THE AGGREGATION CHARACTERISTICS OF COLUMN-PURIFIED RABBIT SKELETAL MYOSIN IN THE PRESENCE AND ABSENCE OF C-PROTEIN AT pH 7.0

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ABSTRACT The aggregation properties of column-purified rabbit skeletal myosin at pH 7.0 were investigated as functions of ionic strength, protein concentration, and time. Filaments prepared by dialysis exhibited the same average length and population distribution at 0.10 and 0.15 M KCl at protein concentrations >0.10 mg/ml; similar results were obtained at 0.20 M KCl, although average filament length was ~0.5 μm shorter. Once formed, these length distributions remained virtually unchanged over an 8-d period. At and below 0.10 mg/ml, average filament length decreased as a function of protein concentration; filaments prepared from an initial concentration of 0.02 mg/ml were half the length of those prepared at 0.2 mg/ml. Filaments prepared by dilution exhibited a sharp increase in average length as the time-course increased up to 40 s, then altered only slightly over a further period of 4 min. Addition of C-protein in a molar ratio of 1–3.3 myosin molecules affected most of these results. Average filament length was affected neither by ionic strength nor by initial protein concentration down to 0.04 mg/ml or over an 8-d period. Filaments formed by dilution in the presence of C-protein exhibited a constant average length and hypersharp length distribution over variable time courses up to 7 min. It is possible that C-protein acts to stabilize the antiparallel intermediate during filamentogenesis, and may also affect subunit addition to this nucleus.

INTRODUCTION

The problem of skeletal muscle thick filament structure is an important one, both functionally in relation to the mechanism of contraction and structurally in terms of an assembly mechanism. X-ray diffraction results (Huxley and Brown, 1967) provided information consistent with a family of possible structures (Huxley and Brown, 1967; Pepe, 1967; Squire, 1974; Pepe and Dowben, 1977) and, although estimates of myosin content per thick filament have been made (Tregear and Squire, 1973; Morimoto and Harrington, 1974; Potter, 1974; Pepe and Drucker, 1979), they are not precise enough to eliminate any but the simple two-strand model unequivocally. The problem is further complicated by the presence of other thick filament proteins (Starr and Offer, 1971; Pepe and Drucker, 1975; Craig and Offer, 1976), which are found in specific bands on either side of the bare zone; their function is unknown, but the possibility of their participation in length determination during filamentogenesis and/or stabilization of the resultant structure cannot be eliminated.

An indirect approach to this problem is the study of myosin aggregation properties in vitro (Huxley, 1963). Unlike many other aggregating systems, such as actin, the resultant synthetic filaments are different in many respects from natural ones, but they resemble each other in both subunit and axial periodicity (Moos et al., 1975; Koretz, 1979*a*). These aggregation properties have been extensively studied as functions of ionic strength, pH, etc. in a number of different laboratories (e.g., Kaminer and Bell, 1966; Josephs and Harrington, 1966; Sanger, 1971; Katsura and Noda, 1971, 1973). However, the largest nonmyosin contaminant in the myosin preparations used in these studies, C-protein, has been shown to bind very strongly to the light meromyosin and subfragment-2 portions of the myosin molecule (Moos et al., 1975; Starr and Offer, 1978), and to disrupt filament structure at high (Moos et al., 1975) and low (Koretz, 1979*b*) molar ratios to myosin. Because of these effects, a reevaluation and extension of earlier studies have been performed with column-purified myosin, as well as comparative studies in the presence of C-protein to specify its effect on the aggregation process.

Our work on column-purified myosin at pH 7.0 is in general agreement with previous results on less pure myosin with certain exceptions related to protein-concentration dependence of filament length and the effects of the time-course of dilution. Filaments prepared in the presence of C-protein are shorter and protein-concentration independent, but exhibit invariant average length and

hypersharp distributions in dilution experiments. Some of the results discussed here were presented in abbreviated form at the 24th Annual Meeting of the Biophysical Society in New Orleans, Louisiana, June 1–5, 1980 (Coluccio and Koretz, 1980).

MATERIALS AND METHODS

Protein and Aggregate Preparation

Myosin and C-protein were prepared from the back and hind-leg muscles of New Zealand white rabbits according to the method of Offer et al. (1973). Further purification of the proteins was accomplished on a DEAE Sephadex A-50 column (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) with a phosphate-EDTA buffer and KCl gradient. Samples from the center of each elution peak were pooled and the remainder discarded. Myosin was precipitated by dialysis against 10 mM imidazole, pH 7.0, and either resuspended at a concentration of ~5 mg/ml in 0.6 M KCl, 10 mM imidazole, pH 7.0, or resuspended in the same buffer at 10 mg/ml and stored in 50% glycerin at −20°C for later use. C-protein was further purified by ammonium sulfate precipitation as described by Offer et al. (1973), resuspended in the KCl-imidazole solution, and clarified in a Beckman Model E ultracentrifuge at 150,000 *g* for 1 h (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.)

