Filamin, a New High-Molecular-Weight Protein Found in Smooth Muscle and Nonmuscle Cells. Purification and Properties of Chicken Gizzard Filamin†

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ABSTRACT: Filamin, a major high-molecular-weight protein of chicken gizzard smooth muscle, was purified to homogeneity by salt extraction, ammonium sulfate precipitation, agarose gel filtration, and diethylaminoethylcellulose ion-exchange chromatography. Purified filamin is an asymmetric oligomer consisting of two large subunits of identical size (2 × 250 000 daltons) as indicated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, chemical cross-linking, sedimentation analysis ($s_{20,w}^0$ = 10 S) and Stokes' radius estimation (a = 120 Å). It has no intersubunit disulfide but appears from oxidation studies to have adjacent thiols near the subunit interface. Filamin contains no amino sugars, methylated lysine, methylated histidine, or hydroxyproline, nor does it exhibit myosin-like ATPase activities. Its amino acid composition and physical properties differ from those of gizzard myosin, for which a purification procedure is described. Filamin and the protein spectrin of erythrocyte membranes have strikingly similar physical properties, but they are chemically distinct.

Vertebrate smooth muscle has been the subject of intense physiological and pharmacological studies. However, relatively little attention has been given until recently to the biochemical and assembly properties of its contractile proteins. In contrast, skeletal muscle and certain invertebrate smooth muscle contractile proteins have been studied in detail. The situation has now changed dramatically, partly because it has been found that some of the proteins of smooth muscle and of many nonmuscle motile cells have similar molecular and antigenic properties. Skeletal muscle contractile proteins, on the other hand, appear to be distinct in many respects (for reviews, see Pollard and Weihing, 1974; Shoenberg and Needham, 1976).

Filamin is a high-molecular-weight protein first isolated from chicken gizzard smooth muscle (Wang et al., 1975). By the use of specific antibodies directed to filamin, antigenically cross-reacting proteins were detected in other organs of chicken and in a wide range of tissue culture cells of other species. Characteristically, these cross-reacting proteins were found associated with filamentous structures in these cells (Wang et al., 1975). Recently we have observed that filamin interacts with and promotes the aggregation of F-actin (Wang and Singer, 1977), and that filamin is closely associated ultrastructurally with plasma membranes of several tissue culture fibroblasts (to be published). It appears that the filamin protein constitutes a family of chemically conserved proteins that might play an important role in the mechanochemical activities of cells through its association with actomyosin and membrane systems.

In this paper, purification procedures and preliminary characterizations of chicken gizzard filamin are reported. Some comparative studies with human erythrocyte spectrin are described.

Experimental Procedure

Purification of Filamin. All steps were carried out in a cold room at 4 °C or on ice. pH adjustment of buffers was done at room temperature.

Step 1: Extraction. Chicken gizzards were removed from the animals within 30 min after exsanguination. The muscle (100 g), trimmed free of fat and connective tissues, was ground in a meat grinder. Muscle fragments were washed once and then homogenized in 300 mL of extraction buffer (0.3 M KCl, 2 mM K_2ATP, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM imidazole, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.9) in a Waring blender with two to three 15-s bursts at 1-min intervals. The foamy homogenate was spun at 18 000 rpm (43 500g_{max}) for 20 min in a Beckman 21 rotor. The supernatant (S_1) was decanted and then filtered through a Whatman number one filter paper.

Step 2: Ammonium Sulfate Fractionation. Finely ground solid ammonium sulfate (16 g per 100 mL of supernatant) was added slowly to the extract (S_1) with stirring. After 30 min the suspension was spun at 12 000 rpm (17 300g_{max}) for 10 min in a Sorvall SS-34 rotor. The supernatant (S_{0-29}) was saved for myosin preparation and the pellet (P_{0-29}) was used for filamin preparation.

Step 3: Agarose Gel Filtration. The rubbery pellet (P_{0-29}) was dissolved in a minimum volume (~10 mL) of buffer A (0.6 M KCl-2 mM $MgCl_2$-1 mM EDTA[1]-0.1 mM dithiothreitol-10 mM Tris-Cl, pH 7.4). Soaking the pellet for 20-30 min and gentle stirring with a glass rod facilitated this process (homogenization should be avoided). The solution was spun at 45 000 rpm (183 000g_{max}) for 45 min in a Beckman Ti 50 rotor and the clear supernatant was applied to a 2.5 × 90 cm column of 4% agarose (Bio-Gel A-15m, 100-200 mesh) equilibrated with buffer A. The column was eluted with the same buffer at a flow rate of 30 mL/h. Fractions (9 mL per fraction) were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Peak fractions containing filamin were pooled.

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[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

Step 4: DEAE-Cellulose Ion-Exchange Chromatography. The pool of fractions was brought to 50% ammonium sulfate saturation (29.1 g of solid ammonium sulfate per 100 mL of solution). After 30 min the suspension was spun at 12 000 rpm for 10 min in a SS-34 rotor. The pellet was then dissolved in ~5 mL of 20 mM Tris–acetate, pH 7.6, spun at 45 000 rpm for 45 min in a Ti 50 rotor and then applied to a 2.5 × 20 cm column of DEAE-cellulose (Whatman DE52). The column was washed with 20 mL of the Tris–acetate buffer and then eluted with a linear gradient of 200 mL of 20 mM Tris–acetate and 200 mL of 0.5 M KCl in 20 mM Tris–acetate, pH 7.6, at a flow rate of 20 mL/h. Filamin was eluted as the major peak between 0.10 and 0.14 M KCl.

Concentration and Storage. Filamin solution could be concentrated by Amicon ultrafiltration and by vacuum dialysis. However, appreciable amounts were lost because of the formation of an insoluble gelatinous layer on the membranes. Protein concentrations of ~4 mg/mL could be obtained by reapplying the filamin solution to a small DE-52 column and eluting with 1.0 M KCl in 20 mM Tris–acetate, pH 7.6. Ammonium sulfate precipitation caused irreversible aggregation of DEAE-cellulose purified filamin but could be safely used to concentrate filamin before that step.

For storage, an equal volume of glycerol was added to filamin in buffer A and the solution was stored at −20 °C.

