Self-Association of Chicken Gizzard Filamin and Heavy Merofilamin†

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ABSTRACT: Filamin is a high molecular weight (subunit M_r 250 000) actin-binding protein isolated from smooth muscle. The protein forms a gel when mixed with solutions of F-actin. A proteolytic fragment of filamin, heavy merofilamin (subunit M_r 240 000), generated by the action of Ca^{2+}-activated protease binds to actin but does not produce gelation. We have studied the self-association properties of filamin and heavy merofilamin by direct examination in the electron microscope and by equilibrium sedimentation distribution studies in the ultracentrifuge. Filamin self-associates reversibly to form dimers; the free energy of dimerization is approximately 7 kcal/mol. Further association to form tetramer and multimer appears to be irreversible. Warming of filamin solutions accelerates aggregation. Heavy merofilamin does not appear to self-associate but is entirely monomeric. These studies suggest that filamin produces gelation of F-actin by binding to actin and then self-associating to cross-link actin filaments into a gel.

Filamin is a high molecular weight actin-binding protein first isolated from smooth muscle (Wang et al., 1975; Shizuta et al., 1976; Wang, 1977) and later found to be present in a variety of other tissues and cell types (Wallach et al., 1978). Filamin is very similar to a macrophage actin-binding protein (ABP)[1] identified by Stossel and his colleagues (Hartwig & Stossel, 1975). The precise role that filamin and ABP play in cell function remains to be elucidated, but the protein shows a great tendency to bind to and cross-link F-actin (Stossel & Hartwig, 1976a; Shizuta et al., 1976; Wang & Singer, 1977; Davies et al., 1978). The cross-linking of F-actin filaments produces a mechanically stable gel, and this structure may play a role in the motility of nonmuscle cells (Stossel & Hartwig, 1976b; Korn, 1978). The study of the interactions of actin and filamin has been complicated by the gelation of solutions and production of insoluble aggregates. To simplify the problem we digested chicken gizzard filamin with purified Ca^{2+}-activated protease and obtained two fragments, heavy merofilamin (M_r 240 000) and light merofilamin (M_r 10 000) (Davies et al., 1978). Heavy merofilamin bound to F-actin but produced no gelation. Also, heavy merofilamin, like native filamin, inhibited actin activation of myosin ATPase (Davies et al., 1977a, 1978).

The studies in this report were undertaken to compare the self-association properties of chicken gizzard filamin and heavy merofilamin. We find that chicken gizzard filamin exists in solution predominantly as a dimer and these solutions progressively accumulate irreversible aggregates. Heavy merofilamin, in contrast, exists predominantly as a monomer with no evidence of aggregation. These studies have led to the conclusion that self-association and aggregation of filamin bound to actin plays a critical role in the gelation of actin by filamin.

Experimental Procedures

Materials

Ca^{2+}-activated protease purified from porcine skeletal muscle prepared by the method of Dayton et al. (1976) was a generous gift from Drs. Robson and Yamaguchi, Iowa State University.

Methods

Preparation of Filamin and Heavy Merofilamin. Filamin was purified from fresh chicken gizzard according to the procedure of Shizuta et al. (1976), with previously described modifications (Davies et al., 1977b). Solutions of filamin were stored at 0–4 °C in 50 mM potassium phosphate, pH 7.5, 100 mM KCl, 1 mM EDTA, and 1 mM DTT.

To prepare heavy merofilamin, we dialyzed chicken gizzard filamin (7.3 mg/mL) for 6 h against 20 mM imidazole hydrochloride, pH 7.5, 100 mM KCl, 1 mM EDTA, and 1 mM DTT. One milliliter of the filamin solution was treated for 30 min at 23 °C with 73 μg of Ca^{2+}-activated protease (CAF) in the presence of 5 mM Ca^{2+}, and the reaction was stopped by addition of EGTA to a final concentration of 10 mM. The sample was then passed over a 0.9 × 30 cm column of Sephadex G-100 to separate heavy and light merofilamin. The void volume fraction containing heavy merofilamin was redigested for 30 min at 23 °C with 40 μg of CAF. After the second digestion the purity of the heavy merofilamin was checked by $NaDodSO_4$ gel electrophoresis and was greater than 95%. The major contaminant was a small amount of undigested filamin. The concentration of KCl was then adjusted to 0.6 M with 2 M KCl, and the sample was dialyzed for 24 h at 5 °C against 50 mM potassium phosphate, pH 7.5, 600 mM KCl, 1 mM EDTA, and 1 mM DTT.

Electron Microscopy of Filamin and Heavy Merofilamin. Freshly made formvar-coated copper 200-mesh grids were glow discharged. Solutions of proteins (5–50 μg/mL) in buffer were added to the surface of the formvar and treated either with 1% glutaraldehyde (Tousimis) in water, followed by 3% phosphotungstic acid (Fisher), or with 1% uranyl acetate (EM Sciences) in water for 1 min each at 23 °C. After being fixed and stained, the samples were air-dried. Observations were performed by using a Hitachi HU-12A electron microscope, and photographs were made at 75 kV by using a 20-μm objective aperture.

Ultracentrifuge Studies. (1) Procedures. Sedimentation–equilibrium experiments were performed in a Beckman Model E analytical ultracentrifuge which had been modified by the addition of a multiplexed He–Ne laser to the Rayleigh in-

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[1] Abbreviations used: ABP, actin-binding protein; CAF, Ca^{2+}-activated protease; DTT, dithiothreitol; rms, root mean square.

terference optical system. The optical system used Kodak 3414 high-resolution aerial photography film in a motor-driven 70-mm magazine to record the data. The individual interference patterns were measured on a Nikon 6C comparator equipped with a digital readout and with a fringe-locating device which permitted a reproducibility of ±0.002 mm on the y axis. The recorded x–y coordinates were transformed to radii and concentrations in the centrifuge cells. These concentration distributions were then analyzed by fitting appropriate mathematical models by using the nonlinear least-squares curve-fitting procedure available through the MLAB program operating on a DEC-10 computer.