The myosin stock solution was diluted with 0.6 M KCl, 10 mM imidazole, pH 7.0, to final protein concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml, as determined by the extinction coefficient for column-purified myosin of Offer et al (1973). Similar solutions were prepared with the addition of 10% C-protein by weight, for a molar ratio of C-protein to myosin of ~ 1:3.3; each myosin solution containing C-protein thus contained 10% more total protein. Some experiments were performed using myosin that had been preserved in glycerin.

Samples of myosin or the myosin-C-protein mixture were dialyzed for at least 18 h against large volumes of KCl-imidazole solutions, pH 7.0, at 4°C with gentle stirring, with dialysis tubing of 12,000 exclusion weight. The 10-mm tubing was prepared by boiling in distilled water twice, followed by two treatments in 1 mM EDTA, ending with a final boil in distilled water. Whereas imidazole concentration was maintained at 10 mM for all experiments, KCl concentrations were varied from 0.05 to 0.25 M in steps of 0.05 M. For long-term dialysis, a single dialysis bag held shut with a clip was used. Daily samples were removed from the bag, which was then closed and replaced in the dialysis medium.

For dilution experiments, myosin solutions of various concentrations in either 0.6 m or 0.3 M KCl were reduced in ionic strength to either 0.2 or 0.1 M KCl with a microburette and gravity feed with rapid stirring. The time-course of dilution was varied from "0" (estimated at ~2 s) to 120 s, with some samples taking as long as 10 min. Data for each gradient were plotted as a function of time.

Turbidity Measurements

Absorption measurements at 280 nm and turbidity measurements at 310 nm were performed on a Beckman DB spectrophotometer or a Gilford modernization system (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) containing a Beckman DU-2 monochromator with quartz microcells. Samples were allowed to come to room temperature for all readings, and those turbidity data where, for a given protein concentration, $A_{280} - A_{310}$ was not a constant, were discarded.

Electron Microscopy and Length Distributions

Samples of aggregates prepared under the various conditions described above were placed on Formvar- and carbon-coated copper grids, blotted, briefly washed with 0.1 M ammonium acetate, pH 7.0, dried, and then negatively stained with 2% uranyl acetate. Electron micrographs at a variety of magnifications dependent on average filament length were taken with a JEOL 100S transmission electron microscope using a 100-kV accelerating voltage and a liquid nitrogen anticontamination device (JEOL USA, Electron Optics Div., Medford, Mass.). Each magnification step was calibrated to true magnification with a grating supplied by Ernest F. Fullman, Inc., Schenectady, N.Y.

The 3¼" × 4¼" negatives were magnified by a factor of exactly three in an Omega photographic enlarger (Omega Engineering, Inc., Stamford, Conn.) and filaments were traced end-to-end. Only well stained aggregates whose entire length was visible in the photographic field were considered. Length measurements were made using a Numonics 1250 planimeter (Numonics Corp., Lansdale, Pa.), and the results displayed in modified histogram form. For each condition, the number of samples was ≥100, but all data sets were normalized to 100 for easy visual comparison of peak height and width. Statistical analyses of the distributions were performed with standard equations for the calculations of moments of grouped data, the first moment being $\bar{x}$, the average of the distribution, and the square root of the second moment being the standard deviation. The third and fourth moments are used in calculations of deviations from a normal distribution.

RESULTS

18-h Dialysis

Turbidity readings of myosin samples (0.2–1.0 mg/ml) dialyzed against varying salt concentrations at pH 7.0 are shown in Fig. 1, plotted as a function of myosin concentration. A sharp increase in both slope and absolute turbidity is apparent between 0.25 and 0.20 M KCl, whereas little difference is observed between 0.20 and 0.10 M. Below 0.10 M, another very sharp increase in slope and turbidity values can be seen. Although all these lines must pass through the origin at zero myosin concentration, no curvature is observed in this particular protein concentration range.