Purification of Myosin. Myosin was purified from the 29 to 55% saturated ammonium sulfate fraction of the extract (S_1). The supernatant after the first ammonium sulfate cut ($S_{0\text{-}29}$) was brought to 55% saturation by adding 14.9 g of ammonium sulfate per 100 mL of solution. After 30 min, the suspension was spun at 12 000 rpm for 10 min in a SS-34 rotor. The pellet was resuspended in a small volume of buffer A and then an equal volume of a KI–ATP solution (1.2 M KI–4 mM ATP–4 mM $MgCl_2$–2 mM EDTA–20 mM Tris-Cl (pH 7.4)) was added. The suspension was homogenized with a Dounce homogenizer and spun at 45 000 rpm for 45 min in a Ti 50 rotor. The clear supernatant was applied to a 2.5 × 90 cm column of Bio-Gel A-15m (100–200 mesh) in buffer A after prerunning the column with 30 mL of a twofold dilution of the above KI–ATP solution. The column was eluted with buffer A at a flow rate of 30 mL/h. Myosin was eluted immediately after the cloudy void volume peak and was located by the maximum ratio of A_{280} to A_{260}. Peak fractions were pooled and dialyzed overnight against two changes of 1 L of low ionic strength buffer (0.05 M KCl–5 mM $MgCl_2$–1 mM EDTA–0.2 mM dithiothreitol–10 mM imidazole, pH 6.4). The precipitated myosin was collected by centrifugation at 12 000 rpm for 10 min, redissolved in buffer A, and spun at 45 000 rpm for 45 min in the Ti 50 rotor. The clear supernatant was dialyzed against 1 L of 0.15 M KH_2PO_4–10 mM EDTA, pH 7.5, for several hours and then applied to a DEAE-Sephadex (A-50) column (2 × 18 cm) equilibrated with the same buffer. The column was washed with 20 mL of the starting buffer and then eluted at 20 mL/h with a linear gradient of 100 mL of starting buffer and 100 mL of 1.0 M KCl in the same buffer. Myosin was eluted as the major peak after the flow through peak.

For storage, an equal volume of glycerol was added to the myosin and the solution was stored at −20 °C.

Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Fairbanks et al. (1971) on 4% acrylamide gels (0.6 × 8.5 cm) using 0.2% sodium dodecyl sulfate in the gel and electrode buffers, unless otherwise indicated. The low concentration of acrylamide was necessary to resolve the large polypeptide chains of filamin and myosin. Most protein samples contained high concentration of potassium salts which precipitated dodecyl sulfate. However, it was not necessary to remove potassium from the sample before electrophoresis. Instead, the samples were boiled for 5 min in sodium dodecyl sulfate sample buffer and then immediately transferred to the polyacrylamide gels when hot (a procedure suggested by Dr. J. F. Ash). Potassium dodecyl sulfate was soluble at high temperature but reprecipitated at room temperature as a disc on top of gels. Electrophoresis was performed first at 2 to 3 mA/tube for 30 min to dissolve all precipitates, then at 6 mA/tube for 2 more h. Under these conditions the electrophoretograms were indistinguishable from those of samples in which potassium had been removed by dialysis.

The gels were stained with 0.05% Coomassie blue in 25% 2-propanol–10% acetic acid for 8 to 10 h, and destained in 10% 2-propanol–10% acetic acid for 12 h.

Stain intensity was measured at 550 nm on a Gilford spectrophotometer equipped with a gel scanner. The relative protein content was determined by measuring weight ratios of individual peaks cut off the chart paper, on the assumption that all proteins have the same color yield. A variation of ~20% was usually found for different loadings of the same sample.

Polypeptide chain weight was estimated by measuring relative mobility and comparing it with a calibration plot of log of molecular weights vs. relative mobility established with the following proteins: human spectrin (240 000 and 220 000), chicken skeletal myosin (heavy chain, 200 000), *E. coli* RNA polymerase (165 000, 155 000, and 95 000), *E. coli* β-galactosidase (130 000), bovine serum albumin (68 000), and chicken skeletal actin (43 000).

Purification of Human Spectrin. Human spectrin was isolated from erythrocytes of outdated human blood according to Nicolson and Painter (1973), except that a Bio-Gel A-15m column was used in the gel filtration step. Spectrin was eluted in two peaks: one in the cloudy void volume and another in the included volume. This latter peak of human spectrin was free from actin and was used in the present study.

Chemical Cross-linking. (1) Dimethyl Adipimidate Dihydrochloride. Purified filamin and human spectrin at various concentrations from 0.1 to 2.0 mg/mL in 0.5 M KCl–0.1 M triethanolamine–1% β-mercaptoethanol at pH 8.5 were treated at room temperature with 2 mg/mL of dimethyl adipimidate dihydrochloride. At different intervals, aliquots of sample solution (400 μL) were removed and added to 250 μL of three times concentrated sodium dodecyl sulfate sample buffer (Fairbanks et al., 1971) containing 0.25 M NH_4Cl to quench the reaction. Samples were boiled for 5 min and then analyzed by electrophoresis on 3.2% or 4% gels. The running times (~4 h) for these gels were typically twice as long as usual to allow cross-linked complexes to enter the gels.

(2) Cu^{2+}–(O-Phenanthroline)$_2$-Catalyzed Oxidation. Protein solutions at various concentrations (0.1 to 0.5 mg/mL) in 0.5 M KCl–0.1 M Tris-Cl at pH 8.5 were treated with Cu^{2+}–(O-phenanthroline)$_2$ complexes (50–100 μM final concentration) at room temperature. At different intervals, aliquots (150 μL) were added to 100 μL of quenching medium containing 20 mM EDTA and 20 mM *N*-ethylmaleimide. After 10 min, solutions were brought to 2% sodium dodecyl sulfate–10% glycerol and then incubated at 50 °C for 30 min before being electrophoresed on 3.2 or 4% gels. Thiols were omitted in all solutions.

Analytical Ultracentrifugation. Sedimentation velocity determinations were performed in a Spinco Model E analytical ultracentrifuge using Schlieren optics. Experiments were

carried out at 20 °C at 60 000 rpm.

Purified filamin in buffer A was concentrated to the desired concentration by treatment in an Amicon PM30 filter and then dialyzed against the same buffer overnight. The dialyzate was used to dilute samples when necessary.

Sedimentation coefficients (s_{20}) were calculated according to Schachman (1957). Correction to standard conditions ($s_{20,w}^0$) was made using a partial specific volume of 0.728 cm^3/g for filamin, as calculated from its amino acid composition according to Schachman (1957).

