Homogeneity of Myosin Subfragments by Equilibrium Centrifugation†

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ABSTRACT: A number of enzymes are currently in use for obtaining proteolytic subfragments of rabbit skeletal muscle myosin. Subfragment-1 can be obtained by papain digestion of polymeric myosin in the presence (Mg·S1) or absence (EDTA·S1) of divalent cations [Margossian, S. S., Lowey, S., & Barshop, B. (1975) *Nature (London) 258*, 163–166]. Subfragment-1 prepared by chymotrypsin is readily fractionated according to its alkali light-chain content into S1(A1) and S1(A2) [Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56]. Digestion of soluble myosin by trypsin or chymotrypsin leads to heavy meromyosin (HMM) and light meromyosin (LMM). Many of these subfragments show extensive cleavages in the heavy- and/or light-chain region by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In view of the widespread use of proteolytic subfragments in kinetic and structural studies, it was of interest to establish the extent of heterogeneity of these preparations under nondenaturing conditions by equilibrium centrifugation. Analysis of the fringe displacements by the computer programs of Roark & Yphantis [Roark, D. E., & Yphantis, D. A. (1969) *Ann. N.Y. Acad. Sci. 164*, 245–278] showed that for three initial loading concentrations, the molecular weight averages M_n, M_w, and M_z were superimposable across the entire solution column for all S1 and HMM species. The same applied for the initial molecular weight averages of LMM and rod, except that with these highly asymmetric molecules, a small drop in molecular weight was observed toward the cell bottom as would be expected from excluded volume effects. We conclude that the subfragments of myosin are remarkably homogeneous in benign solvents, despite the existence of some cleavages in their primary structure.

Proteolytic subfragments of myosin have been used extensively to investigate both the physical and kinetic parameters of the energy transduction mechanism of muscle and other actomyosin-dependent contractile systems. Although myosin has been characterized in a number of laboratories (Holtzer & Lowey, 1959; Lowey & Holtzer, 1959; Holtzer et al., 1962; Dreizen et al., 1966; Richards et al., 1967; Gershman et al., 1969; Godfrey & Harrington, 1970a,b; Szuchet, 1977; Emes & Rowe, 1978) and criteria for its purity and homogeneity are well established, less is known about the molecular weight distribution and heterogeneity of its proteolytic subfragments. Heavy meromyosin (HMM) and light meromyosin (LMM) produced by limited tryptic or chymotryptic digestion migrate as single peaks in the ultracentrifuge; the same degree of homogeneity is observed for subfragment-1 (S1) and rod prepared with papain or chymotrypsin (Lowey et al., 1969; Weeds & Pope, 1977). When analyzed by sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis, however, both S1 and HMM can show extensive cleavage of the light and/or heavy chain depending on the conditions of the digestion. In recent years, the procedures for preparing myosin subfragments have been improved and defined more clearly with the goal of minimizing proteolytic degradation. For instance, depending upon whether Mg^{2+} is included in the digestion mixture or is removed with EDTA, two distinct species of papain subfragment-1 are obtained: Mg·S1 has a full complement of the DTNB light chain, whereas EDTA·S1 lacks this class of light chain almost completely (Margossian et al., 1975). The heavy chains of both types of papain S1 show multiple cleavages. In contrast, when myosin filaments are digested by chymotrypsin in the absence of divalent cations, the S1 heavy chain remains intact, but the DTNB light chain is totally degraded as judged by sodium dodecyl sulfate gel electrophoresis (Weeds & Taylor, 1975; Weeds & Pope, 1977). HMM prepared by digesting soluble myosin with chymotrypsin also exhibits an intact heavy chain, but the intensity of the DTNB light-chain band is reduced compared to undigested myosin. This can be accounted for by assuming that the DTNB light chain is partially degraded into a smaller fragment which comigrates with the alkali-2 light chain (Weeds & Pope, 1977). In spite of the widespread use of these subfragments in kinetic, binding, and other experiments, few reports exist on the extent of their heterogeneity except in accounts concerning the shape of S1 (Yang & Wu, 1977; Kretzschmar et al., 1978; Mendelson & Kretzschmar, 1980).