Electron microscope grids prepared at each ionic strength for the 0.2- and 0.4-mg/ml samples were examined to determine the sensitivity of average filament length to slight changes in ionic strength. (Higher protein concentrations result in an overload of the field of view, and could

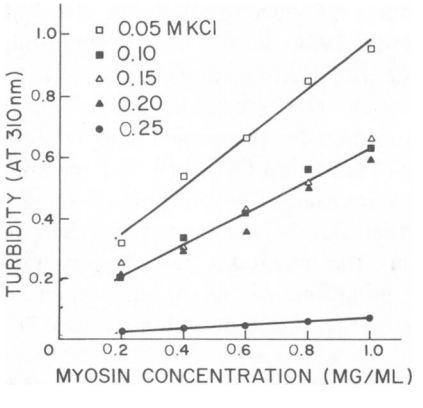

FIGURE 1 Turbidity measurements at 310 nm from samples of column-purified myosin dialyzed for 18 h at 4°C and pH 7.0, plotted as a function of myosin concentration. All solutions contain 10 mM imidazole.

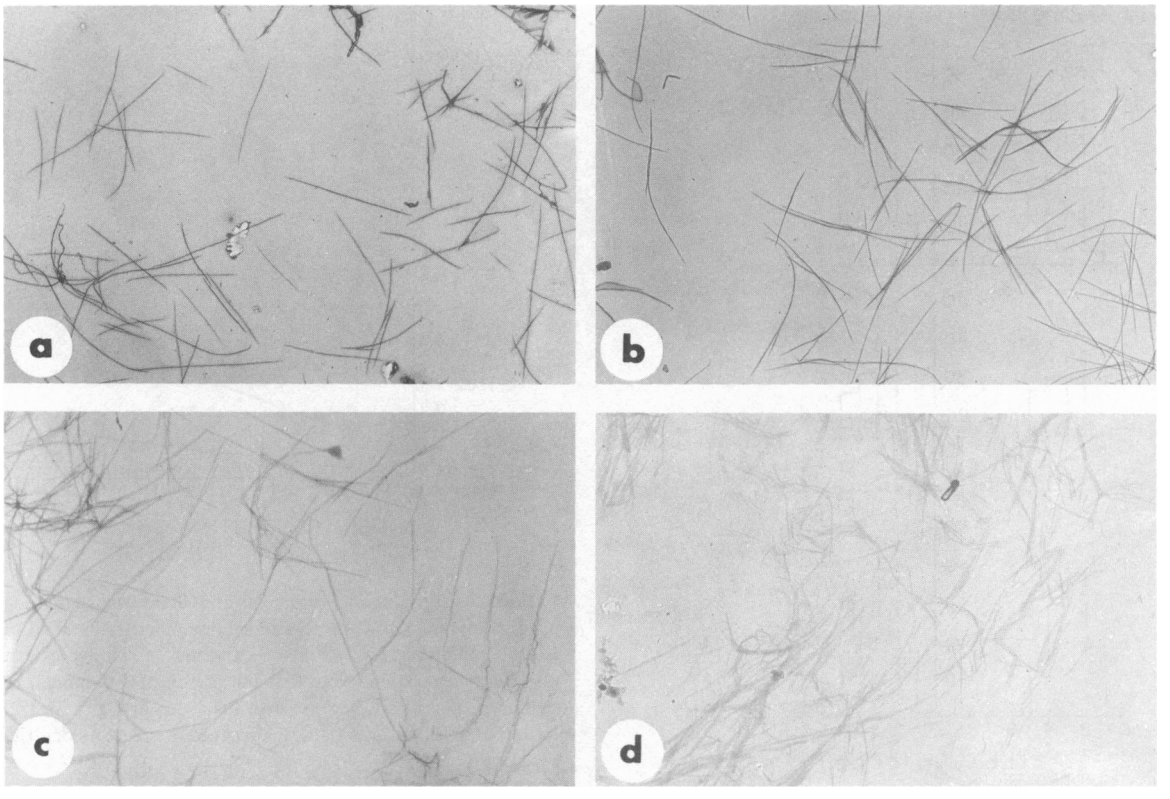

FIGURE 2 Electron micrographs of synthetic thick filaments negatively stained with uranyl acetate. The aggregates were formed by 18-h dialysis of 0.2 mg/ml myosin solutions against various KCl concentrations at pH 7.0 and 4°C. ×4,300 magnification. (*a*) 0.20 M KCl, (*b*) 0.15 M KCl, (*c*) 0.10 M KCl, (*d*) 0.05 M KCl.

not therefore be characterized.) Fig. 2 shows micrographs for the 0.2-mg/ml samples at four different ionic strengths. All of the micrographs are at the same magnification of ×5,700, and superficial examination of Fig. 2 *a-c* suggests that the populations of filaments exhibit approximately the same length characteristics.