(2) *Analysis of Data.* Since filamin is a large protein, subunit molecular weight 250 000, and polymers of filamin sedimented very rapidly, it was necessary to centrifuge filamin solutions to equilibrium at low rotor speeds. The selection of a rotor speed that did not produce an excessively steep gradient of tetramer near the bottom of the solution column resulted in significant concentrations of monomer and dimer at the meniscus. Since some small, but significant loss of mass was sometimes observed during centrifugation runs, conservation of mass calculations was impractical. The meniscus concentration was therefore determined during the curve-fitting procedure. For convenience, we have defined a concentration distribution function of radial position $G_i(r)$ as

$$G_i(r) = \exp[iAM_r(r^2 - r_b^2)] - \exp[iAM_r(r_m^2 - r_b^2)] \quad (1)$$

where i has the value of 1, 2, or 4 for monomer, dimer, and tetramer, respectively. M_r is the monomer molecular weight (250 000), r_m and r_b are the radii of the solution column meniscus and bottom, respectively, and $A = (1 - \bar{v}\rho_0)\omega^2/2RT$.[2] It was assumed that the value of $\bar{v}$, calculated from the amino acid composition, was the same for all species present. The second exponential term in $G_i(r)$ reflects the fact that we actually measured and fit the difference between the true concentration (c_r) and the meniscus concentration (c_m). If the quantity measured is denoted c_r', then

$$c_r' = c_r - c_m = c_{b,1}G_1(r) + c_{b,2}G_2(r) + c_{b,4}G_4(r) \quad (2)$$

where $c_{b,1}$, $c_{b,2}$, and $c_{b,4}$ are, respectively, the concentrations of monomer, dimer, and tetramer at $r = r_b$. This equation is valid regardless of whether we are dealing with an associating or a nonassociating system. The first model we tested, model I, assumed that there was a rapidly reversible equilibrium, i.e.

$$4F_1 \underset{}{\overset{K_{12}}{\rightleftharpoons}} 2F_2 \overset{K_{24}}{\rightleftharpoons} F_4 \qquad \text{(model I)}$$

where F_1, F_2, and F_4 represent the monomer, dimer, and tetramer of filamin, respectively. The equilibrium constants K_{12} and K_{24} are defined as

$$K_{12} = [F_2]/[F_1]^2 \qquad K_{24} = [F_4]/[F_2]^2 \quad (3)$$

where $[F_1]$, $[F_2]$, and $[F_4]$ are the molar concentrations of monomer, dimer, and tetramer, respectively. For this model, the equilibrium constants k_{12} and k_{24} are given by

$$k_{12} = c_{r,2}/c_{r,1}^2 \quad (4)$$

$$k_{24} = c_{r,4}/c_{r,2}^2 = c_{r,4}/k_{12}^2c_{r,1}^4 \quad (5)$$

or

$$k_{14} = c_{r,4}/c_{r,1}^4 \quad (6)$$

Thus, at $r = r_b$

$$c_{b,2} = k_{12}c_{b,1}^2 \quad (7)$$

$$c_{b,4} = k_{24}k_{12}^2c_{b,1}^4 = k_{14}c_{b,1}^4 \quad (8)$$

Substitution in eq 2 gives

$$c_r' = c_{b,1}G_1(r) + k_{12}c_{b,1}^2G_2(r) + k_{14}c_{b,1}^4G_4(r) \quad (9)$$

where $c_{b,1}$, k_{12}, and k_{14} are now used as fitting parameters to be varied in the nonlinear least-squares curve-fitting procedure. Only the values of $c_{b,1}$ were unique to each cell; the values of k_{12} and k_{14} applied to all three cells in an experiment. k_{24} is determined by k_{12} and k_{14}. The equilibrium constants are on an arbitrary weight/volume scale (fringes) and may be readily converted to molar equilibrium constants. Since the concentration in grams per liter is obtained by dividing the concentration (in fringes) by the specific refractive increment (SRI = 3.42 fringes L/g at 633 nm), then

$$K_{12} = (\text{SRI} \times M_r/2)k_{12} \quad (10)$$

$$K_{24} = (\text{SRI} \times M_r)k_{24} \quad (11)$$

and the values for the change in standard free energy were calculated by using

$$\Delta G^\circ = -RT \ln K \quad (12)$$

The second model tested presumed that reversible equilibrium was not present and fixed proportions of monomer, dimer, and tetramer were present, i.e.

$$F_1 + F_2 + F_4 \qquad \text{(model II)}$$

The relationship among these species may be described by

$$R_{12} = [F_2]/[F_1] \qquad R_{24} = [F_4]/[F_2] \quad (13)$$

where the R's represent molar ratios of the species. For this model, the initial concentrations of each component prior to distribution in the centrifugal field will be in fixed ratios regardless of total concentration. This may be expressed as

$$c_{0,t} = c_{0,1} + R_{12}c_{0,1} + R_{14}c_{0,1} \quad (14)$$

where $c_{0,t}$ is the total initial concentration, $c_{0,1}$ is the initial concentration of monomer, and R_{12} and R_{14} are the weight ratios of dimer to monomer and tetramer to monomer, respectively.

