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Monomers, Dimers, and Minifilaments of Vertebrate Skeletal Myosin in the Presence of Sodium Pyrophosphate†

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Received June 19, 1985

ABSTRACT: The self-assembly of myosin in the presence of sodium pyrophosphate was studied in the pH range between 7.0 and 8.5. As evidenced by sedimentation velocity ($s^0_{20,w}$ = 6.30 S) and light-scattering measurements (molecular weight of 470 000; radius of gyration = 45 nm), myosin existed in a predominantly monomeric form in the presence of 5 mM sodium pyrophosphate at pH 8.5 and above. The concentration-dependent monomer–dimer equilibrium could be easily shifted toward dimeric species at pH 8.0 in the presence of 5 mM sodium pyrophosphate and 5 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol. The estimated parameters of the dimeric particles were $s^0_{20,w}$ between 10 and 11 S, molecular weight of 1.1×10^6, and radius of gyration = 52 nm. These results are consistent with a head to tail (parallel) arrangement of staggered myosin molecules in the dimer. At lower pH values (7.5), and in the presence of 10 mM sodium pyrophosphate, the monomer–dimer species were in dynamic equilibrium with myosin minifilaments. At pH 7.0, the minifilaments appeared to be the only detectable species present in solutions of myosin in 5 mM sodium pyrophosphate. The molecular parameters of these minifilaments, including sedimentation and viscosity coefficients, molecular weight, radius of gyration, and morphological appearance, were almost indistinguishable from those obtained for myosin minifilaments prepared in 10 mM citrate–tris(hydroxymethyl)aminomethane at pH 8.0 [Reisler, E., Smith, C., & Seegan, G. (1980) *J. Mol. Biol. 143*, 129-145]. The equilibrium polymerization reactions of myosin in sodium pyrophosphate are discussed in the context of minifilament assembly.

It is frequently desirable to block or inhibit the self-association of myosin under physiological or low ionic strength conditions. The dissociated myosin could be particularly useful in studies on filament assembly, in comparative work aimed at assessing the role of myosin superstructure in its functional and structural properties, and in experiments analyzing the flexible regions and the behavior of the entire myosin molecule. Earlier work demonstrated the importance of ionic interactions to the assembly of myosin (Huxley, 1963; Josephs & Harrington, 1966; Kaminer & Bell, 1966; Katsura & Noda, 1971). The same conclusion about the dominant contribution of electrostatic forces to the formation of thick filaments was recently derived on the basis of the distribution of charged amino acids in the myosin rod (McLachlan & Karn, 1982, 1983). In view of these observations, charged ligands offer an obvious choice of reagents for suppressing the myosin polymerization reaction. In one study (Reisler et al., 1980), such a goal was achieved by substituting citrate–tris(hydroxymethyl)aminomethane (Tris) (10 mM) for more conventional buffer systems. In 10 mM citrate–Tris, the self-assembly of myosin is terminated upon formation of small bipolar minifilaments made of 16–18 myosin molecules. These minifilaments have indeed become useful in studies on catalytic, conformational, and assembly properties of myosin (Oriol-Audit et al., 1981; Reisler et al., 1982; Cheung & Reisler, 1983; Pastra-Landis et al., 1983; Margossian et al., 1983; Strzelecka-Golaszewska & Piwowar, 1984).

Among the better known and understood effectors of myosin assembly are nucleotides, nucleotide analogues, and pyrophosphate. These ligands bind with low affinity to the rod portion of myosin and shift its polymerization equilibrium toward monomeric species. The dissociation of both myosin filaments and minifilaments by nucleotides is highly cooperative and appears to involve a direct polymer–monomer transition (Harrington & Himmelfarb, 1972; Oriol-Audit et al., 1981). The reverse reaction, i.e., association of myosin in the presence of nucleotides or pyrophosphate, has been studied only by electron microscopy, and little is known about the reaction course except for its final products (Pinset-Harstrom & Triffy, 1979).