Stokes' Radius Determination. Stokes' radius was estimated by the method of Laurent and Killander (1964). A 1.5 × 60 cm column of Bio-Gel A-15m (100–200 mesh) equilibrated with buffer A was calibrated by determining the elution volumes (V_i) of standard proteins of known Stokes' radii (Andrew, 1970): human fibrinogen (107 Å), bovine thyroglobulin (85 Å), horse spleen ferritin (79 Å), rabbit muscle aldolase (45 Å), bovine serum albumin (35 Å), and cytochrome *c* (17 Å). The void volume (V_0) and column volume (V_e) were determined by blue dextran and dinitrophenyllysine, respectively. The Stokes' radii of gizzard filamin, myosin, and human spectrin were determined by extrapolation of the linear plot of $(-\log K_{av})^{1/2}$ vs. Stokes' radius.

Protein Determination. Relative protein concentration was determined by the method of Lowry et al. (1951), as modified by Hartree (1972). Crystalline bovine serum albumin was used as the standard. The concentration of bovine serum albumin was determined by measuring the absorbance at 280 nm ($E_{280nm}^{1\%} = 6.6$). The extinction coefficient $E_{280nm}^{1\%}$ of filamin was determined to be 6.8 (based on Lowry protein determination) and was used to calculate the protein concentration of purified filamin.

Amino Acid Analysis. Samples of purified filamin were dialyzed exhaustively against distilled water and lyophilized. Performic acid oxidation was carried out according to Hirs et al. (1956). Oxidized samples were hydrolyzed in 6 N HCl in sealed tubes at 105 °C for 24, 48, and 72 h. The hydrolyzates were analyzed on a Beckman Model 120B amino acid analyzer.

In separate experiments, tryptophan content was estimated by the method of Bencze and Schmid (1957), and free thiol content was determined by the use of Ellman's reagent in 1% sodium dodecyl sulfate according to Ellman (1959). In both determinations, readings were followed until they became constant.

ATPase Assay. Myosin-like ATPase activities were assayed according to Clarke and Spudich (1974).

Other Proteins. Rabbit skeletal muscle actin was prepared according to Spudich and Watt (1971). *E. coli* RNA polymerase and β-galactosidase were gifts of Dr. J. Duffy. Human fibrinogen was a gift of Dr. R. Doolittle. Other proteins were purchased from Sigma.

Chemicals. Dimethyl adipimidate dihydrochloride was synthesized according to Davies and Stark (1970) by Mrs. M. Adams in this laboratory. Orthophenanthroline, phenylmethanesulfonyl fluoride, dithiothreitol, and ATP were purchased from Sigma.

Results

Filamin Purification. When whole chicken gizzard smooth muscle was analyzed on sodium dodecyl sulfate gels, two major bands were observed near the top (Figure 1a): the lower one corresponded to the heavy chain of myosin (200 000 daltons) and the upper one comigrated with purified filamin (250 000 daltons, see below). On the assumption that myosin and filamin

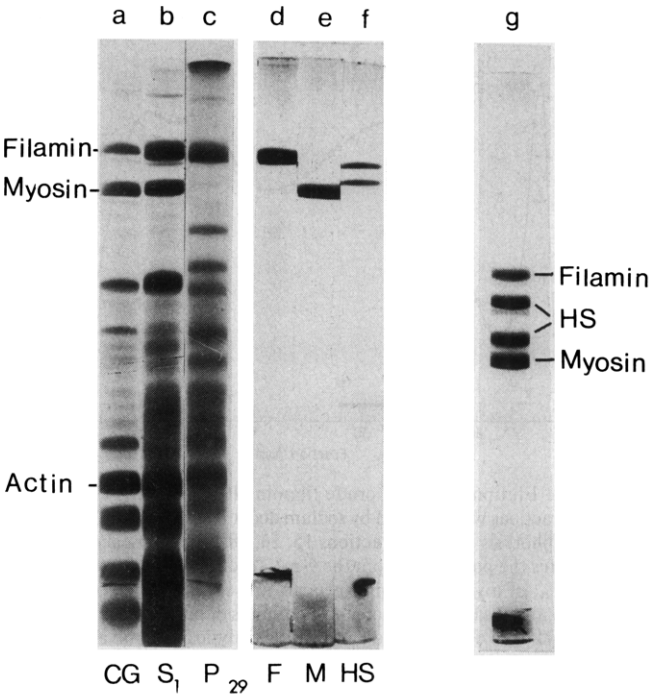

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of (a) chicken gizzard smooth muscle, CG (~100 μg); (b) salt extract of gizzard muscle, S_1 (~100 μg); (c) pellet of ammonium sulfate fractionation, $P_{0\text{-}29}$ (~75 μg); (d) purified gizzard filamin, F (~10 μg); (e) purified gizzard myosin, M (~10 μg); (f) purified human spectrin, HS (~5 μg); and (g) a mixture (5 μg of each) of filamin, spectrin, and myosin. Gel g was obtained by prolonged electrophoresis.

were the major components in these bands, it was estimated (Table I) from these gels in several experiments that filamin comprised 6–7% of the total muscle protein and represented 30–50% as much protein as myosin (the relative amount was variable for unknown reasons). It was found that myosin and filamin were extracted simultaneously by salt solutions near neutral pH. In the clear red extract, filamin was enriched relative to myosin. Only ~30% of total filamin and ~15% of total myosin was present in the extract. Attempts to reextract the residue produced an unworkable solution of gel-like consistency.

The ammonium sulfate fractionation step (29% saturation) quantitatively separated filamin from myosin (Figures 1b and c).[2]

Gel filtration on a 4% agarose column efficiently separated filamin from most contaminating smaller proteins. The elution profile and sodium dodecyl sulfate gels of fractions of the agarose column are shown in Figure 2. Sodium dodecyl sulfate gels of the cloudy void volume fractions showed the presence of a small amount of filamin, which appeared to be the leading edge of the major peak with $K_{av} = 0.34$. The peak fractions were pooled with care to exclude fractions (e.g., fraction 38 in Figure 2) containing a peptide with an apparent chain weight of 240 000 daltons (which moved slightly faster than filamin on sodium dodecyl sulfate gels). At this stage, pooled fractions

[2] When finely powdered ammonium sulfate was added slowly to a stirred protein solution, filamin precipitated as a slightly brown, firm, and rubbery pellet free of myosin. If myosin coprecipitated with filamin, a loose pellet was obtained. In this case, only half of the precipitated filamin and myosin was soluble in buffer A. However, subsequent purification steps removed myosin from filamin. Filamin was found sticky to glass surfaces; therefore, plastic beakers and centrifuge tubes were used in this and later stages of purification.