We have analyzed, in detail, the molecular weight distribution and homogeneity of myosin subfragments prepared by several methods. The incentive for such an analysis arose from a study of the binding of HMM and S1 to F-actin (Margossian & Lowey, 1978). In the initial experiments with S1, it became apparent that the binding of S1 to actin was affected by the level of divalent cations in the medium, and, as a consequence, the state of aggregation of S1 in various solvents was investigated by equilibrium centrifugation (Margossian & Lowey, 1977). In later studies, which compared the binding of HMM and S1 to actin, the homogeneity of HMM acquired special significance. In these studies, the concentration of bound subfragment was obtained by measuring the absorbance of free subfragment remaining in the supernatant after sedimentation of the complex in the analytical ultracentrifuge (Margossian

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DDT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; ATP, adenosine 5′-triphosphate.

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& Lowey, 1978). Since the possible presence of aggregates cosedimenting with actin or the existence of single-headed species in HMM would lead to an overestimate or an underestimate of the binding constant, respectively, it was essential to determine whether or not these subfragments were monodisperse in the solvents used. A preliminary report of these results has been presented (Margossian & Stafford, 1979).

Materials and Methods

Preparation of Proteins. Myosin was prepared from the back and leg muscles of male, white New Zealand rabbits by the method of Holtzer & Lowey (1959). Papain subfragment-1 was obtained by digesting myosin at 10–15 mg/mL with 0.03 mg/mL papain (Worthington Biochemical Corp.) in 0.2 M ammonium acetate (pH 7.2) with added 2 mM $MgCl_2$ or 2 mM EDTA. Insoluble material was removed by centrifugation, and the supernatant was purified by ion-exchange chromatography as described by Margossian et al. (1975).

Heavy meromyosin was prepared by digesting a 15 mg/mL solution of myosin in 0.6 M KCl and 0.05 M potassium phosphate (pH 6.5) with 0.05 mg/mL trypsin (Worthington) for 5 min at room temperature (Lowey & Cohen, 1962). The HMM was further purified by chromatography on a 2.5 × 60 cm DEAE-cellulose (Whatman DE52) column equilibrated in 0.05 M Tris-HCl (pH 7.9) and 1 mM DTT. The protein was eluted with a linear gradient to 0.5 M KCl in a total volume of 1 L. The front two-thirds of the peak was pooled, salted out between 43 and 55% saturated ammonium sulfate, and dialyzed exhaustively against 0.1 M KCl, 0.01 M imidazole (pH 7.0), 1 mM $MgCl_2$, 1 mM DTT, and 0.3 mM EGTA (Margossian & Lowey, 1978; Lowey et al., 1969).

In all the equilibrium sedimentation experiments reported here, 0.3 mM EGTA was included to chelate calcium. In preliminary experiments it was noticed that 0.1 mM $CaCl_2$ in the buffer induced aggregation of myosin subfragments. Although this effect was reversible by dialysis against EGTA, the stoichiometry of association was not reproducible, and further investigation of this phenomenon is required.

Chymotryptic subfragment-1 was prepared by digesting a suspension of myosin at 10–15 mg/mL in 0.12 M NaCl, 0.02 M sodium phosphate (pH 7.0), and 1 mM EDTA for 7 min with α-chymotrypsin as described by Weeds & Taylor (1975). The two isoenzymes, S1(A1) and S1(A2), were separated by ion-exchange chromatography on a 2.5 × 60 cm DEAE-cellulose column equilibrated in 0.05 M imidazole–HCl (pH 7.0). The proteins were eluted with a linear gradient to 0.12 M NaCl in a total volume of 1 L. Both S1(A1) and S1(A2) were further purified on a Bio-Gel A-5m gel column, 1 × 100 cm, equilibrated in 0.1 M KCl, 0.01 M imidazole (pH 7.0), 1 mM DTT, 1 mM $MgCl_2$, and 0.3 mM EGTA. The peak fractions were used directly in the centrifugation experiments.

