide bonds also cross-link the three tail subunits. Although the precise stoichiometry of disulfide bonds on each tail subunit has not been determined, n is ~ 5 (Rosenberry et al., 1980; P. Barnett and T. L. Rosenberry, unpublished experiments). Pepsin degrades the catalytic subunits to small fragments and removes about one-third of each tail subunit, apparently a non-triple-helical region in contact with the catalytic subunits (Rosenberry et al., 1980). The lower structure represents the pepsin-resistant tail subunit fragments, which appear to be trimers of $\sim 72\,000$ molecular weight that are largely, although not exclusively, triple helical. At low digestion temperatures these trimers retain a sufficient number m of disulfide cross-links to maintain each 24 000 molecular weight fragment monomer (F_1) as a cross-linked trimer (F_3) in which $n \geq m \geq 2$. At higher digestion temperatures the disulfide cross-linkage region in the trimer can be degraded (m decreases) with only a slight decrease in the monomer molecular weight.

and 14S acetylcholinesterase forms appears as a 72 000 molecular weight species on sodium dodecyl sulfate gels and has a largely collagen-like amino acid composition (Figure 7). We have labeled this fragment F_3. F_3 retains interpolypeptide disulfide linkages, but disulfide reduction converts it primarily to F_1 components with molecular weights of $\sim 24\,000$. Thus, F_3 is presumably a disulfide-linked trimer of F_1. Further pepsin digestion of F_3 results in proteolytic cleavage inside the intersubunit disulfide linkage such that the predominate species prior to disulfide reduction on sodium dodecyl sulfate gels become the dimeric F_2 and monomeric F_1 forms. This residual F_1 component does not stain as intensively as F_2 on these gels prior to reduction, but F_1 staining can be detected in pool I samples in which F_3 has been largely converted to F_2, and a quantitative estimate of the relative amount of F_1 generated can be obtained by hydroxylysine analysis like that in Figure 5. Pool I samples in which either F_3 or F_2 predominate give both CD spectra typical of triple-helical collagen and amino acid compositions that are virtually identical, and the sizes of the reduced F_1 polypeptides from these samples do not differ significantly. Thus the intersubunit disulfide bonds in F_3 must occur close to the end(s) of the polypeptide chains, because pepsin cleavage inside these bonds does not significantly affect either the triple-helical content or the reduced polypeptide molecular weights. The data do not directly indicate whether these disulfide bonds are in a triple-helical domain, but they presumably occur in a non-triple-helical region that is partially protected from pepsin degradation by close juxtaposition to the triple helix. Pepsin cleavage of F_3 to F_2 and F_1 occurs at a greatly increased rate between 25 and 30 °C, suggesting that the region of at least one of the intersubunit disulfide linkages undergoes thermal destabilization in this temperature range. Continued pepsin cleavage of F_2 to F_1 between 30 and 40 °C indicates that the entire linkage region becomes completely susceptible to pepsin at these temperatures. The presumably triple-helical F_1 structures devoid of intersubunit disulfide bonds retain some pepsin resistance, but their degradation to small fragments can be readily detected at 40 °C.

The relatively specific localization of A_{12} acetylcholinesterase forms to neuromuscular junctions together with both the cytochemical localization of a significant fraction of junctional enzyme in the basement membrane and the selective release of junctional enzyme by collagenase has been widely interpreted as indicating that these acetylcholinesterase forms are localized through the direct interaction of their collagen-like tail structures with the junctional basement membrane [see Lwebuga-Mukasa et al. (1976)]. Thus these tail structures are de facto junctional basement membrane collagens and are the first such collagens to be specifically identified and isolated. Basement membrane collagens isolated from a variety of sources by pepsin digestion consist of a very heterogeneous class of structures that includes type IV polypeptides corresponding in size to the 94 000 molecular weight $\alpha 1$ chain of type I collagen as well as to larger polypeptides, among them A, B, and C chains, many of which retain interpolypeptide disulfide bonds (Dehm & Kefalides, 1978; Kresina & Miller, 1979; Sage & Bornstein, 1979; West et al., 1980). In addition, certain disulfide-linked aggregates containing smaller polypeptides have been characterized following pepsin digestion. These include a 7S collagenous structure, obtained from a variety of basement membranes, with unusual resistance toward bacterial collagenase (Risteli et al., 1980) and a 280 000 molecular weight aggregate from human placental tissue (Furuto & Miller, 1980). Following reduction and alkylation, this aggregate dissociated to yield two classes of collagen-like polypeptides with apparent molecular weights of 40 000 as well as a heterogeneous mixture of much smaller noncollagenous peptides. These two classes resemble the acetylcholinesterase tail subunit fragments, not only by their involvement in a disulfide-linked assembly that included noncollagenous peptides but also by their somewhat less than one-third glycine content and their similar levels of proline and lysine hydroxylation. Analyses of cyanogen bromide fragments of the placental collagen-like polypeptides showed many fragments with less than one-third glycine, indicating that noncollagenous domains were distributed at several points along the polypeptides. Such a distribution remains to be demonstrated for the pepsin-resistant acetylcholinesterase tail subunit fragments, although the 27% glycine content of these fragments suggests a larger proportion of noncollagenous domains than in the placental polypeptides. The presence of noncollagenous domains in the tail subunit fragments is also indicated by a comparison of the 72 000 F_3 molecular weight with the 50-nm length of the tail structure estimated from electron micrographs (Cartaud et al., 1975). The calculated length of F_3 if it were completely

triple helical would be 68 nm (based on a mean residue weight of 100 and a triple helix rise of 0.286 nm/residue). Thus the noncollagenous domains in F_3 contribute considerably less to its length.