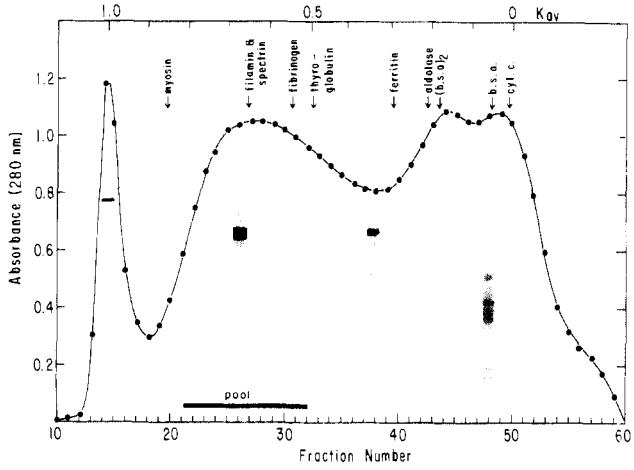

FIGURE 2. Elution profile of crude filamin ($P_{0\text{-}29}$) from a 4% agarose column. Fractions were assayed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (gels for fractions 15, 26, 38, and 45 are shown). The bar indicates the pool made from the 9-mL fractions. The partition coefficients, K_{av}, of myosin, filamin, spectrin, and several standard proteins (where bsa and $(bsa)_2$ are monomers and dimers of bovine serum albumin, respectively) were determined on a separate analytical column and plotted on the top portion of the figure. Details are given in the text.

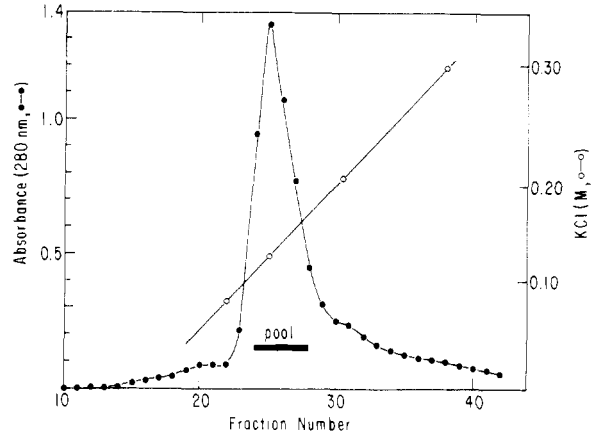

FIGURE 3: Elution profile of partially purified filamin from a DEAE-cellulose column. Proteins (●—●) were eluted with a linear salt gradient (O—O). The bar indicates the pool made from the 7-mL fractions. See the text for details.

were 85–90% pure, major contaminants being peptides of chain weight 120 000, 55 000, and 44 000 daltons. (The last peptide is presumably actin. Attempts to remove it, however, by dissolving the ammonium sulfate pellet and eluting the column with 0.6 M KI, failed to decrease the amount appreciably.) These contaminants were removed by the final ion-exchange chromatography step.

Filamin in the pooled fractions of the gel filtration step was recovered by precipitation with 50% saturated ammonium sulfate as described in the Experimental Procedure section, or dialyzed directly against the starting buffer (20 mM Tris-acetate (pH 7.6)) and then applied to a DE-52 column. A typical elution profile is shown in Figure 3. All the protein was bound to the column. Filamin was eluted as a major peak with a sharp leading edge. Fractions eluted between 0.10 and 0.14 M KCl were pooled. Myosin was eluted at 0.16 M KCl under this condition.

The final yield was about 50 mg of filamin from 100-g wet weight of smooth muscle. Major loss occurred during the first extraction and during the concentration of filamin solutions. The results of a typical purification experiment are shown in Table I.

Fresh filamin preparations showed only one band on sodium dodecyl sulfate gels (Figure 1d). Upon storage at 4 °C, a set of finely spaced bands with faster mobilities gradually appeared, presumably due to proteolysis. Storage at −20 °C in 50% glycerol prevented or greatly retarded this degradation.

Myosin. Myosin was recovered from the 29–55% saturated ammonium sulfate fraction of the extract (S_1) and was purified by the KI-ATP gel filtration method (Clarke and Spudich, 1974; Stossel and Pollard, 1973) and then by the DEAE-Sephadex method of Richards et al. (1967), as modified by Godfrey and Harrington (1970). The yield was ~15 mg from 100-g wet weight of gizzard muscle. The final preparation consisted of a heavy chain at 200 000 daltons and two light chains at 20 000 and 17 000 daltons, in agreement with previous results in the literature (Shoenberg and Needham, 1976). A minor band near 170 000 daltons was present at the amount of 1–2% of the total stain intensity. It could be a product of proteolysis of myosin heavy chain, or a protein impurity similar to the minor contaminates present in skeletal muscle myosin preparations (Offer, 1973). The calcium-activated ATPase activity of the gizzard myosin preparation is 0.09 μmol of P_i mg^{-1} min^{-1} at 25 °C (cf. Barany et al., 1966). No further characterization was made. This highly purified myosin was used as an antigen to prepare specific antibodies in rabbits.

Polypeptide Chain Weight of Filamin. The apparent chain weight of filamin was estimated from its relative mobility on 4% acrylamide–sodium dodecyl sulfate gels. As shown in Figure 1, filamin (F) had a lower mobility than the larger chain of human spectrin (HS). Extrapolation from the calibration straight line yielded an apparent chain weight of 250 000–255 000 daltons for filamin. Prolonged coelectrophoresis of a mixture of filamin, human spectrin, and gizzard myosin gave better separations of these large chains and confirmed this estimated value (Figure 1g). These values required an assumption that the empirical relationship of chain weight and electrophoretic mobility holds for these unusually large peptides. Independent molecular weight estimation by hydrodynamic measurements described below suggests that this value is probably within 10% of the actual chain weight.