LMM and rod were obtained from the precipitates of myosin digested for HMM or S1 with trypsin or chymotrypsin, respectively. After solubilization of the LMM and rod precipitates in 0.6 M KCl and 0.05 M potassium phosphate (pH 7.0), undigested myosin was denatured by adding 3 volumes of 95% ethanol in the cold. The precipitates were collected by centrifugation, and after they were dispersed in 0.6 M KCl and 0.05 M potassium phosphate, alcohol was removed by exhaustive dialysis against the same buffer. The protein solutions were clarified by centrifugation and dialyzed overnight against 0.03 M KCl and 0.01 M potassium phosphate buffer (pH 7.0). The precipitated protein was collected by centrifugation, dissolved in 0.6 M KCl and 0.05 M potassium phosphate (pH 7.0), and further purified by gel permeation chromatography (1 × 100 cm column) on a Bio-Gel A-15m column equilibrated in the same buffer. The fraction with the highest optical density was used in the sedimentation equilibrium runs.

Protein concentrations were obtained by measuring absorbance at 280 nm. Values for $E_{280}^{1\%}$ of 6.0, 8.3, 8.1, 7.4, 7.5, 3.0, and 2.2 were used, respectively, for HMM, Mg·S1, EDTA·S1, S1(A1), S1(A2), LMM, and rod (Margossian & Lowey, 1978).

Ultracentrifugation. Meniscus depletion sedimentation equilibrium runs were carried out on a Beckman Instruments Model E analytical ultracentrifuge equipped with Rayleigh optics and an externally adjustable Rayleigh aperture assembly (Stafford, 1978). Usually, each subfragment preparation was examined at three loading concentrations (0.10, 0.30, and 1.0 mg/mL) in a 12-mm, six-channel externally loading cell with sapphire windows (Ansevin et al., 1970). The camera was focused at the $^2/_3$ plane (Yphantis, 1964). All samples were dialyzed exhaustively against their respective buffers (Casassa & Eisenberg, 1964). The buffer for HMM and S1 contained 0.10 M KCl, 0.01 M imidazole (pH 7.0), 1 mM $MgCl_2$, 1 mM DTT, and 0.3 mM EGTA. In the case of LMM and rod, a 0.6 M KCl, 0.05 M potassium phosphate (pH 7.0) and 10 mM EDTA buffer was used. The values of the apparent partial specific volume (ϕ') were assumed to be the same for S1 and HMM. A value of 0.720 cm^3/g was used for HMM (Young et al., 1964) at 5 °C, giving a buoyancy factor of 0.276. A value of 0.701 cm^3/g was used for LMM and rod (Young et al., 1964) at 5 °C, giving a buoyancy factor of 0.279.

Rayleigh interferograms were taken on Kodak Nuclear Medicine film (NMC). The fringes were scanned by using a flat-bed densitometer according to the Fourier transform method described by DeRosier et al. (1972). The data were then analyzed by using computer programs on a PDP 11/40 and DEC system 20 computer (Roark & Yphantis, 1969; Pollard et al., 1978).

Criteria of Homogeneity. In the context of this paper, when we say that a macromolecular solution is homogeneous, we mean that it is composed of a single macromolecular component. This component may consist either of a single macromolecular species or of a series of polymers of a single chemical entity which are in reversible equilibrium with each other. In the case of an incompressible solution composed of a single ideal macromolecular species, all types of apparent molecular weight averages will be equal to each other and to the molecular weight of the species. For a solution of polymers in rapidly reversible equilibrium, the molecular weight moments will tend to range from that of the smallest species to that of the largest species across the centrifuge cell. The thermodynamic restrictions on the system (Squire & Li, 1960; Yphantis, 1964) require that the molecular weight moments be a function strictly of the local cell concentration and not of the radial position in the field. Therefore, any given molecular weight moment for a single-component system will have the same value at any given local macromolecular concentration at all loading concentrations. In contrast, a heterogeneous system (i.e., one composed of several species which are not in chemical equilibrium with each other) will be fractionated in the centrifugal field in such a way that the molecular weight moments will be strictly a function of the position in the field independent of the local macromolecular concentration. Therefore, one may use a plot of any one of the molecular weight moments as a function of local cell concentration at several loading concentrations as a test for