At equilibrium, the initial concentration of any species may be expressed in terms of the concentration at the cell bottom. Conservation of mass requires that

$$\int_{r_m}^{r_b} c_{0,i}\,dr^2 = \int_{r_m}^{r_b} c_{r,i}\,dr^2 = \int_{r_m}^{r_b} c_{b,i}\exp[AM_{r,i}(r^2 - r_b^2)]\,dr^2 \quad (15)$$

and thus

$$c_{0,i}(r_b^2 - r_m^2) = c_{b,i}[1 - \exp[AM_{r,i}(r_m^2 - r^2)]]/(AM_{r,i}) \quad (16)$$

Defining $\Delta r^2 = r_b^2 - r_m^2$ and $M_{r,i} = iM_r$, eq 16 gives

$$c_{b,i} = c_{0,i}iAM_r\Delta r^2/[1 - \exp(-iAM_r\Delta r^2)] \quad (17)$$

Using eq 17, defining $X_i = iAM_r\Delta r^2$, and using the definitions of R_{12} and R_{14} in eq 14 gives

$$c_r' = c_r - c_m = c_{0,1}X_1G_1(r)/[1 - \exp(-X_1)] + R_{12}c_{0,1}X_2G_2(r)/[1 - \exp(-X_2)] + R_{14}c_{0,1}X_4G_4(r)/[1 - \exp(-X_4)] \quad (18)$$

where $c_{0,1}$, R_{12}, and R_{14} are now used as fitting parameters to be varied in the nonlinear least-squares curve-fitting procedure. The values of $c_{0,1}$ were unique for each cell; the values

[2] $\bar{v}$ = partial specific volume (0.734); ρ_0 = solvent density; ω = rotor angular velocity (radians per second), R = gas constant; T = absolute temperature.

of R_{12} and R_{14} applied to all three cells in an experiment.

The third model we tested assumed a rapid and reversible equilibrium between monomer and dimer and an irreversible conversion of dimer to tetramer

$$4F_1 \rightleftharpoons 2F_2 \rightarrow F_4 \qquad \text{(model III)}$$

which may then be described by

$$c_r = c_{b,1}G_1(r) + k_{12}c_{b,1}^2G_2(r) + c_{b,4}G_4(r) \qquad (19)$$

where $c_{b,4}$ is the concentration of tetramer at $r = r_b$, and the values of the parameters $c_{b,1}$ and $c_{b,4}$ are unique to each cell and only the value of k_{12} is common to all three cells in an experiment.

Three criteria were used in evaluating the quality of fit of a given model. First, the parameters obtained in fitting had to be physically meaningful, i.e., having nonnegative values. Second, a minimum value of the root mean square (rms) error denoted a superior fit. Since the rms error obtained by using MLAB takes the number of degress of freedom of a model into account, the comparison of rms values for models having differing numbers of parameters is valid. The third criterion used was a qualitative evaluation of the deviation of experimental data points from the fitting line. If two models satisfy the first criterion and have essentially equal rms errors, a lesser degree of systematic deviation can become an important selection criterion.

Results

Comparison of Filamin and Heavy Merofilamin. (*1*) *Electron Microscopic Studies.* Negatively stained images of filamin solutions show predominantly amorphous aggregates of protein (Figure 1A). Using very dilute solutions of filamin, it is possible to discern individual globular protein molecules of approximately 170–180-Å diameter (Figure 1B). Negatively stained solutions of heavy merofilamin, on the other hand, contain much smaller globular protein molecules of 80–85-Å diameter (Figure 1C). This discrete pattern is maintained even at high concentrations of protein; the heavy merofilamin in Figure 1C is at the same concentration as the filamin in Figure 1A (50 μg/mL) and 10 times more concentrated than in Figure 1B. These studies show that heavy merofilamin aggregates less than filamin.

The low contrast, poorly defined appearance of filamin aggregates and separated filamin elements shown in Figure 1A,B is characteristic of this protein, since in parallel experiments glutamine synthetase and ferritin gave characteristic crisp well-defined images (results not shown). Filamin has this same appearance when examined by other negative-stain procedures such as uranyl acetate with or without fixation in glutaraldehyde. Titration of filamin solutions to lower concentrations did not show a corresponding linear decrease in the amount of protein visualized by negative staining until a concentration of 1–5 μg/mL was reached. At this point, small islands of protein similar to those seen in Figure 1B were found. This suggests that filamin sticks to the hydrophilic formvar surface. Single angle or rotary shadowing of filamin also showed no discernible image, suggesting that the filamin molecules collapse on drying. This is also likely from the negative-stained images and makes accurate calculations of molecular size from these images impossible.

Previously, the electron microscopic image of a negatively stained preparation of a protein similar to filamin (macrophage actin-binding protein) was published (Stossel & Hartwig, 1976a). Our data indicate that under the conditions of those experiments, filamin would have a low contrast, amorphous appearance which would be difficult to see. The images shown