Chemical Cross-linking of Filamin. The question of whether filamin is an oligomeric protein consisting of more than one subunit was approached by the chemical cross-linking method of Davies and Stark (1970). As shown in Figure 4, gels a and b, treatment of native filamin in high ionic strength buffer with the cross-linking reagent dimethyl adipimidate resulted in the gradual disappearance of the monomer band and the appearance of a new band corresponding in mobility to the dimer of filamin (430 000 daltons).[3] More than 70% of the original monomer band was cross-linked to this new band within 1 h, while little increase in the amount of the minor band with higher chain weight was detected. This result was independent of the protein concentration (from 0.1 to 2.0 mg/mL). For comparison, parallel cross-linking experiments were carried out on human spectrin under identical conditions. Similar qualitative results were obtained. As shown in Figure 4, gels c and d, the spectrin monomer doublet disappeared simultaneously, with the almost exclusive formation of a new band

[3] It has been observed that the apparent chain weight of a cross-linked complex is usually smaller than the sum of chain weights of its components (Wang and Richards, 1974).

TABLE I: Purification of Chicken Gizzard Filamin.[a]

Purification Step	Total Protein (mg)	Purity[b] (%)	Total Filamin[c] (mg)	Recovery[d] (%)	Purification (fold)
Muscle homogenate	15 000[e]	6	900	100	1
Salt extract (S_1)	3 375	8	270	30	1.3
Ammonium sulfate pellet (P_{0-29})	520	35	180	20	6
Agarose gel filtration (A15-m)	137	85	116	13	14
DEAE-cellulose chromatography (DE52)	45	99	44	5	16

[a] Results from a typical preparation using 100-g wet weight of smooth muscle. [b] Calculated from the relative stain intensity of protein peaks on sodium dodecyl sulfate gels. [c] Total protein × purity. [d] Based on the total filamin content in whole muscle. [e] The amount of sodium dodecyl sulfate (5%) soluble proteins of gizzard muscle was estimated to be 150 mg of protein per g wet weight. (A small amount of white connective and elastic tissue was not solubilized.)

having closely similar mobility to that of cross-linked filamin.

In control experiments in which the adipimidate reagent was added to sodium dodecyl sulfate denatured filamin and spectrin, no such changes were observed (data not shown).

These results, taken together, suggest that native filamin is a dimeric protein consisting of two subunits of identical size. These cross-linking data do not rule out the possible presence of small amounts of monomeric subunits or aggregates of dimers in filamin solution.

To determine if any interchain disulfide bond is present in the filamin dimer, filamin was alkylated with *N*-ethylmaleimide, denatured with sodium dodecyl sulfate, and analyzed on sodium dodecyl sulfate gels without reduction with thiols. As shown in Figure 4, gel e, unreduced samples gave one major band at 250 000 daltons and a small and variable amount of a band corresponding to the dimer of filamin subunit. This putative dimer band disappeared when samples were reduced with thiols. It therefore appears that no significant amount of interchain disulfide is present in native filamin. The small amount of dimer complex seen in unreduced samples presumably arose as a result of air oxidation of adjacent free thiols between subunits during the purification process.

The possible presence of such adjacent thiols was tested by treating native filamin with Cu^{2+}-(*O*-phenanthroline)$_2$ complex, a reagent which catalyzes the air oxidation of thiols to disulfides (Wang and Richards, 1974). As shown in Figure 4, gel f, such treatment of native filamin caused the nearly complete disappearance of the subunit band and generated a dimer band (~60% of the total stain intensity) plus some material which stayed near the top of the gels. If treated samples were reduced with thiols before analysis, no such pattern was observed, suggesting that these cross-linked complexes were disulfide linked. In control experiments where Cu^{2+}-(*O*-phenanthroline)$_2$ was added to filamin in the presence of EDTA (which destroys the catalytic activity of the reagent by chelating the copper ion), no cross-linking was detected (data not shown). Parallel oxidation experiments carried out on human spectrin resulted in the formation of cross-linked subunits (Figure 4, gels g and h), confirming an earlier report (Steck, 1972). These results suggest that filamin, as is the case with spectrin, has free thiols near its subunit interface and that these thiols are close enough to form disulfides upon oxidation.

These cross-linking experiments provided suggestive evidence for the subunit structure of filamin. Independent hydrodynamic measurements to be described below supported this conclusion. Together, these measurements have provided some interesting information on the size, shape, and flexibility of the molecule.

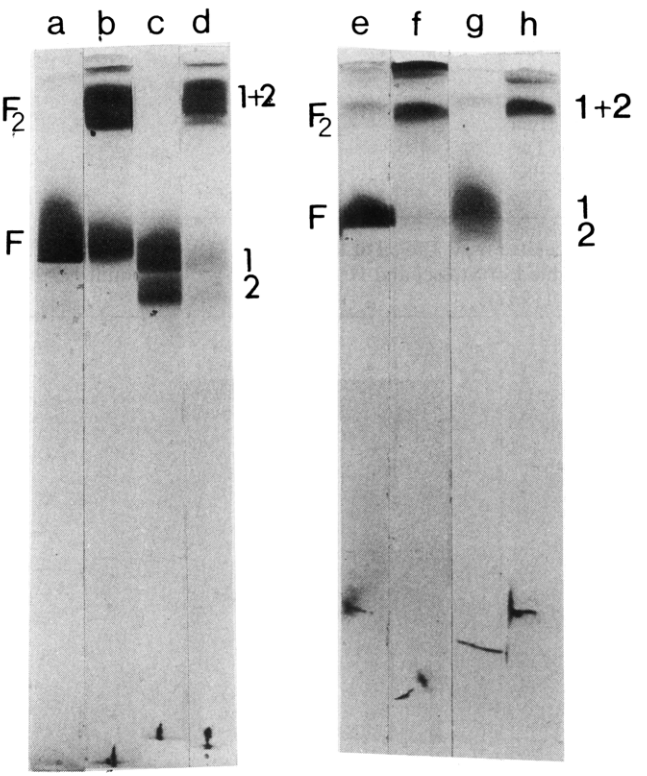

FIGURE 4: Chemical cross-linking of filamin and spectrin. Gels a and b: a solution of filamin at 0.2 mg/mL (gel a) was treated with dimethyl adipimidate dihydrochloride (2 mg/mL) at room temperature for 60 min (gel b). Samples were denatured, reduced, and analyzed on 4% acrylamide gels by prolonged electrophoresis. F: subunit of filamin, F_2: dimer complex of filamin. Gels c and d: A solution of spectrin at 0.2 mg/mL (gel c) was treated with dimethyl adipimidate dihydrochloride (2 mg/mL) at room temperature for 60 min (gel d). The doublet of spectrin is labeled as 1 and 2, and the cross-linked complex as 1 + 2. Gels e and g: filamin (gel e) and spectrin (gel g) were alkylated, denatured, and analyzed on 3.2% acrylamide gels. Thiols were omitted. Gels f and h: filamin (gel f) and spectrin (gel h) at 0.2 mg/mL were first treated at room temperature with Cu^{2+}-(*O*-phenanthroline)$_2$ (final concentration 50–100 μM) for 5 min and then alkylated, denatured, and analyzed on 3.2% acrylamide gels. Thiols were omitted.