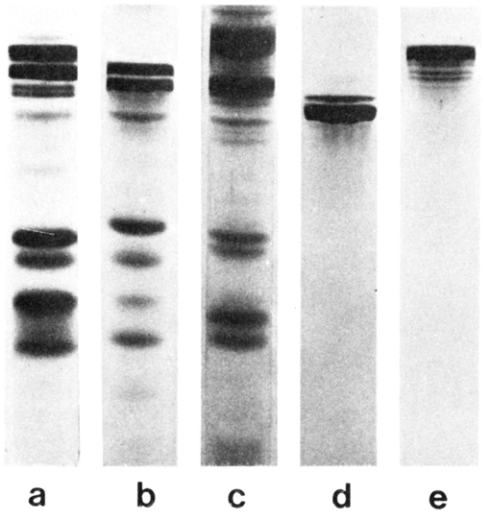

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of myosin subfragments used in high-speed equilibrium runs: (a) Mg·S1; (b) EDTA·S1; (c) tryptic HMM; (d) LMM; (e) rod.

a single-component system. If the several plots do not superimpose, then one may safely conclude that the system is heterogeneous as long as pressure effects have been ruled out. High-speed equilibrium ultracentrifugation, therefore, is a sensitive technique for detecting heterogeneity (Yphantis, 1964).

Thermodynamic nonideality generally makes analysis of multispecies systems difficult. A series of molecular weight moments which are insensitive to the effects of nonideality have been derived by Yphantis & Roark (1972). These moments, which are combinations of two or more of the ordinary molecular weight moments, are variously independent of the second, third, or fourth virial coefficients. The two most useful moments for our purposes, which are independent of the second virial coefficient, are the moments M_{y1} and M_{y2}, defined by the relationships

$$1/M_{y1,app} = 2/M_{n,app} - 1/M_{w,app} = 1/M_{y1,id} + Oc^2$$

$$1/M_{y2,app} = M_{z,app}/M_{w,app}^2 = 1/M_{y2,id} + Oc^2$$

These molecular weight moments are especially useful for studying nonideal systems and were used in the case of LMM and rod, both of which have significant second virial coefficients.

$NaDodSO_4$ Gel Electrophoresis. All the preparations were monitored by sodium dodecyl sulfate gel electrophoresis (Weber & Osborn, 1969) as described in Margossian & Lowey (1978).

Results

Polypeptide Composition and Homogeneity. We have analyzed many of the subfragments of myosin that are used commonly in kinetic and structural analyses to determine the extent of their heterogeneity. Subunit composition and polypeptide heterogeneity were examined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Mg·S1 and EDTA·S1 (parts a and b of Figure 1, respectively) are similar in appearance except that in EDTA·S1 the DTNB light chain is digested extensively. There is ~1 mol of this light chain/mol of Mg·S1, while in EDTA·S1 ~80% of the DTNB light chain is degraded (Margossian et al., 1975). In tryptic HMM (Figure 1c), the DTNB light chain appears to have undergone limited proteolysis as indicated by its increased mobility on $NaDodSO_4$–polyacrylamide gels. Chymotryptic S1(A1) contains a heavy chain and only the alkali-1 light chain, whereas S1(A2) contains predominantly the alkali-2 light chain contaminated slightly by alkali 1 (see Figure 1f,g; Margossian & Lowey, 1978). The heavy chain of these isoenzymes appears to be fairly intact, with no detectable cleavages, in contrast to the papain S1 heavy chain (Figure 1a,b) which shows extensive proteolysis. However, these cleavages do not seem to influence the solution homogeneity under nondenaturing conditions, as shown below. Both LMM and rod appear as a single component with a small satellite band on $NaDodSO_4$–polyacrylamide gels (Figure 1d,e).