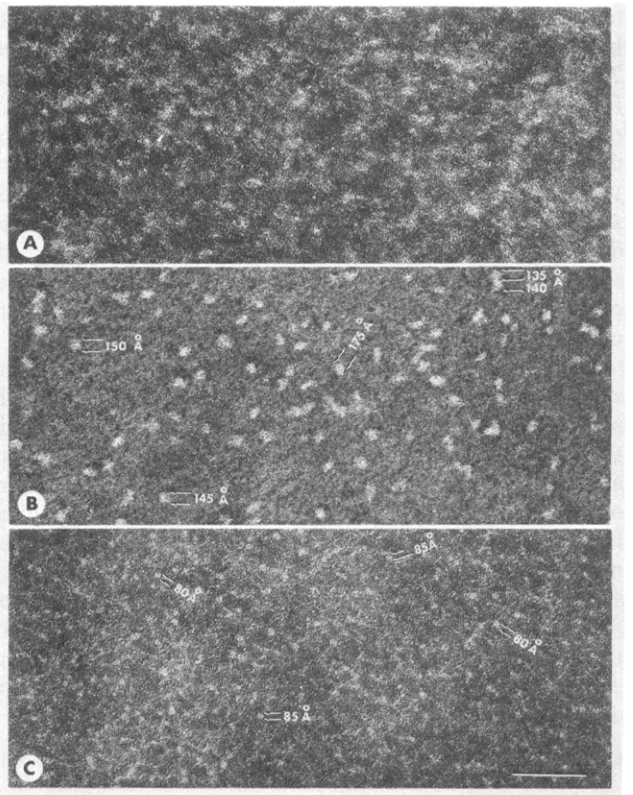

FIGURE 1: Negatively stained electron microscopic appearance of filamin and heavy merofilamin. (A) 50 μg/mL filamin (glutaraldehyde–phosphotungstic acid); (B) 5 μg/mL filamin (uranyl acetate); (C) 50 μg/mL heavy merofilamin (glutaraldehyde–phosphotungstic acid). (All were 98 550× magnifications; bar = 0.1 μm.)

were of a "120-Å diameter hollow cylindrical molecule" almost identical with negative stained images of ferritin (results not shown). We have never seen any structures of this type in our filamin preparations.

(*2*) *Ultracentrifuge Studies.* To examine the difference between filamin and heavy merofilamin in a quantitative manner, we compared the sedimentation equilibrium distribution of the two proteins. As will be discussed under Studies on the Self-Association of Filamin in more detail, it is only feasible to characterize the self-association of filamin in solutions of high ionic strength. At low salt concentrations filamin aggregates progressively during the course of the ultracentrifuge run, and the system cannot be quantitatively analyzed. In Figure 2A,C the distribution of proteins at equilibrium is shown by the open circles. The solid line in each figure is the "best fit" line obtained as described under Methods. In the case of the heavy merofilamin (Figure 2A), the experimental data are well fitted assuming 96.6% monomer ($M_r = 2.4 \times 10^5$) plus a small amount (3.4%) of high molecular weight aggregate ($M_r = 1.3 \times 10^6$). Figure 2B is the difference plot showing the deviation of the experimental data from the fitting curve. The low magnitude of the rms error, 0.024 fringes, and the random distribution of the experimental points about the calculated curve indicate a good fit for this model of heavy merofilamin. Warming heavy merofilamin to 10 °C did not perturb the equilibrium sedimentation distribution, 97.6% monomer and 2.3% aggregate.

Solutions of native filamin are polydisperse. Under Studies on the Self-Association of Filamin, we will discuss in more detail the self-association properties of filamin, but Figure 2C,D presents data from a sample of filamin prepared and run in parallel with heavy merofilamin. The data in Figure 2C are well fitted assuming the filamin solution contains a mixture