Sedimentation Coefficient of Filamin. As shown in Figure 5, filamin in high salt buffer (buffer A) yielded a single symmetric Schlieren peak in sedimentation velocity experiments. Occasionally at high (~10 mg/mL) protein concentration traces of faster and slower sedimenting peaks were detected.

TABLE II: Amino Acid Composition of Chicken Gizzard Filamin; Comparison with Values Reported for Myosin, Spectrin, and Actin Binding Protein.

Amino Acid	Chicken Gizzard Filamin (mol %)	Chicken Gizzard Myosin[a] (mol %)	Human Erythrocyte Spectrin[b] (mol %)	Rabbit Macrophage Actin Binding Protein[c] (mol %)
Lys	4.8	9.9	6.6	6.0
His	2.3	1.6	3.0	2.2
Arg	5.5	5.4	5.8	4.1
Asp	7.6	10.0	9.4	8.7
Thr	5.3	5.0	4.6	6.2
Ser	6.8	5.3	5.0	6.8
Glu	9.5	18.7	15.8	11.4
Pro	8.0	2.8	3.3	7.1
Gly	12.5	5.4	5.8	11.8
Ala	9.3	8.6	8.0	7.4
½-Cystine	2.0[d]	1.1	0.54	0.46
Val	10.0	4.6	5.9	8.5
Met	0.8	2.3	1.8	1.3
Ile	3.3	4.1	3.4	4.4
Leu	6.0	9.8	13.9	6.2
Tyr	2.6	1.8	2.4	3.1
Phe	2.9	3.0	4.5	3.2
Trp	1.0[e]	0.74		1.2

[a] Calculated from Table I of Barany et al. (1966). [b] Fuller et al. (1974). Similar values were reported by Marchesi et al. (1970). [c] Calculated from Table I of Stossel and Hartwig (1976). [d] Determined as cysteic acid. [e] Determined spectrophotometrically according to Bencze and Schmidt (1957).

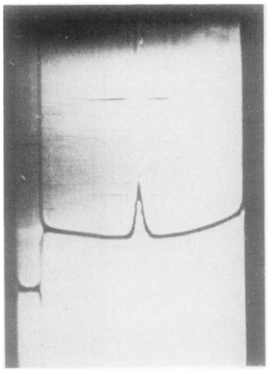

FIGURE 5: Sedimentation velocity pattern of filamin in buffer A. Schlieren pattern of filamin (3.0 mg/mL) at 48 min after reaching speed. Phase plate angle was 55 °C. See the text for details.

The sedimentation coefficients showed marked protein concentration dependence above 0.5 mg/mL as described by the equation $s_{20} = 9.6S - 0.57c$ where c is the protein concentration in mg per mL. At protein concentration below 0.5 mg/mL, the s_{20} seemed to become constant at 9.2 S. Because of this uncertainty of extrapolation, the s_{20}^0 value is given here as 9.4 ± 0.2 S ($s_{20,w}^0 = 10 \pm 0.3$ S).

Stokes' Radius of Filamin. The Stokes' radius of filamin was estimated by determining the elution volume of filamin on a 4% agarose gel filtration column calibrated by standard proteins of known Stokes' radii (see Experimental Procedure and Figure 2). A value of 120 ± 5 Å was obtained for native filamin in buffer A. In separate experiments, the Stokes' radii for chicken gizzard myosin and human spectrin were estimated to be 180 ± 5 and 120 ± 5 Å, respectively. It is interesting that spectrin, in contrast to myosin, was eluted in a position indistinguishable from that of filamin. The value for gizzard myosin is in reasonable agreement with those (170 to 190 Å) reported for other myosins (Pollard and Weihing, 1974).

Molecular Weight and Subunit Structure of Filamin. An approximate molecular weight of 480 000 to 520 000 daltons for native filamin was calculated from independently measured values of sedimentation coefficient and Stokes' radius by the method of Siegel and Monty (1966).

The molecular weight estimated by these hydrodynamic measurements is in good agreement with that independently derived from the subunit composition, deduced from chemical cross-linking data, and the subunit chain weight, estimated by sodium dodecyl sulfate gel electrophoresis (2 × 250 000 to 2 × 255 000).

These data, taken together, strongly suggest that native filamin is a dimer consisting of two subunits of identical size.

Frictional Ratio of Filamin. The frictional ratio of filamin, a parameter related to the shape and flexibility of the protein hydrodynamic particle, was calculated from the molecular weight and the Stokes' radius according to Siegel and Monty (1966). A value of 2.2 to 2.3 was obtained.

Amino Acid Composition. Table II shows the amino acid composition of purified filamin. The compositions of a number of high-molecular-weight proteins are also included for comparison. No amino sugar or unusual amino acids characteristic of actin, myosin, and collagen such as *N*-methyllysine, *N*-methylhistidine, or hydroxyproline could be detected. The free thiol content of 66 mol/mol (1.5 mol %) is similar to the total cysteic acid content of oxidized samples (2.0 mol %), suggesting that the cystine content is zero or low.

The composition data suggest that filamin is not a glycoprotein (no carbohydrate could be detected on sodium dodecyl sulfate gels by periodate–Schiff base stain) and indicate that filamin has a composition distinct from that of gizzard myosin or human spectrin.

UV Spectrum. Filamin yielded a typical protein UV spectrum with λ_{max} at 277 nm and no absorption above 300 nm. No evidence could be detected in its spectrum to suggest the

presence of nucleotide or prosthetic groups such as flavin or NAD.