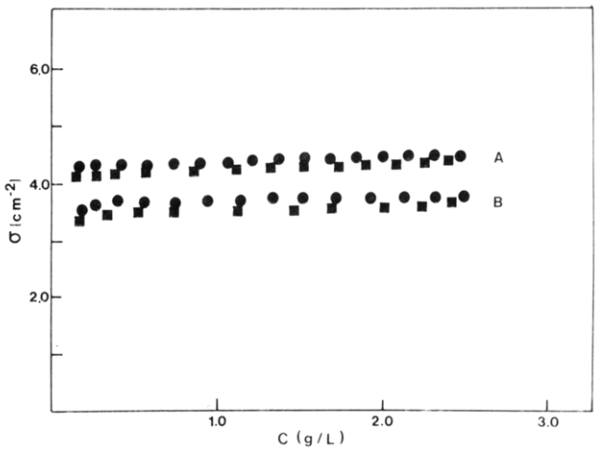

FIGURE 2: Molecular weight vs. concentration plots for S1 prepared by papain digestion. (A) Mg·S1; (B) EDTA·S1. σ_w (●); σ_n (■). Conversion factor = 2.98×10^4 daltons cm^2 (rpm = 16000). σ's are related to molecular weight by the following relation: $\sigma = M(1 - \phi'\rho)\omega^2/(RT)$, where ϕ' is the apparent partial specific volume defined by Casassa & Eisenberg (1964), ρ is the solution density, ω is the angular velocity, R is the gas constant, and T is the absolute temperature.

Molecular Weight of S1 and HMM. Homogeneity of both the papain and the chymotryptic subfragments was investigated by equilibrium centrifugation under nondenaturing conditions. Samples were examined for low molecular weight fragments due to proteolysis and for the presence of aggregates. Both Mg·S1 and EDTA·S1, which showed cleavages on $NaDodSO_4$ gels, appeared homogeneous by the criteria of equilibrium ultracentrifugation (Figure 2). The number and weight average molecular weights superimposed across the length of the cell giving a value of 130000 for Mg·S1 and 110000 for EDTA·S1, the difference reflecting the absence of the DTNB light chain in EDTA·S1. The chymotryptic isoenzymes S1(A1) and S1(A2), on the other hand, which showed no cleavages on $NaDodSO_4$ gels, did seem to contain both lower molecular weight material and some aggregates (not shown). Attempts to remove these extraneous components by further purification on a Bio-Gel A-5m gel filtration column were partially successful. A small amount of lower molecular weight material was still evident after gel filtration as a slight downward curvature at the meniscus. The molecular weight of S1(A1) and S1(A2) extrapolated to 110000 and 100000, respectively (Figure 3A,B), consistent with the results obtained from $NaDodSO_4$ gels.

Although HMM obtained by tryptic digestion revealed cleavages in the heavy-chain region on $NaDodSO_4$ gels, it was remarkably homogeneous by the criteria of equilibrium ultracentrifugation under native conditions (Figure 3C), ruling out any contamination by significant amounts of single-headed HMM or S1. The possible presence of very large aggregates which would not be seen by this technique was unlikely, in view of their absence in earlier sedimentation velocity runs. A value of 350000 was found for the molecular weight of HMM in