Analysis of these micrographs (Fig. 3 *a-f*, solid lines and Table I) indicates that myosin filaments formed at 0.10 and 0.15 M KCl are about the same length, a little more than 3 μm on the average. This observation holds true for initial protein concentrations of both 0.2 and 0.4 mg/ml. Filaments formed by dialysis against 0.20 M KCl tend to be somewhat shorter (~2.5–2.6 μm), although again there is no significant difference due to protein concentration. No clear-cut trend in the sharpness of the distribution peaks is exhibited by the data, but the histograms generally tail off to the right to a greater or lesser extent.

It was not possible to perform the same type of length analysis with filaments formed at 0.05 M KCl (Fig. 2 *d*). The clumping that can be seen in the photographic field appears to be characteristic of filaments at this ionic strength, and it is impossible to determine whether these filaments show the same length characteristics as those formed at higher ionic strength without altering environ-

TABLE I
DIALYSIS AGAINST VARIABLE IONIC STRENGTH

KCl	$\bar{x}$	σ
0.1 M		
0.20 mg/ml	3.45	1.30
0.40 mg/ml	3.13	1.35
0.20 mg/ml + C	2.26	0.74
0.40 mg/ml + C	2.20	0.68
0.15 M		
0.20 mg/ml	3.26	1.47
0.40 mg/ml	3.34	1.29
0.20 mg/ml + C	2.50	0.93
0.40 mg/ml + C	2.47	0.78
0.20 M		
0.20 mg/ml	2.55	0.93
0.40 mg/ml	2.72	1.66
0.20 mg/ml + C	2.42	0.93
0.40 mg/ml + C	2.45	0.83

Analysis of the dialysis experiment illustrated in Fig. 3. Filament populations containing C-protein exhibit both shorter average lengths and narrower band widths than in C-protein's absence.

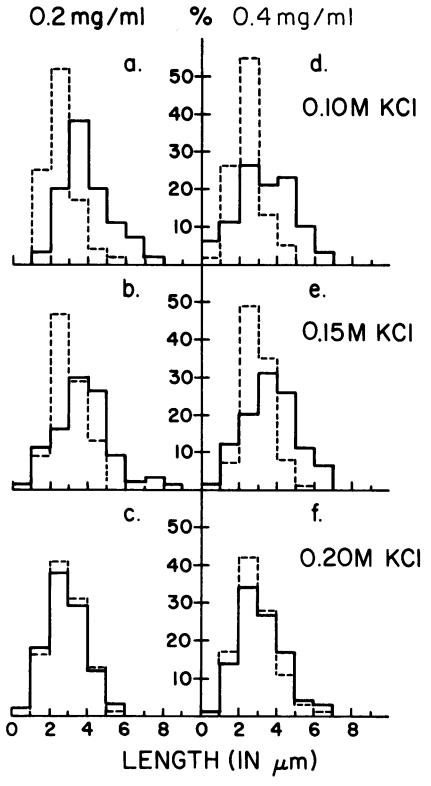

FIGURE 3 Length distributions of myosin filaments prepared from 18-h dialysis. Solid lines indicate myosin solutions, while broken lines represent a 3.3:1 mixture of myosin and C-protein. All histograms have been normalized to 100 for easy comparison. (*a*) 0.2 mg/ml myosin ± C-protein, 0.10 M KCl, (*b*) 0.2 mg/ml myosin ± C-protein, 0.15 M KCl, (*c*) 0.2 mg/ml myosin ± C-protein, 0.20 M KCl, (*d*) 0.4 mg/ml myosin ± C-protein, 0.10 M KCl, (*e*) 0.4 mg/ml myosin ± C-protein, 0.15 M KCl, (*f*) 0.40 mg/ml myosin ± C-protein, 0.20 M KCl.

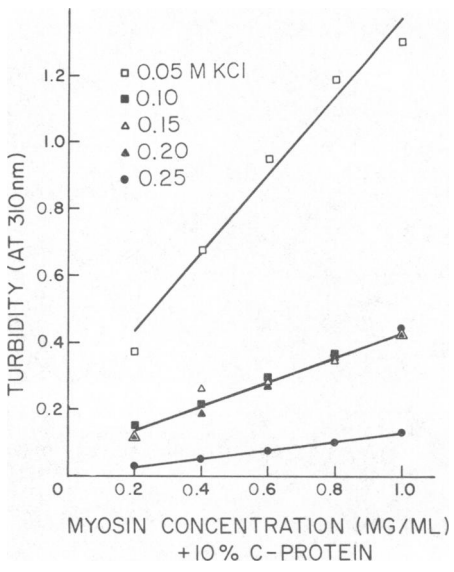

FIGURE 4 Turbidity measurements at 310 nm from samples of column-purified myosin with C-protein added in a molar ratio of 3.3 myosin to 1 C-protein dialyzed for 18 h at 4°C and pH 7.0, plotted as a function of myosin concentration. Note that the presence of C-protein results in a shallower slope and lower intercept than seen in Fig. 1 for 0.10–0.20 M KCl. The 0.05 M KCl data demonstrate a steeper slope than seen in Fig. 1.