The dissociation of assembled myosin by pyrophosphate at alkaline pH has been particularly useful in probing the flexibility of the hinge region in myosin (Highsmith et al., 1977, 1982) and as a preliminary step in the preparation of the minifilament particles (Reisler et al., 1980). Our goal in this study was to characterize the assembly of myosin in the presence of pyrophosphate and to provide a well-defined structural basis for the application of this ligand in future work. We show that under low ionic strength conditions, and in the presence of 5 mM sodium pyrophosphate at pH 8.5, myosin

† This work was supported by U.S. Public Health Service Grants AM22031 (to E.R.) and GM-24034 (to J.A.L.) and by National Science Foundation Grant PCM 83-16926 (to J.A.L.).
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0006-2960/86/0425-0326$01.50/0

exists in a monomeric form. At pH 7.0, in the same buffer, myosin forms minifilament particles which are indistinguishable from the minifilaments obtained in 10 mM citrate–Tris at pH 8.0. At intermediate pH values, we detect the presence of myosin dimers, which at pH 7.5 appear to be in dynamic equilibrium with the minifilaments.

Materials and Methods

Preparation of Proteins. Rabbit skeletal myosin was prepared as described previously (Godfrey & Harrington, 1970). Prior to use, myosin was exhaustively dialyzed against 0.5 M KCl and 10 mM potassium phosphate (pH 7.0) and clarified by centrifugation. The protein was then dialyzed against solutions of 5 or 10 mM pyrophosphate adjusted to an appropriate pH. Following this dialysis, myosin was adjusted to a concentration between 4 and 5 mg/mL and centrifuged for 20 min at 27000*g*. The supernatant was used without any further purification. Although no attempt was made to remove the C protein, myosin processed in this way showed a somewhat reduced content of C protein on sodium dodecyl sulfate (SDS) gels [see also Reisler et al. (1980)].

Protein Concentration. The concentration of myosin solutions was determined spectrophotometrically by employing an extinction coefficient of $E^{1\%}_{280nm} = 5.55$ cm^{-1} (Godfrey & Harrington, 1970).

Analytical Ultracentrifugation. The sedimentation experiments were carried out at rotor temperatures close to 20 °C in a Spinco Model E analytical ultracentrifuge. At high protein concentrations (above 2.0 mg/mL), the sedimentation process was monitored by using the schlieren optical system. At low protein concentrations, the sedimentation measurements were done with the photoelectric scanning system of the Model E ultracentrifuge. In most cases, the sedimentation velocity experiments were run in 12-mm double-sector cells at rotor speeds between 20×10^3 and 30×10^3 rpm. These rotor speeds did not cause any dissociation of minifilaments obtained in 5 mM pyrophosphate at pH 7.0. The calculated sedimentation coefficients were reduced to standard conditions of water at 20 °C, and protein concentrations were corrected for radial dilution.

Viscosity Measurements. The viscosities of myosin solutions were measured as described previously (Reisler et al., 1980), in an Ostwald-type glass viscometer with a shear rate of 180 s^{-1} and an outflow time of 150 s for water at 15 °C. All protein solutions were clarified by centrifugation (12000*g* for 15 min) prior to viscosity measurements. The outflow time was measured 4–5 times for each solution. The temperature of the viscosity bath was regulated at 15 ± 0.02 °C with a Braun 1440 Thermomix and Lauda RC3 cooling unit. Reduced viscosities, η_{sp}/c, were calculated as described before (Reisler et al., 1980).