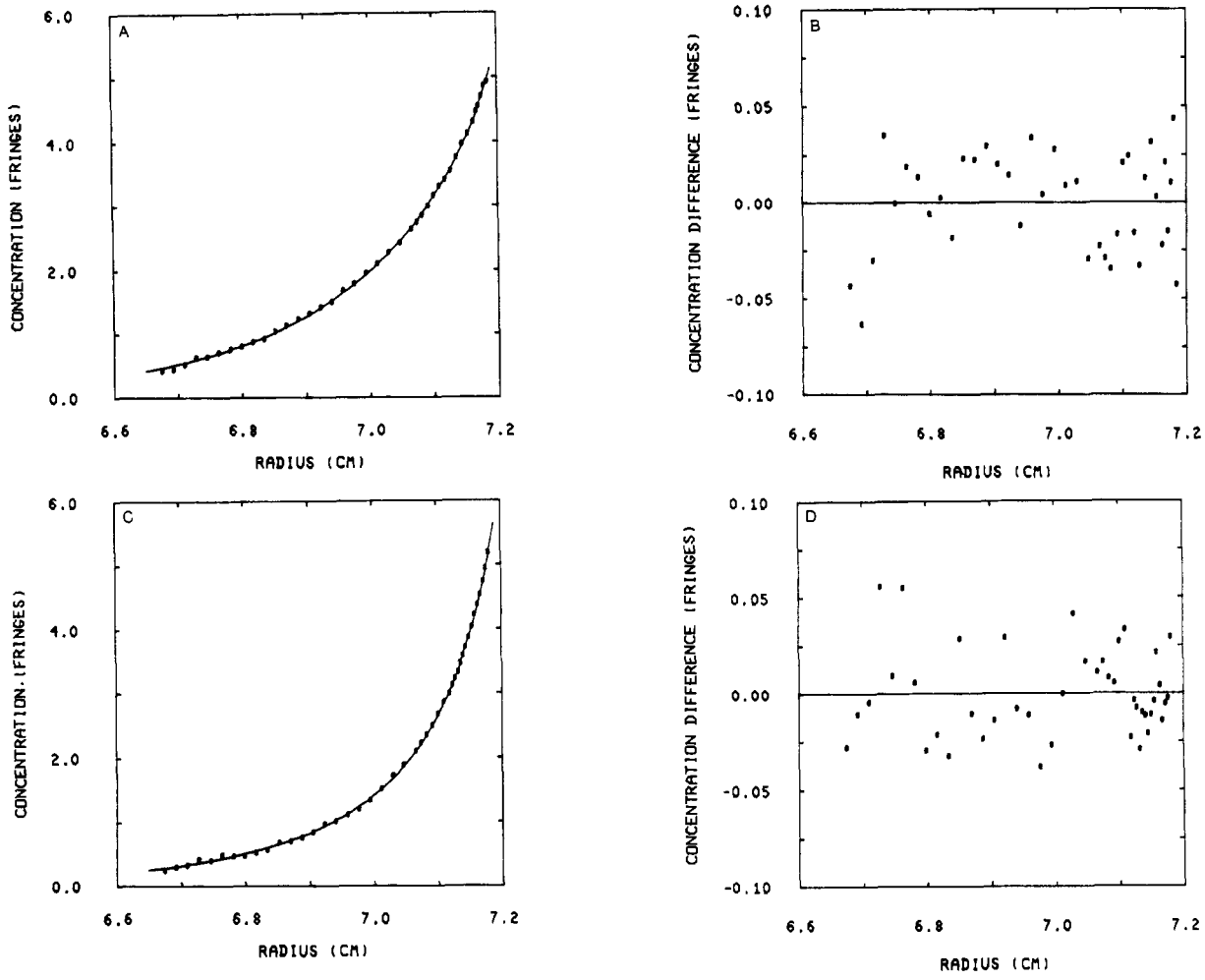

FIGURE 2: Equilibrium sedimentation distribution of filamin and heavy merofilamin. (A) Radial distribution of heavy merofilamin (open circles). Heavy merofilamin (0.73 mg/mL) was centrifuged at 7200 rpm in a An-G rotor at 5 °C for 95 h. The concentration distribution of protein was measured as described under Experimental Procedures. The solid line is the best fit line obtained assuming 96.6% monomer and 3.4% aggregate. (B) Difference plot of fitting curve for heavy merofilamin. Plot shows the deviation of the experimental data from the calculated fitting curve. rms error is 0.024 fringes. (C) Radial distribution of filamin (0.75 mg/mL) (open circles). Experimental procedure was identical with that of (A). The solid line is the best fit line obtained assuming 77.1% monomer, 15.2% dimer, and 8.0% tetramer. (D) Difference plot of fitting curve for filamin data. rms error is 0.033 fringes.

of monomer (M_r = 2.5 × 10^5, 77.1%), dimer (15.2%), and tetramer (8.0%). The rms error for this fit, 0.033 fringes, and the random distribution of the data points about the fitting curve signify a good fit of the data. When the solution of filamin was warmed to 10 °C, the distribution shifted to higher molecular weight species, monomer constituted 47% of the solution, dimer was 50.2%, and tetramer was 2.3%. Thus, in contrast to heavy merofilamin, warming of filamin solutions produced increased self-association.

Studies on the Self-Association of Filamin. The studies comparing the ultracentrifugal behavior of filamin and heavy merofilamin indicated considerable complexity in the behavior of filamin in solution. We therefore have studied in some detail the solution properties of chicken gizzard filamin.

(1) Identification of the Species Present in Solution. Chicken gizzard filamin has a subunit molecular weight of 250 000 as judged by $NaDodSO_4$ gel electrophoresis (Shizuta et al., 1976). In solution, at low concentrations of KCl, equilibrium ultracentrifugation of fresh preparations of filamin gives a molecular weight of 500 000 (Shizuta et al., 1976; Wang, 1977). Studies with filamin in low salt concentrations, however, were complicated by the formation of significant quantities of high molecular weight aggregates and the resulting loss of observable mass during centrifugation. Higher salt concentrations, such as 0.6 M KCl, minimized the formation of aggregates greater than tetramer and greatly reduced the loss of observable mass during centrifugation. For this reason the studies presented here were carried out in 50 mM potassium phosphate, pH 7.5, 0.6 M KCl, 1 mM EDTA, and 1 mM dithiothreitol.

In this buffer the concentration distribution of filamin could not be fitted by models that assume only a single component. A two-component model using monomer and dimer gave a better fit to the data. Assuming a model of monomer, dimer, and tetramer gave further improvement (Figure 2C,D). The inclusion of higher molecular weight components gave no improvement in the quality of fit. In all the solutions of filamin in 0.6 M KCl buffers we have examined, there have been no cases where the data could be satisfactorily fit assuming a single molecular species; we always have observed varying proportions of monomer, dimer, and tetramer.

(2) Studies on the Interconversion of Filamin Species. Having established the presence of monomer, dimer, and tetramer in solutions of filamin in 0.6 M KCl buffer, it was desirable to define the relationship among these species. Two possibilities were considered initially. The first possibility was that all of the species present were in rapidly reversible equilibrium, i.e.

$$4F_1 \underset{}{\overset{K_{12}}{\rightleftharpoons}} 2F_2 \overset{K_{24}}{\rightleftharpoons} F_4 \qquad \text{(model I)}$$