mental conditions. It should be noted, however, that there is some degree of higher order apparent in this and other micrographs. Rather than forming a tangled network, which might be expected for very long filaments suddenly dropped onto a planar surface, the filaments seem to align themselves longitudinally in small bundles. These semi-ordered bundles of filaments, with an average filament-to-filament distance of ~14 nm, will in turn interact with other bundles to form the clumps visible in Fig. 2 *d*. The source of this interaction, and the reason for it appearing only below 0.1 M KCl, is unclear, but probably involves the myosin heads.

Because C-protein has been shown to disrupt filament structure at high molar ratios to myosin (Moos et al., 1975) and to alter filament structure at a ratio approximately that found in the banded region of the natural thick filament (Koretz, 1978, 1979*b*), its effect on the aggregation process was investigated under the same conditions used for studying myosin alone. Fig. 4 shows the turbidity data for the various conditions plotted as a function of myosin concentration. In general, the results are very similar to those obtained with myosin alone. Comparing Figs. 1 and 4, one sees that myosin aggregation in the presence of C-protein follows the same pattern of a sharp increase in turbidity between 0.25 and 0.20 M KCl, little change between 0.2 and 0.1 M KCl, and a second sharp increase in turbidity below 0.1 M.

Electron micrographs of the aggregates show that filaments formed in the presence of C-protein in the ionic range of 0.10–0.20 M KCl tend to exhibit the same length characteristics, whereas those formed at 0.05 M KCl exhibit the clumping seen with myosin alone in Fig. 2*d*. Histograms of the length distributions (Fig. 3*a–f*, broken lines) and statistical analysis of these distributions (Table I) indicate that filament length is approximately independent of the protein concentration and ionic strength under the conditions considered, with filaments prepared against 0.10 M KCl being ~0.2 μm shorter than those formed against higher ionic strength on the average. Again, distributions tend to tail off to the right under all conditions; however, the sharpness of the distributions generally decreases with increasing ionic strength.

One further point about these results should be noted. We have found that with careful control of pH and dialysis bag preparation both the turbidity and length data are reproducible from preparation to preparation. In addition, experiments performed using myosin that had been kept at −20°C in 50% glycerin gave the same results as those performed with fresh myosin. This indicates that whatever groups interact during the aggregation process are not adversely affected by low temperature storage.

Filament Formation at Low Myosin Concentrations

Filaments were prepared according to the same procedure described above for 18-h dialysis and with the same variety of ionic strengths, but at 1/10 the protein concentration range in the presence and absence of C-protein. Both turbidity readings at all ionic strengths and electron microscope studies for 0.1 M KCl were performed.

Although the turbidity data are very similar in the presence and absence of C-protein, curving downward to the origin below about 0.08 mg/ml for all ionic strengths, it is clear from the electron microscopy studies (Fig. 5 and Table II) that two very different processes are occurring. As myosin concentration increases, there is a concommitant increase in average filament length from 1.60 μm at 0.02 mg/ml to 2.38 μm at 0.10 mg/ml; although the distributions tend to be skewed to the right, they also tend to be much sharper than the distributions observed at higher protein concentrations (Table I). In contrast, samples containing C-protein are approximately constant in average length over the same concentration range, as well as exhibiting the same average length seen for much higher concentrations of the mixture. In other words, average myosin filament length appears to increase with increasing protein concentrations, approaching the observed 3-μm size at 0.2 mg/ml asymptotically; in the presence of C-protein, average filament length is constant over a concentration range of 0.04–0.40 mg/ml, dropping slightly only at 0.02 mg/ml.