Light Scattering. Total intensity light scattering and diffusion coefficients were determined during the visit of one of us to the laboratory of Dr. H. Eisenberg in the Polymer Department at the Weizmann Institute of Science, Rehovot, Israel. The scattering data were collected at 20 °C by using a modified laser autocorrelation instrument (Malvern 4300, Precision Devices and Systems Ltd., Malvern, U.K.) as described by Jolly & Eisenberg (1976). All measurements were done with vertically polarized light ($\lambda = 514.5$ nm) and covered the angular range between 12° and 150°. The experimental system was routinely tested for linearity and alignment. The total intensity scattering data were presented in the form of Zimm plots (Zimm, 1948) in which $Kc/R(\theta)$ is plotted vs. $\sin^2(\theta/2) + kc$, where K is the optical constant given below, c is the protein concentration, $R(\theta)$ is the reduced scattering intensity (corrected for solvent contribution), and k is a constant chosen for a convenient presentation of the analyzed concentration range. In the limit of vanishing concentrations of macromolecules ($c \rightarrow 0$), $Kc/R(\theta) = 1/M_w$, where M_w is the weight-average molecular weight of the protein. The optical constant K is given by $K = 4\pi^2 n_B^2 (\partial n/\partial c)^2/\lambda^4 N_A R_B$, where n_B is the refractive index of benzene, $(\partial n/\partial c)_{T,\mu}$ is the refractive index increment for myosin at constant temperature and chemical potential (Herbert & Carlson, 1971), N_A is Avogadro's number, and $R_B = 29.9 \times 10^{-6}$ cm^{-1} is the absolute Rayleigh ratio of benzene for vertically polarized light at $\lambda = 514$ nm. From the angular dependence of scattering data, it is also possible to evaluate the radius of gyration, R_g, of the scattering particles according to the equation:

$$Kc/R(\theta) = 1/M_w P(\theta) \qquad (c \rightarrow 0)$$

where $P(\theta)$ is the particle scattering factor (Eisenberg, 1971). For small particles, R_g is obtained from a limiting slope (at $c \rightarrow 0$) of the Zimm plot (Eisenberg & Reisler, 1971).

Diffusion coefficients were determined from quasi-elastic laser light-scattering measurements as described previously (Jolly & Eisenberg, 1976; Reisler et al., 1980) and were corrected to standard conditions.

Electron Microscopy. Myosin minifilaments (in 5 mM pyrophosphate, pH 7.0) were cross-linked with dimethyl suberimidate prior to grid preparation. The cross-linking reaction was carried out for 2 h at 0 °C and at final protein and reagent concentrations of 1.0 and 2.0 mg/mL, respectively. The cross-linking reaction was terminated by the addition of ethanolamine and by a 40-fold dilution of the sample during its application onto the grid. The cross-linking prevented minifilament dissociation during their negative staining and, as verified by measurements of scattered light, did not change the size distribution of protein particles. Similar attempts to cross-link myosin species present in pyrophosphate at pH 7.5 or 8.0 were unsuccessful and caused rapid conversion of these particles into minifilaments.

Electron micrographs of cross-linked protein samples were taken at an operating voltage of 80 kV on a Phillips 400 microscope. Samples were negatively contrasted by a soluble-layer carbon procedure described by Lake (1979).

Results

The assembly of myosin in the presence of sodium pyrophosphate depends strongly on the concentration of H^+ ions in the solvent. Reproducible and homogeneous forms of assembly were routinely observed at pH 7.0 and 8.5, whereas the association of myosin between pH 7.5 and 8.0 was characterized by great sensitivity to experimental variables.

pH 8.5: Myosin Monomers. Sedimentation velocity experiments were used as the main probe for detecting the presence of myosin monomers in solutions of 5 mM sodium pyrophosphate. Typical results of such experiments carried out at pH 8.5 are shown in Figure 1. Under these conditions, the sedimentation coefficient of myosin extrapolates to $s^0_{20,w} = 6.30 \pm 0.2$ S, value close to that observed for monomeric myosin (Lowey, 1971). The positive concentration dependence of $1/s$ vs. c extends to protein concentrations of up to 2.0 and 3.0 mg/mL, indicating that the polymerization of myosin must be greatly reduced under these conditions. Both the intrinsic sedimentation coefficient and the $1/s$ vs. c dependence of myosin solutions are highly reproducible at pH 8.5. Increasing the concentration of pyrophosphate to 10 or 20 mM (at pH 8.5) does not affect the sedimentation velocity results.