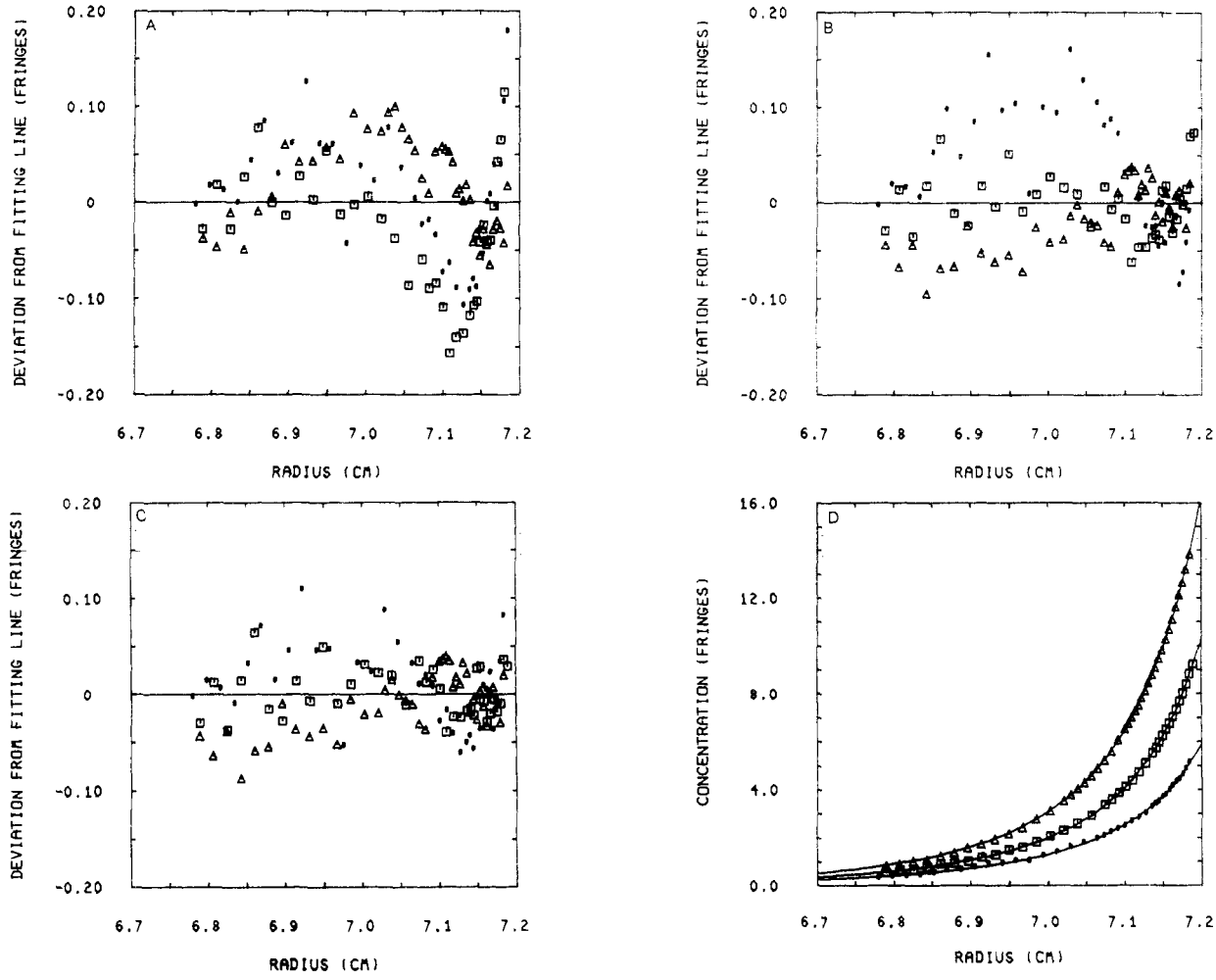

FIGURE 3: Comparison of models I–III in simultaneous fitting of filamin equilibrium sedimentation distribution data. Three concentrations of filamin (1.1 mg/mL, closed circles; 0.74 mg/mL, squares; 0.37 mg/mL, triangles) were centrifuged to equilibrium at 5 °C at 4800 rpm in a An-G rotor. The data were then fitted simultaneously to each of three models, and the difference between the experimentally derived data points and the calculated fitting curve is plotted as a function of radial distance in the centrifuge cell. (A) The difference plot of the data from the curve assuming model I (complete interconvertibility of monomer, dimer, and tetramer, $4F_1 \rightleftharpoons 2F_2 \rightleftharpoons F_4$). rms error is 0.071 fringes. (B) The difference plot of the data from the curve assuming model II (no interconvertibility, $F_1 + F_2 + F_4$). rms error is 0.051 fringes. (C) The difference plot of the data from the curve assuming model III (partial interconvertibility, $4F \rightleftharpoons 2F_2 \rightarrow F_4$). rms error is 0.035 fringes. (D) The actual comparison of the experimental data and the fitting curve using model III.

where F_1, F_2, and F_4 represent the monomer, dimer, and tetramer of filamin, respectively. An alternative model was that there was no interconversion of filamin species and that a fixed proportion of monomer, dimer, and tetramer was present, i.e.

$$F_1 + F_2 + F_4 \qquad \text{(model II)}$$

As demonstrated by Svedberg (Svedberg & Pedersen, 1940), it is not possible to distinguish between these two systems by a sedimentation–equilibrium experiment at a single loading and a single rotor speed. The best resolution would be obtained by examining different initial loading concentrations at several rotor speeds (Yphantis, 1964). To have used more than one rotor speed would have required relatively short solution columns in order to avoid excessive times to reach equilibrium. Our choice of the superior resolution afforded by longer solution columns precluded this option. The multiplexed He–Ne laser in this refractometric optical system made possible the examination of several initial concentrations under identical experimental conditions. The experiments involved the loading of three different initial concentrations of filamin and centrifuging to equilibrim at a single rotor speed. The resulting data were then analyzed by simultaneously fitting the concentration distributions of all three cells with the model under consideration.