References

Anglister, L., & Silman, I. (1978) *J. Mol. Biol. 125*, 293–311.

Anglister, L., Leibovich, S. J., & Silman, I. (1979) 7th Meeting of the International Society for Neurochemistry, Jerusalem, Israel.

Barnett, P., & Rosenberry, T. L. (1979) *Biochim. Biophys. Acta 567*, 154–160.

Betz, W., & Sakmann, B. (1973) *J. Physiol. (London) 230*, 673–688.

Birk, Y., Gertler, A., & Khalef, S. (1963) *Biochem. J. 87*, 281–284.

Bon, S., & Massoulié, J. (1976) *FEBS Lett. 71*, 273–278.

Bon, S., Huet, M., Lemonnier, M., Rieger, F., & Massoulié, J. (1976) *Eur. J. Biochem. 68*, 523–530.

Bon, S., Cartaud, J., & Massoulié, J. (1978) *Eur. J. Biochem. 85*, 1–14.

Bon, S., Vigny, M., & Massoulié, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2546–2550.

Brodsky-Doyle, B., Leonard, K. R., & Reid, K. B. M. (1976) *Biochem. J. 159*, 279–286.

Cartaud, J., Rieger, F., Bon, S., & Massoulié, J. (1975) *Brain Res. 88*, 127–130.

Dehm, P., & Kefalides, N. A. (1978) *J. Biol. Chem. 253*, 6680–6686.

Dudai, Y., Silman, I., Kalderon, N., & Blumberg, S. (1972) *Biochim. Biophys. Acta 268*, 138–157.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

Fietzek, P. P., & Kuhn, K. (1976) *Int. Rev. Connect. Tissue Res. 7*, 1–60.

Furuto, D. K., & Miller, E. J. (1980) *J. Biol. Chem. 255*, 290–295.

Hall, Z. W. (1973) *J. Neurobiol. 4*, 343–361.

Hall, Z. W., & Kelly, R. (1971) *Nature (London), New Biol. 232*, 62–63.

Houston, L. L. (1971) *Anal. Biochem. 44*, 81–88.

Johnson, C. D., Smith, S. P., & Russell, R. L. (1977) *J. Neurochem. 28*, 617–624.

Kefalides, N. A. (1968) *Biochemistry 7*, 3103–3112.

Kresina, T. F., & Miller, E. J. (1979) *Biochemistry 18*, 3089–3097.

Lwebuga-Mukasa, J. S., Lappi, S., & Taylor, P. (1976) *Biochemistry 15*, 1425–1434.

Massoulié, J. (1980) *Trends Biochem. Sci. (Pers. Ed.) 5*, 160–164.

Massoulié, J., & Rieger, F. (1969) *Eur. J. Biochem. 11*, 441–455.

McCann, W. F. X., & Rosenberry, T. L. (1977) *Arch. Biochem. Biophys. 183*, 347–352.

McMahan, U. J., Sanes, J. R., & Marshall, L. M. (1978) *Nature (London) 271*, 172–174.

Miller, E. J., Lane, J. M., & Piez, K. A. (1969) *Biochemistry 8*, 30–39.

Rieger, F., Bon, S., Massoulié, F., Cartaud, J., Picard, B., & Benda, P. (1976) *Eur. J. Biochem. 68*, 513–521.

Risteli, J., Bachinger, H. P., Engel, J., Furthmayr, H., & Timpl, R. (1980) *Eur. J. Biochem. 108*, 239–250.

Rosenberry, T. L., & Richardson, J. M. (1977) *Biochemistry 16*, 3550–3558.

Rosenberry, T. L., Chang, H. W., & Chen, Y. T. (1972) *J. Biol. Chem. 247*, 1555–1565.

Rosenberry, T. L., Chen, Y. T., & Bock, E. (1974) *Biochemistry 13*, 3068–3079.

Rosenberry, T. L., Barnett, P., & Mays, C. (1980) *Neurochem. Int. 2*, 135–137.

Rotundo, R. L., & Fambrough, D. M. (1979) *J. Biol. Chem. 254*, 4790–4799.

Rubin, A. L., Pfahl, D., Speakman, P. T., Davison, P. F., & Schmidt, F. O. (1963) *Science 139*, 37–38.

Sage, H., & Bornstein, P. (1979) *Biochemistry 18*, 3815–3822.

Trelstad, R. L., Kang, A. H., Toole, B. P., & Gross, J. (1972) *J. Biol. Chem. 247*, 6469–6473.

Watkins, M. S., Hitt, A. S., & Bulger, J. E. (1977) *Biochem. Biophys. Res. Commun. 79*, 640–647.

Webb, G. (1978) *Can. J. Biochem. 56*, 1124–1132.

West, T. W., Fox, J. W., Jodlowski, M., Freytag, J. W., & Hudson, B. G. (1980) *J. Biol. Chem. 255*, 10451–10459.