To verify the monomeric nature of myosin in 5 mM pyrophosphate at pH 8.5, we have measured the angular depen-

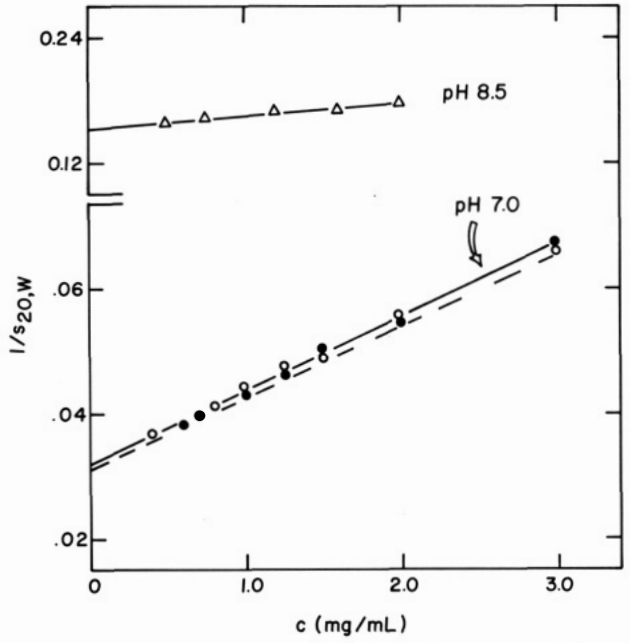

FIGURE 1: Sedimentation coefficients of myosin monomers (Δ) and minifilaments (O) in 5 mM sodium pyrophosphate at pH 8.5 and 7.0, respectively. For comparison, we show the sedimentation coefficients of myosin minifilaments prepared in 10 mM citrate–Tris at pH 8.0 (●, dashed curve).

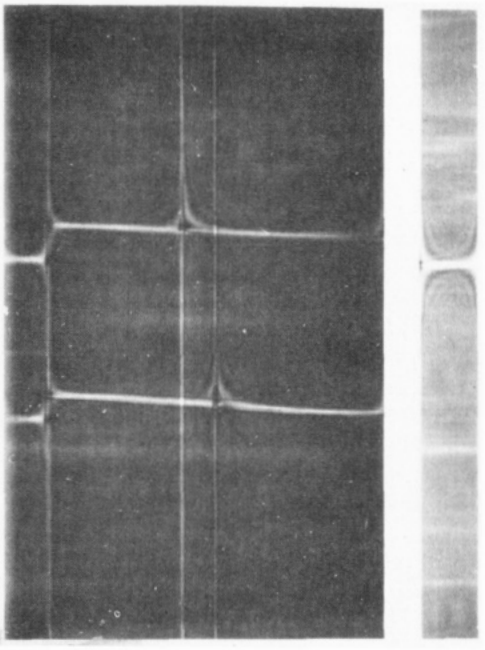

FIGURE 2: Velocity sedimentation patterns of myosin minifilaments in 5 mM sodium pyrophosphate at pH 7.0. Myosin solutions were run at concentrations of 2 mg/mL (lower, regular cell) and 3 mg/mL (upper, wedge cell) at 20 °C and at rotor speeds of 20000 rpm. The respective sedimentation coefficients were 18.0 and 15.2 S.

Table I: Molecular Properties of Myosin Assemblies

assembly form	solvent	hydrodynamic coeff	$M_w \times 10^{-6}$ [a]	R_g (nm)[a]
monomer	5 mM PP_i, pH 8.5	$s^0_{20,w}$ = 6.30 S	0.47 ± 0.02	45 ± 2
dimer	5 mM PP_i, 5 mM Bis-Tris, pH 8.0	$s^0_{20,w}$ = 10–11 S[b]	1.1 ± 0.05[b]	52 ± 3[b]
minifilament	5 mM PP_i, pH 7.0	$s^0_{20,w}$ = 31.5 S $[\eta]$ = 3.4 dL/g	8.6 ± 0.5	120 ± 10
minifilament	10 mM citrate–Tris, pH 8.0	$s^0_{20,w}$ = 32.2 S $[\eta]$ = 3.4 dL/g[c]	8.0 ± 0.4	99 ± 8