Initially, we analyzed solutions of filamin at 5 °C, comparing models I and II, in each case simultaneously fitting the data from three different initial loading concentrations. Both models gave realistic parameter values. However, the rms error of 0.0705 fringes for the associating system (model I) was significantly inferior to the rms error of 0.0509 obtained for the nonassociating system (model II). Additionally, it can be seen in Figure 3A,B that the systematic deviation for the associating system is more marked than that exhibited by the nonassociating system. We concluded that no interconvertibility of the filamin species gave a better description of the data than assuming complete interconvertibility.

Three factors led us to consider a third model, one that involved partial interconvertibility. First, inspection of Figure 3B clearly shows systematic deviation of the data about the fitting line. The highest and lowest loading concentrations (triangles and circles, respectively) show consistent and opposite deviations from the fitting line. Second, filamin, although a dimer in low salt, can be dissociated to monomer in 0.6 M KCl buffers. Also, warming of filamin solutions leads to increased dimer formation. Therefore, it seemed likely that

filamin monomers and dimers are in fact interconvertible. Third, when different preparations of filamin were analyzed by using the self-associating monomer–dimer–tetramer model, it was found that while the values of K_{12} in a number of experiments were comparable, the values of K_{24} varied quite widely for the different experiments. It was also found that when the data from individual cells were analyzed singly, the values of K_{12} from each cell were consistent, while the values of K_{24} were widely variable.

These considerations suggested that while the concentrations of monomer and dimer were related in a rapidly reversible equilibrium, the concentration of tetramer might be individually variable in each cell, i.e.

$$4F_1 \overset{K_{12}}{\rightleftharpoons} 2F_2 \rightarrow F_4 \qquad \text{(model III)}$$

Using this model gave a substantially better fit as reflected in the reduction of the rms error to 0.0354 and in the improved deviation plot (Figure 3C). For illustrative purposes, the actual fit of this model to the data is shown in Figure 3D. Our confidence in this model was enhanced by the fact that it gave a better fit for all the data that we analyzed at 5 °C and at 10 °C than either of the other two models.

Substituting trimer for tetramer gave fits which were always inferior. Models which included trimer as well as tetramer did not give better fits than models without trimer and usually gave zero or small negative values for the concentration of trimer. These results led us to conclude that it was improbable that there was any significant concentration of trimer present. Model III assumes the presence of tetramer which is not in equilibrium with monomer and dimer. We have also considered models where some of the monomer or dimer were not in equilibrium with the other species present, i.e., incompetent monomer or dimer. In no case was there any evidence which supported the presence of any appreciable concentration of either of these species. We also considered the possibility of thermodynamic nonideality in the behavior of filamin solutions. Because of the greatly increased complexity of these computations which involved the use of a root finder, only the experiments at the highest concentrations were analyzed, since these were the most likely to exhibit nonideality if it was present. No evidence of the presence of thermodynamic nonideality was found.

(3) Effects of Temperature and Storage on Filamin Self-Association. Having concluded that filamin solutions can be described by a model involving a reversible monomer–dimer equilibrium (with equilibrium constant K_{12}) and variable amounts of tetramer, we next examined several preparations of filamin to determine the value for these parameters (Table I).

In our initial experiment, we studied a preparation of filamin at 5 °C and observed a value for K_{12} of 3.56×10^5 L/mol. The preparation contained a small, but variable fraction of tetramer (8–10%). The calculated free energy for dimerization under these conditions was −7.14 kcal/mol. We repeated the experiment (experiment II) with a different preparation of filamin and found very similar values both for K_{12} and for the percent fraction of tetramer (<10%) in the sample. In this experiment after reaching equilibrium at 5 °C, the temperature of the rotor was increased to 10 °C and the sample was reequilibrated. Analysis showed a substantial increase in K_{12} (to 13.5×10^5 L/mol), indicating an increase in the magnitude of the change of free energy of dimerization; there was also a substantial increase in the fraction present as tetramer. Further increasing the temperature to 15, 20, and 25 °C resulted in formation of high molecular weight aggregates and irreversible aggregation of the sample.

Table I: Comparison of Equilibrium Constants and Change of Free Energy of Association and Percent of Tetramer Formation with Four Preparations of Filamin

expt	cell	type of prepn	temp (°C)	%[a] tetramer	K_{12}[a] (L/mol)	ΔG° (kcal/mol)	rms error[d] (fringes)
A	1	stored[b]	5	8	3.56×10^5	−7.14	0.045
	2			10			
	3			7			
B	1	stored[b]	5	10	2.79×10^5	−6.90	0.053
	2			0			
	3			3			
	1		10	25	1.35×10^6	−7.91	0.049
	2			8			
	3			15			
C	1	fresh	5	0	5.96×10^4	−6.05	0.190
	2			24			
	3			26			
	1		10	7	1.95×10^5	−6.83	0.224
	2			45			
	3			46			
D	1	fresh	5	8	1.15×10^4	−5.15	0.075
	2			15			
	3			6			
	1		10	0	6.30×10^5	−7.49	0.088
	2			10			
	3			14			
	1		5[c]	0	2.84×10^5	−6.92	0.068
	2			7			
	3			11			

[a] Calculated by the curve-fitting procedure described under Experimental Procedures by simultaneously fitting all data to model III. [b] Stored for 2 weeks at 0–5 °C. [c] After being warmed to 10 °C the sample was recooled to 5 °C and centrifuged until equilibrium was achieved. [d] Root mean square error in fringes was at a wavelength of 633 nm for the fit of the complete data set to model III. Figures 2 and 3 permit a correlation of the magnitude of the rms error and the appearance of the difference plot.