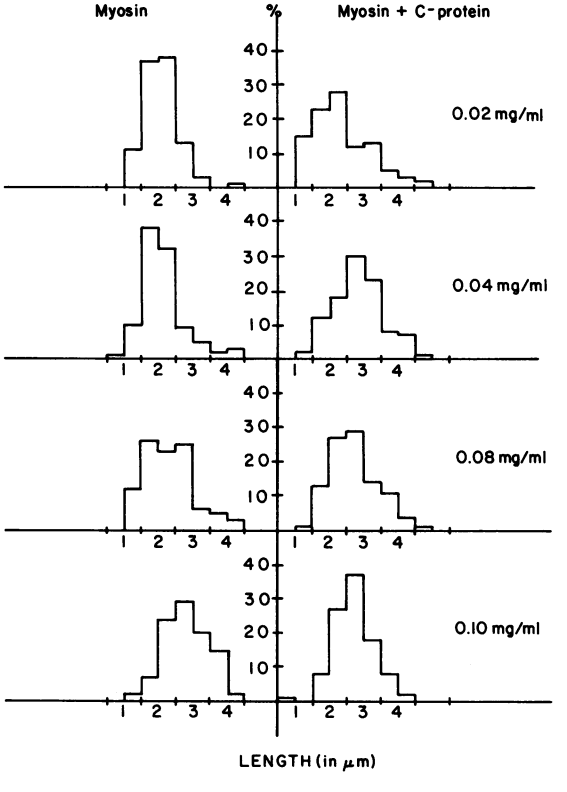

FIGURE 5 Length distributions of myosin filaments prepared from initial low concentrations of myosin with or without C-protein. (*a*) 0.02 mg/ml myosin, (*b*) 0.04 mg/ml myosin, (*c*) 0.08 mg/ml myosin, (*d*) 0.10 mg/ml myosin, (*e*) as (*a*), with C-protein, (*f*) as (*b*), with C-protein, (*g*) as (*c*), with C-protein, (*h*) as (*d*), with C-protein.

TABLE II
DIALYSIS: LOW MYOSIN CONCENTRATIONS

	$\bar{x}$	σ
Myosin		
0.02 mg/ml	1.60	0.43
0.04 mg/ml	1.70	0.65
0.08 mg/ml	1.83	0.67
0.10 mg/ml	2.38	0.83
Myosin + C		
0.02 mg/ml	1.90	0.79
0.04 mg/ml	2.33	0.71
0.08 mg/ml	2.30	0.69
0.10 mg/ml	2.37	0.67

Dialysis experiment illustrated in Fig. 5 against 0.10 M KCl, 10 mM imidazole, pH 7.0. In the absence of C-protein, myosin filament length gradually increases with increasing protein concentration. In C-protein's presence, filament length is invariant at all concentrations above 0.02 mg/ml.

Long-term Dialysis

Some aggregating systems (e.g., actin) trend to undergo a redistribution of length with time, whereas others (e.g., flagellin) exhibit no length redistribution over a period of weeks or months. Myosin aggregates are apparently in dynamic equilibrium with monomers or dimers at pH 8.0 and low-to-intermediate ionic strength, and can change distribution characteristics with a reduction in pH. To test whether aggregated myosin will undergo a length redistribution with time at pH 7.0 at constant environmental conditions, myosin at two concentrations with and without C-protein was dialyzed for a period of 8 d, with samples for turbidity measurements and electron microscopy taken daily.

Neither turbidity measurements nor filament-length distributions appear to change significantly over the experimental time-course. Statistical analyses of filament lengths for days 1, 3, and 6 are listed in Table III. The shapes of the curves, as well as peak location and height, are unchanged over the time-course of the experiment for each condition. Comparison between the curves of samples with and without C-protein at the two concentrations show the same relationships discussed earlier for the 18-h dialyses, and are statistically indistinguishable from those results. Thus, if myosin aggregates can demonstrate redistribution with time, the time-course for this reaction is more similar to flagellin than to actin.

TABLE III
LONG-TERM DIALYSIS

KCl	$\bar{x}$	σ
Day 1		
0.20 mg/ml	3.09	1.06
0.40 mg/ml	3.32	1.43
0.20 mg/ml + C	2.43	0.94
0.40 mg/ml + C	2.47	0.84
Day 3		
0.20 mg/ml	3.14	1.23
0.40 mg/ml	2.78	1.53
0.20 mg/ml + C	2.73	0.91
0.40 mg/ml + C	2.51	0.79
Day 6		
0.20 mg/ml	2.83	0.93
0.40 mg/ml	3.07	1.01
0.20 mg/ml + C	2.36	1.05
0.40 mg/ml + C	2.14	0.74

Analysis of the filament length on days 1, 3, and 6. No clear indication of change over the time-course of the experiment is observed.

Dilution Experiments

Samples of myosin at varying concentrations and in either 0.6 or 0.3 M KCl were diluted to 0.2 or 0.1 M KCl over short time periods (2–120 s) with 10-mM imidazole, pH 7.0. The ionic gradient during dilution was kept as close to linear as possible, and grids for electron microscopy were immediately prepared.