Enzyme Activity. Filamin has no detectable myosin-like ATPase activities (K^+-EDTA ATPase < 0.002 μmol of P_i mg^{-1} min^{-1} at 37 °C), nor actin-stimulated ATPase activities. These data also indicate that the filamin preparation is essentially free of myosin. No effort was made to search for other possible enzyme activities. It should be noted that filamin is purified as a protein and assayed only by sodium dodecyl sulfate gels. It is possible that intrinsic enzyme activities have lost during purification. However, an important functional property is at least partially preserved since the filamin preparation is capable of interacting with actin (Wang and Singer, 1977).

Discussion

Purity of Filamin Preparation. The following criteria were used to establish the purity of the filamin preparation: (a) On sodium dodecyl sulfate gels, a single band was observed at various sample loadings (from 0.5 to 25 μg) and under prolonged electrophoresis conditions (Figure 1d). (b) In sedimentation velocity experiments, a single symmetric peak throughout the run was observed for filamin in high ionic strength medium (Figure 5). (c) In double immunodiffusion tests, a single precipitin line was observed when specific antibody directed against filamin was tested against both purified filamin and the salt extract of gizzard muscle, suggesting its immunological purity (Wang et al., 1975).

Analysis of the purity of native filamin by electrophoresis or isoelectric focusing was inconclusive since filamin tended to aggregate to a variable extent under most electrophoresis and isoelectric focusing conditions (see below). Frequently, filamin did not enter the gels; occasionally it did, but the mobility toward the cathode under alkaline pHs was extremely low and multiple bands were observed (unpublished observation).

Molecular Properties of Filamin. A systematic study of the solubility properties of filamin has not yet been made. Freshly prepared filamin was found to be soluble in solutions containing 0.002 M KCl to 0.8 M KCl, from pH 6.2 to 10.0. Addition of up to 15 mM of Ca^{2+} or Mg^{2+} did not cause any visible precipitation. In preliminary experiments, it was observed that filamin was soluble but aggregated at physiological ionic strength as revealed by multiple boundaries of high sedimentation coefficients in sedimentation velocity experiments (unpublished observation). Occasionally opalescence was observed in filamin solutions of lower ionic strength after prolonged storage at 4 °C, suggesting an even higher extent of aggregation of filamin. Therefore, high ionic strength buffers in which filamin is monodisperse were used in most of the purification steps and in hydrodynamic characterizations to simplify interpretation of results.

The conclusion that native filamin is a dimeric protein consisting of two large subunits with identical size was supported by both chemical cross-linking data and hydrodynamic measurements. Interestingly, its subunits are not linked together by interchain disulfide, but there are adjacent thiols on each subunit that could easily be oxidized to such disulfides.

The hydrodynamic data also indicate that filamin is an asymmetric molecule: the low and markedly concentration-dependent sedimentation coefficient (10 S), the large Stokes' radius (120 Å), and high frictional ratio (2.2) are all consistent with such a view. The sedimentation coefficient is between that of cold insoluble globulin, a more or less globular protein of similar molecular weight (14 S) (Mosher, 1975) and that of the rod-like myosin (6.1 S) (Barany et al., 1966). The frictional ratio is much higher than those of most globular proteins (with values from 1.1 to 1.3), smaller than those of rod-like molecules such as tropomyosin (3.2) and myosin (3.5) (Tanford, 1961), but similar to those of fibrinogen (2.3) (Doolittle, 1975) and human spectrin (2.1–2.2) (Gratzer and Beaven, 1975). Since the frictional ratio is determined by both shape and solvation factors, no quantitative conclusion about the hydrodynamic shape of the filamin molecule can be made without further independent measurements. However, by assuming a reasonable hydration value of 0.2 g/g and a prolate ellipsoid shape, an axial ratio of 13–15 was calculated (Tanford, 1961). Alternatively, the large frictional ratio suggests a certain degree of flexibility of the filamin molecule.

Comparison of Filamin with Myosin and Spectrin. Because of the unusually high molecular weight of filamin, efforts were made to explore possible relationships of filamin to other proteins of similar size, in particular, to myosin and spectrin. The comparison was considered essential to establish an identity for filamin. For example, it is possible that a peptide band of 250 000 daltons on sodium dodecyl sulfate gels could be an artifact resulting from the incomplete dissociation of aggregated peptides (e.g., of one myosin heavy chain plus several light chains or plus one actin). Furthermore, unusually large heavy chains of myosins (220 000 to 250 000 daltons) have been reported (Nachmias, 1973; Clarke and Spudich, 1974).

(1) Comparison with Myosin. The chemical, physical, enzymatic, and immunological evidence available so far strongly suggests that filamin and myosin are distinct proteins. Aside from the larger chain size, no peptides near 20 000 daltons corresponding to the light chains of myosin could be detected in filamin. Amino acid compositions differ and no methylated amino acid was found in filamin. Structurally, although both are oligomers containing two large peptide chains and of similar molecular weights, filamin behaves quite differently from myosin in solutions of high ionic strength: it has a sedimentation coefficient of 10 S and a frictional ratio of 2.2. In contrast, myosin has an s value of 6.1 and a frictional ratio of 3.5 (Tanford, 1961). Thus, filamin is not as asymmetric and/or rigid as myosin. Furthermore filamin has no myosin-like ATPase activities, when purified under conditions where myosin retains its enzyme activities. The tendency to aggregate at low ionic strength is similar; however, no myosin-like bipolar filaments have been observed for filamin under such conditions. Finally, filamin and myosin are antigenically distinct: antifilamin antibodies do not cross-react with myosin, nor do antimyosin antibodies cross-react with filamin (Wang et al., 1975).