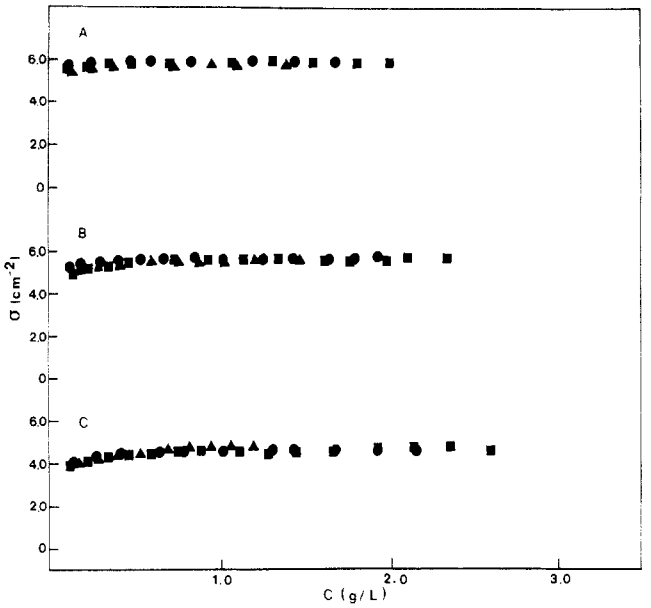

FIGURE 3: Plots of weight-average molecular weight distribution, σ_w, as a function of local cell concentration for (A) S1(A1), (B) S1(A2), and (C) HMM at initial loading concentrations of 0.1 mg/mL (▲), 0.3 mg/mL (●), and 1.0 mg/mL (■); conversion factor for S1 = 1.91 × 10^4 daltons cm^2 (rpm = 20000); conversion factor for HMM = 7.63 × 10^4 daltons cm^2 (rpm = 10000).

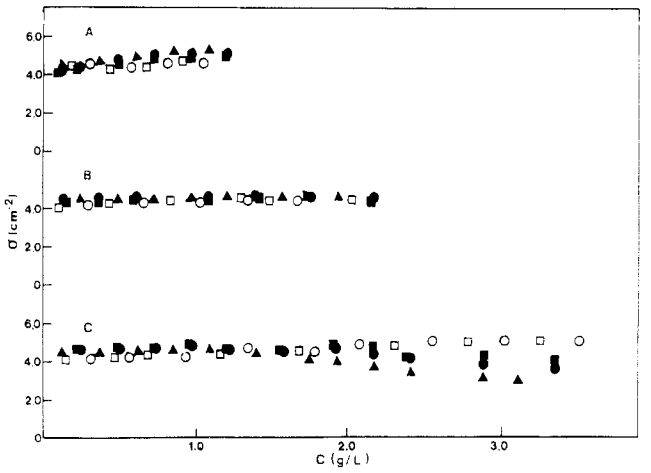

FIGURE 4: Molecular weight of LMM vs. local cell concentration at three initial loading concentrations: (A) 0.1 mg/mL; (B) 0.3 mg/mL; (C) 1.0 mg/mL. σ_n (■); σ_w (●); σ_z (▲); σ_{y1} (○); σ_{y2} (□). Conversion factor = 2.95 × 10^4 daltons cm^2 (rpm = 16000).

agreement with values reported previously (Holtzer et al., 1962; Lowey & Cohen, 1962).

The homogeneity of these preparations can be appreciated best when the molecular weight average for the different loading concentrations is plotted on the same graph. Figure 3 shows plots of the weight average molecular weight as a function of local cell concentration for three different loading concentrations spanning a 10-fold range for S1(A1), S1(A2), and HMM, respectively. The superimposition of these graphs in conjunction with their lack of significant curvature demonstrates that these three preparations each consist of a single, ideal macromolecular species.

Molecular Weight of LMM and Rod. The molecular weights of LMM and rod were found to be 130000 and 220000, respectively. Both preparations appeared to be fairly homogeneous as evidenced by initial overlap of the various molecular weight moments as a function of local cell concentration (Figures 4 and 5 at each cell loading concentration). The downward curvature of the plots for M_n, M_w, and M_z at