Typical data for a set of experiments diluting a 1.0-mg/ml myosin solution from 0.3 to 0.2 M KCl are shown in Fig. 6. As the dilution time is increased from "0" (~2 s), the turbidity rapidly increases in exponential fashion, reaching an apparent plateau by ~35–40 s. Similar turbidity data are obtained with different myosin concentrations and/or ionic end points. If the time-course for dilution is extended over minutes rather than seconds, however, it is clear that the plateau obtained with short dilution times is itself an intermediate rather than an end state. The increased dilution times result in turbidities that eventually approach those observed for dialyzed samples prepared under the same ionic conditions.

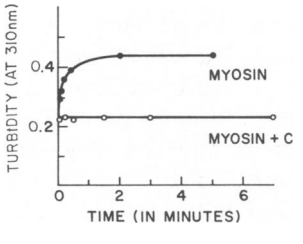

FIGURE 6 Representative results of dilution experiments. Initial conditions: 1.0 mg/ml myosin or myosin with added C-protein, 0.30 M KCl, 10 mM imidozole pH 7.0. Final conditions: 0.67 mg/ml protein, 0.20 M KCl, 10 mM imidozole, pH 7.0. Turbidity after sample dilution at 310 nm is plotted against the time-course of dilution. Myosin ± C-protein as labeled.

Electron microscope examination of samples taken from solutions diluted over different time periods reveals that average filament length increases with increasing dilution time (Fig. 7*a–c* and Table IV). The average filament length is 0.46 μm at t = "0", 0.71 μm at t = 15 s, and 0.74 μm at t = 4.35 min, whereas filaments prepared by dialysis to the same ionic strength are ~2.5 μm, more than three times as long.

Dilution experiments similar to those previously described for myosin were also performed in the presence of C-protein. Fig. 6 also shows a typical plot of turbidity vs. time of dilution for one set of experiments. No change in either turbidity or average filament length (Fig. 7*d–f* and Table IV) is observed over time ranges up to 7 min, which suggests that filamentogenesis in the presence of C-protein is complete within the fastest dilution time. It should also be noted that these distributions are hypersharp (kurtosis much greater than zero), and remain unaltered over the time periods considered.

DISCUSSION

The strongest influences on filamentogenesis have been shown to be pH and the time-course of ionic strength reduction (Kaminer and Bell, 1966; Josephs and Harrington, 1966; Sanger, 1971; Katsura and Noda, 1971, 1973). Although pH was held constant in the present work, there

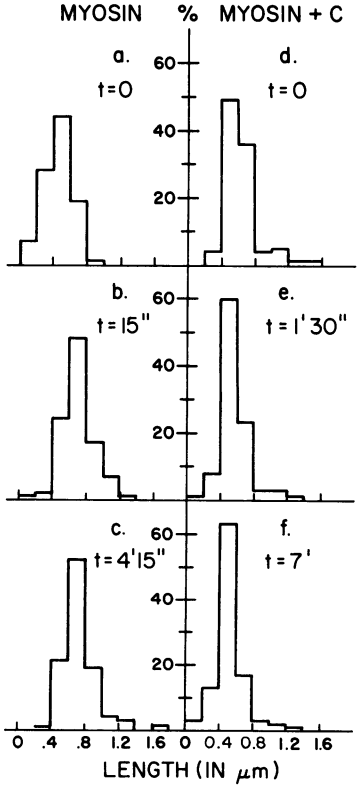

FIGURE 7 Length distributions of myosin or myosin + C-protein mixtures used in dilution experiments. Time of dilution as labeled. (*a–c*) myosin, (*d–f*) myosin plus C-protein.

TABLE IV
DILUTION EXPERIMENTS

Time	$\bar{x}$	σ
(s)		
Myosin		
0	0.46	0.16
15	0.71	0.22
255	0.74	0.19
Myosin + C		
0	0.60	0.13
75	0.57	0.14
420	0.54	0.14

Analysis of the dilution experiments illustrated in Fig. 6.

is no way of determining whether our dialysis time-course is directly comparable to those of other workers, since length distributions are dependent on the particular dialysis tubing used and would be different under different formative conditions. Because we are also using a preparative procedure that results in much purer myosin, our observations cannot be quantitatively compared to earlier studies. However, the aggregation characteristics of column-purified myosin as functions of ionic strength, protein concentration, and time at pH 7.0 are qualitatively similar to observations reported for less pure rabbit skeletal myosin. That is, bipolar well formed filaments are observed in the electron microscope at 0.20 M KCl and below (Kaminer and Bell, 1966); their average length is dependent on the time-fourse of ionic strength decrease (Sanger, 1971), and is partially independent of protein concentration.