(2) Comparison with Spectrin. In contrast to the differences between myosin and filamin, the similarities between gizzard filamin and human spectrin are indeed striking.[4] Aside from their similar but nonidentical chain weights, they both are oligomers of two subunits. They both lack interchain disulfides but have adjacent free thiols near the subunit interface. Their hydrodynamic properties are indistinguishable at the present resolution. Thus, they both have very similar sedimentation coefficients (a range of 8.7 to 10.2 has been reported for human spectrin), Stokes' radii (120 Å), and frictional ratios (2.2). These data, taken together, strongly suggest that filamin and spectrin have similar size, shape, and flexibility. Neither fi-

[4] Although conflicting data exist in the literature as to the solubility and hydrodynamic properties of human spectrin (for review, see Kirkpatrick, 1976), we found that spectrin purified from the included volume of 4% agarose column is soluble in both high and low ionic strength solutions.

lamin nor spectrin have detectable myosin-like ATPase activities (however, see Kirkpatrick, 1976). The aggregation property of spectrin is still controversial; therefore, meaningful comparison with filamin in this regard is not yet possible. It is important to emphasize that, despite these similarities, filamin and spectrin appear to be chemically distinct, as their amino acid compositions differ and their subunits are clearly distinguishable on sodium dodecyl sulfate gels. Species difference (chicken filamin vs. human spectrin) might contribute to some of the differences discussed above. Unfortunately, purified chicken erythrocyte spectrin is not yet available for direct comparison. It was observed, however, that chicken erythrocytes contained no peptide of the size of filamin, and that chicken spectrin comigrated with human spectrin on sodium dodecyl sulfate gels (unpublished observation). Immunological tests have so far failed to detect any cross-reactivity between filamin and spectrin (to be published).

Elsewhere we will present immunological evidence that filamin does not cross-react with a large number of known contractile proteins and other high-molecular-weight proteins such as cold insoluble globulin (Mosher, 1975) and microtubule associated protein (Shelanski et al., 1973).

Possible Functions of Filamin. The function of filamin remains to be established. A detailed study of the interaction of filamin with other contractile proteins in vitro and of the localization of filamin in smooth muscle and nonmuscle cells in vivo is in progress. Available evidence (to be published) supports the idea that filamin is a regulatory contractile protein which might play a role in the assembly of the actomyosin system in cells. Briefly (1) filamin is not present in the blood plasma and striated muscle; (2) filamin is localized intracellularly in smooth muscle and nonmuscle cells as a component of the filamentous system containing actin and myosin (Wang et al., 1975); (3) purified filamin interacts with and aggregates actin at physiological ionic strength (Wang and Singer, 1977); and (4) filamin is associated ultrastructurally with plasma membranes of several tissue culture fibroblasts (to be published).

The absence of filamin in striated muscle suggests that filamin is not essential for the basic sliding filament mechanism of contractile systems (Huxley, 1969). The membrane association of filamin raises the intriguing possibility that filamin and spectrin share not only some molecular properties, but also similar roles in the structure and function of biological membranes (Singer, 1974). In this connection, it is significant that spectrin also interacts with actin (Tilney and Detmers, 1975; Pinder et al., 1975).

Related Studies. While this work was in progress, several papers appeared reporting the detection of high-molecular-weight proteins in chicken gizzard smooth muscle (Sobieszek and Bremel, 1975; Sobieszek and Small, 1976) and in extracts of sea urchin eggs (Kane, 1975), sperms (Tilney, 1976), rabbit macrophages (Hartwig and Stossel, 1975; Stossel and Hartwig, 1976), amebas (Pollard, 1976; Taylor et al., 1976), and human fibroblasts (Weihing, 1976).

Hartwig and Stossel purified from rabbit macrophages a high-molecular-weight protein which binds and aggregates actin and have therefore designated it as actin-binding protein (Hartwig and Stossel, 1975). Its reported size (220 000 daltons) and physical properties were distinct from those of filamin described in this work. However, they have recently published an improved purification procedure, revised the chain weight to 280 000 daltons, and reported its amino acid composition (Stossel and Hartwig, 1976). It is of great interest that the amino acid compositions of actin binding protein and filamin are strikingly similar (Table II) and that both proteins interact with actin. It appears likely that actin binding protein may be a member of the family of cross-reacting proteins that we have previously detected in a wide range of nonmuscle cells using antifilamin antibody (Wang et al., 1975). If true, then the similarity of amino acid compositions would strongly support our proposal that filamin-like molecules are chemically conserved (Wang et al., 1975). Further work is necessary to establish the relationship between filamin, actin binding protein, and other reported high-molecular-weight proteins.

Note Added in Proof

After the submission of this manuscript, a paper appeared by Shizuta et al. (1976) on the isolation and characterization of chicken gizzard filamin.

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Investigation of the Aggregation and Activation of Prothrombin Using Quasi-Elastic Light Scattering†

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ABSTRACT: The technique of quasi-elastic light scattering was used to measure the translational diffusion coefficient, D, of purified human prothrombin in buffered aqueous solutions and to monitor for the first time the fragmentation of this protein as it is converted to thrombin. The values of $D_{20,w}$, measured at two different concentrations, are 4.72×10^{-7} cm^2/s at 2 mg/cm^3 and 4.51×10^{-7} cm^2/s at 5 mg/cm^3; the corresponding molecular weights ($\overline{M}_w$ of 92 000 and 120 000), obtained by combining sedimentation velocity measurements with the diffusion data, confirm the presence of molecular aggregates of prothrombin in these solutions. These results, as well as analysis of the intensity-intensity autocorrelation functions from two-component systems with various dimer conformations, indicate the presence of end-to-end dimers in these prothrombin solutions. The values obtained for D indicate a dimer weight fraction of 0.4 to 0.5 in the 2 mg/cm^3 solution and 0.6 or greater in the 5 mg/cm^3 solution. The fragmentation of prothrombin was monitored in a nonphysiologic activation system, containing taipan snake venom, dihexanoylphosphatidylcholine, and $CaCl_2$. At a temperature of 15 °C, conversion to thrombin proceeded very slowly and was still incomplete after 90 h. A method for determining the percentage of converted prothrombin is an activated system containing aggregates from the average value of D and light scattering data is discussed.

The physical properties of prothrombin (factor II) and its conversion to thrombin (factor IIa) have been investigated extensively during the past few years. Most preparations of this protein have shown a tendency to form aggregates in various solution environments. Cox and Hanahan (1970) observed that bovine prothrombin dimerized at concentrations less than 4 mg/cm^3 and formed larger aggregates at higher concentrations. They also found from diffusion and sedimentation studies that the frictional ratio of the protein increased with concentration and degree of aggregation and that the aggregated form was more asymmetric than its monomer. Similarly, Kisiel and Hanahan (1973) have shown that the weight-average molecular weight of human prothrombin in solutions of moderately high salt concentrations was also a function of pH. To date,

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