The finding that increasing the temperature resulted in an increase in K_{12} suggested that prolonged storage of filamin solutions might lead to an increased tendency to self-association. The preparations used in experiments I and II were preparations that had been stored at 4 °C for 2–3 weeks prior to being used in the experiment. We therefore repeated experiment II, but using a fresh preparation of filamin tested immediately following purification (experiment III). The results were comparable although K_{12} had a slightly lower value (5.96×10^4 L/mol) in this experiment than in the preceding experiments. Again warming the solution produced a marked increase in K_{12} and an increased fraction of tetramer. Experiment IV is a repeat of experiment III using another fresh preparation of filamin. The value of K_{12} is low again and rises markedly on warming to 10 °C. Recooling the samples to 5 °C resulted in a partial reversal of the increased K_{12} and gave a value comparable to the K_{12} observed with stored preparations of filamin.

In summary, the model presented seems to consistently fit the data. Fresh preparations of filamin have the lowest tendency toward dimer formation. Limited warming of filamin solutions or prolonged storage below 5 °C produced an increased tendency toward dimer formation and promoted irreversible tetramerization. Prolonged warming above 10 °C produced marked aggregation of filamin solutions even in 0.6 M KCl buffers.

Discussion

Aggregation Properties of Filamin. Chicken gizzard filamin is a protein with a marked tendency to aggregation. Solutions

of filamin stored at 4 °C in the presence of low concentrations of KCl rapidly accumulate very high molecular weight aggregates (Shizuta et al., 1976; Wang, 1977). In order to study this problem we have found it necessary to resort to high concentrations of KCl (0.6 M) to reduce the formation of polymeric filamin. Under these conditions we have found that solutions of filamin are polydisperse, containing a mixture of monomers, dimers, and tetramers of the protein. Modeling studies have suggested that the monomer and dimer of filamin are readily interconvertible and appear to be in thermodynamic equilibrium with a change of standard free energy of association of approximately −7 kcal/mol. Aggregation of dimers to form tetramers does not appear to be such a simple association. Tetramer formation appears to be largely irreversible or at least to have a very slow rate of reversal so that filamin solutions progressively accumulate tetrameric aggregates. It is not unreasonable to believe that the tetramerization of filamin represents the first step in the formation of very large protein aggregates.

Increasing the temperature of filamin shifts the associations of the protein toward dimer formation and promotes the formation of high molecular weight aggregates. KCl, on the other hand, tends to reduce filamin self-association. These features of filamin self-association are in marked contrast to the behavior of spectrin, another high molecular weight actin-binding protein. Solutions of spectrin consist of monomers, dimers, and tetramers (Gratzer & Beaven, 1975; Schechter et al., 1976; Kam et al., 1977); however, warming of spectrin solutions promotes disaggregation and a shift toward monomer (Kam et al., 1977). Furthermore, high concentrations of KCl have been reported to favor aggregation of spectrin, whereas they inhibit filamin aggregation. Filamin and spectrin are quite similar in some respects: both are high molecular weight actin-binding proteins, both are phosphoproteins, and both appear to play an important role in the function of the submembranous cytoskeleton of the cells in which they are found. It may be that the differences in the association properties of these proteins reflect specialized roles of the proteins in erythrocytes as opposed to motile cells.

Comparison of Filamin and Heavy Merofilamin. A central feature of these studies was the comparison between chicken gizzard filamin and heavy merofilamin, the proteolytic fragment obtained by treatment of filamin with Ca^{2+}-activated protease. Initial studies in the electron microscope revealed that heavy merofilamin showed no aggregate formation. This defect in self-association was confirmed in the ultracentrifuge experiments where solutions of heavy merofilamin were entirely monomeric. The very small amount of high molecular weight material in heavy merofilamin solutions was compatible with the degree of contamination by undegraded filamin.

The differences between filamin and heavy merofilamin are not due to large-scale conformational differences in the two proteins. The CD spectra, between 190 and 300 nm, of filamin and heavy merofilamin are the same, as are the intrinsic fluorescence emission spectra of the two proteins.[3]

The major implication of the difference between filamin and heavy merofilamin is the insight it offers into the mechanism of actin–filamin gelation. Both filamin and heavy merofilamin bind to F-actin (Shizuta et al., 1976; Wang & Singer, 1977; Davies et al., 1978). However, while filamin causes gelation of F-actin solutions, heavy merofilamin does not (Davies et al., 1978). The observation that merofilamin is defective in self-association leads to the conclusion that the process of gelation requires aggregation of filamin molecules. Since the defect in heavy merofilamin can be detected in the absence of actin, it seems reasonable to conclude that the aggregation of filamin is not induced by binding to actin but rather reflects an intrinsic property of the molecule.

We think these studies support the notion that filamin binds to F-actin and then the self-association of filamin molecules cross-link the actin filaments to form a macromolecular aggregate or gel. The dependence of gelation on filamin self-association would explain the inhibition of gelation by high concentrations of KCl (Wang & Singer, 1977; Wallach et al., 1978), low temperatures (Stossel & Hartwig, 1976a), or substitution of heavy merofilamin for filamin (Davies et al., 1978).

In cells the majority of filamin appears to be concentrated beneath the plasma membrane in the microfilament mat.[4] Since actin is also concentrated in the microfilament mat, it would seem likely that this structure is comparable to a loose actin–filamin gel. Physiological processes that disrupt the integrity of the microfilament mat could function not only by depolymerization of F-actin or disruption of actin–filamin binding but also by disruption of filamin self-association. The endogenous regulators of microfilament function are not yet identified, but the demonstration that minor modification of the filamin molecule can greatly change its self-association properties makes this step a potential site of physiologic regulation.

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[3] P. J. A. Davies, unpublished observation.

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