Upon closer examination of the data, some very interesting observations emerge. Filaments prepared by dialysis against varying ionic strengths exhibit the same average length at 0.10 and 0.15 M KCl, and are not much shorter at 0.20 M KCl; these length distributions, once formed, are preserved over a week's time-course of study and are independent of protein concentration above 0.10 mg/ml. If formed by dilution, average filament length increases sharply when the dilution time-course is increased up to ~35–40 s; beyond this, the average length increases imperceptibly with time up to the values observed in dialysis experiments. In addition, the filament populations can all be described in terms of a normal distribution; there is a strong tendency to a right-handed skew, but the shape of the curve is almost always near normal as indicated by analysis of kurtosis.

The presence of C-protein during filamentogenesis affects most of these observations. Although the ionic conditions for filamentogenesis appear to be similar, both average filament length and sensitivity of length to ionic strength have been altered. Under our particular dialysis conditions, filaments containing C-protein are consistently ~2.3–2.5 μm long; this length is also independent of myosin concentration down to 0.04 mg/ml. As with myosin alone, filament lengths are maintained over 8 d time and exhibit a normal distribution, but tend to be somewhat more peaked. In dilution experiments, no alteration of average filament length with time is observed; length distributions are hypersharp (kurtosis coefficient $\gg$3) and maintained whether time of formation is 0 s or 7 m. Our results diverge significantly from those of Miyahara and Noda (1980), who generally used different C-protein-myosin ratios and buffers.

Myosin filamentogenesis is generally assumed to proceed through three stages, each one dependent on ionic strength. As salt is lowered, myosin gradually forms increasing amounts of parallel dimers (Godfrey and Harrington, 1970*a*, *b*), the probable subunit for elongation itself. At some point, different at different pH values, these dimers will aggregate into small antiparallel nuclei; these nuclei may well be similar to, if not identical with, the minifilaments produced by Reisler et al. (1980) at pH 8.0. Then at some critical ionic strength, above 0.20 M KCl at pH 7.0, parallel addition of myosin to either side of the antiparallel nucleus rapidly occurs, which results in biopolar synthetic filaments.

The elongation step, which is extremely fast according to Katsura and Noda (1973), has been thought to occur by dimer addition to a sheet that rolls up and closes upon the binding of the proper number of molecules (Koretz, 1979*a*). The advantage of such a model, simplistic as it might be, is that as closure occurs, the site of subunit addition becomes inaccessible; thus, a well formed filament could not easily exchange subunits with any soluble subunits in the medium for alteration of length distribution over time. The latter implication is consistent with our observation of preserved filament length distribution over a period of 8 d.

If observed filament distributions are in fact locked in in the absence of perturbing environmental changes, such as alteration of pH, then the characteristics of these distributions are determined during filament formation itself. It seems likely that the critical determinant is nucleus formation, since elongation occurs rapidly once the necessary ionic strength is reached. For dialysis experiments, where average length and shape of distribution were independent of protein concentration above ~0.10 mg/ml, the percentage of nuclei would be constant and independent of protein concentration; i.e., doubling protein concentration would double the number of filaments, rather than alter the population characteristics.

The dilution results could then be explained in terms of the conditions necessary for nucleus equilibration. Perhaps nuclei form rapidly and in high numbers at a particular ionic strength, then very gradually decrease in number as ionic strength is lowered and conditions become less favorable for the antiparallel interaction. A slow decrease, as with dialysis, would result in a steadily increasing average length with increased time span of salt removal; a rapid decrease, as in dilution experiments, would favor elonga-

tion before the slow back-reaction had proceeded very far. It should be noted in this context that gizzard smooth muscle myosin, which forms filaments ~0.5 μm long under normal procedures (Kaminer, 1969), will form much longer filaments with a dilution-dialysis procedure that induces a 24-h salt gradient (Hinssen et al., 1978).

Within this basic framework, interpretation of the results in the presence of C-protein is straightforward. C-protein has been shown to bind strongly to both the light meromyosin and subfragment-2 portions of the myosin molecule (Moos et al., 1975; Starr and Offer, 1978) at ionic strengths where myosin is soluble, with a suggested stoichiometry of 1 C-protein to 3 or 4 myosins (Koretz, 1979*b*). If C-protein can act to stabilize the nucleus and/or reduce dissociation of the nucleus at unfavorable ionic strengths, then average filament length in populations prepared by dialysis would be shorter because of increased nuclei number at the time of elongation. In dilution experiments, inhibition of the back reaction would, over a "short" time-course, result in a relatively invariant population. The change in filament structure when C-protein is added to myosin in a ratio of 1 to 3–4 before filamentogenesis (Koretz, 1979*b*) is consistent with some type of organization before elongation occurs. In addition, more structured myosin binding would be consistent with both the hypersharp dilution length distributions observed in the presence of C-protein and with preservation of average filament length down to very low protein concentrations.

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