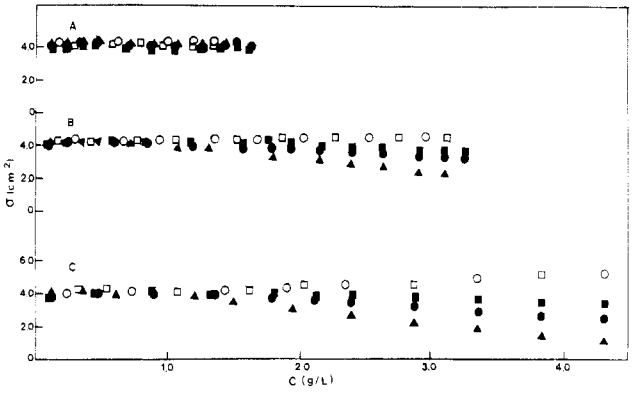

FIGURE 5: Molecular weight of myosin rod vs. concentration at three initial loading concentrations: (A) 0.1 mg/mL; (B) 0.3 mg/mL; (C) 1.0 mg/mL. Symbols are the same as those in Figure 4. Conversion factor = 5.24 × 10^4 daltons cm^2 (rpm = 12000).

Table I: Molecular Weights of Subfragments of Myosin

species	$NaDodSO_4$ gels	M_r (×10^{-3})	σ^a (×cm^2)
Mg·S1		130	4.3 ± 0.15
EDTA·S1		110	3.7 ± 0.10
S1(A1)	90000 (heavy chain)	112	5.9 ± 0.10
S1(A2)		106	5.7 ± 0.10
HMM	140000 (heavy chain)	350	4.6 ± 0.15
LMM	77000	130	4.3 ± 0.15
rod	120000	220	4.1 ± 0.15

[a] See the legend to Figure 2 for the definition of σ. Since there is some uncertainty in the literature values of ϕ', we have chosen to record the experimentally determined values of σ in addition to the molecular weights which depend on the choice of ϕ'. Values of σ for S1's and HMM were obtained by averaging values of σ_w for $c > 1.0$ g/L and for LMM and rod by averaging values of σ_{y1} and σ_{y2} for $c < 1.0$ g/L at the highest loading concentration.

higher concentrations shows the expected nonideality arising from excluded volume effects. Plots for M_{y1} and M_{y2}, which are independent of the second virial coefficient, are constant over nearly the entire cell with slight upward curvature at the base of the cell. This may be attributable to a small amount of dimerization (Harrington & Burke, 1972). These results, which are summarized in Table I, agree well with values measured previously by Lowey et al. (1969) and Harrington & Burke (1972) if one takes into account the different values of ϕ' used by each investigator.

Discussion

The homogeneity of the proteolytic subfragments of myosin in nondenaturing solvents has been investigated by high-speed sedimentation equilibrium. Particular emphasis was placed on the single- and double-headed species prepared either by papain or by chymotryptic digestion, since these subfragments are used most commonly in kinetic analyses and actin-binding experiments. In many studies, the homogeneity of these proteins has been taken for granted, but this assumption is not necessarily warranted. Subfragments of myosin are no more stable than the parent molecule, and myosin is well-known to have a strong tendency toward aggregation (Lowey & Holtzer, 1959).

The experiments reported here show that the subfragments of myosin can be obtained as homogeneous preparations in spite of their being products of proteolysis. The α-helical coiled coils, LMM and rod, appear to be homogeneous under nondenaturing conditions. The plots of apparent molecular weight distribution across the centrifuge cell for these fragments reveal only the nonideal behavior expected from ex-

cluded volume effects. It was rather unexpected to see that the chymotryptic subfragments S1(A1) and S1(A2), both of which appear very uniform and homogeneous on NaDodSO_4–polyacrylamide gels, exhibit slight heterogeneity in sedimentation equilibrium runs. In contrast, the papain subfragments, Mg·S1 and EDTA·S1, are highly homogeneous in nondenaturing solvents, despite the considerable proteolysis observed on NaDodSO_4 gels. Similarly, HMM was found to be very homogeneous in benign solvents, but showed extensive cleavages under denaturing conditions.

Although there have been many studies of the molecular weights of myosin and its subfragments [see reviews by Lowey (1979) and Harrington (1979)], this is the first detailed analysis of the homogeneity of the enzymatically active myosin subfragments. We conclude that they are remarkably uniform in size and by this criterion are suitable models for studying individual properties of myosin, such as the hydrolysis of ATP and the binding to actin.

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