[a] Molecular weights and radii of gyration were obtained from measurements of the angular dependence of light scattered from myosin solutions. Molecular weights were determined from Zimm plots extrapolated to $c = 0$ and $\theta = 0$, and radii of gyration were obtained either from the limiting slope of Zimm plots or from the fit of the experimental data to scattering curves for thin rods. [b] Molecular parameters of myosin dimers were obtained from measurements carried out in the concentration range between 1.0 and 3 mg/mL. [c] Taken from Reisler et al. (1980).

dence of light scattered from protein solutions. The molecular weight, $M_w = 4.7 \times 10^5$, and radius of gyration, $R_g = 45$ nm (Table I), determined in these experiments agree well with the previous data on myosin monomers (Herbert & Carlson, 1971). Also, in analogy with sedimentation velocity experiments, the light-scattering measurements do not reveal any significant protein aggregation in 5 mM pyrophosphate at pH 8.5 or above (up to a myosin concentration of 1 mg/mL).

pH 7.0: Myosin Minifilaments. Sedimentation velocity profiles of myosin dialyzed against 5 mM pyrophosphate at pH 7.0 are analogous to those previously described for myosin minifilaments in 10 mM citrate–Tris at pH 8.0 (Reisler et al., 1980). Schlieren patterns of the pH 7.0 myosin (in pyrophosphate) have the form of hypersharp bars characteristic of a homogeneous solution of asymmetric polymer particles. These patterns do not reveal the presence of any additional myosin species except for the polymeric material (Figure 2).

The *s* vs. *c* dependence of myosin in 5 mM pyrophosphate at pH 7.0 coincides quite remarkably with that of myosin minifilaments in citrate–Tris and extrapolates to almost the same value for the intrinsic sedimentation coefficient, $s^0_{20,w} = 31.5$ S (Figure 1, Table I). Also, like the original minifilaments, the pH 7.0 particles do not dissociate in the analytical ultracentrifuge upon an increase in the hydrostatic pressure due to higher rotor speeds. Thus, according to sedimentation velocity data, myosin in 5 mM pyrophosphate in pH 7.0 is indistinguishable from myosin minifilaments. It should be noted that sedimentation coefficients of myosin at pH 7.0 are highly reproducible and show little experimental variation between different sample preparations.

The reduced viscosities of myosin in 5 mM pyrophosphate at pH 7.0 (not shown) extrapolate to an intrinsic viscosity value of $[\eta] = 3.4$ dL/g which is virtually identical with that of myosin minifilaments. We also observe a very similar concentration dependence of reduced viscosities of myosin in pyrophosphate and citrate–Tris.

Direct molecular weight determinations were made by measuring the light scattered from myosin solutions down to an angle of 12°. These experiments confirm that myosin in 5 mM pyrophosphate at pH 7.0 and the citrate–Tris (pH 8.0) minifilaments have the same mass. A Zimm plot for the former material extrapolates to $M_w = 8.6 \times 10^6$ (Figure 3), whereas the latter species yield $M_w = 8.0 \times 10^6$ (Table I). The pyrophosphate minifilaments appear somewhat more heterogeneous, as evidenced by their higher molecular weight and bigger radius of gyration (Table I). Althoug radii of gyration of such large assemblies could still be determined from the limiting slopes of Zimm plots (e.g., Figure 3), the molecular interpretation of these R_g values in terms of structural models (rods, coils, etc.) requires a more complex analysis of the angular dependence of scattering curves (Eisenberg & Reisler, 1971).

In excellent agreement with the physical evidence, electron micrographs of myosin assembled in pyrophosphate at pH 7.0 show that these particles are indistinguishable from the

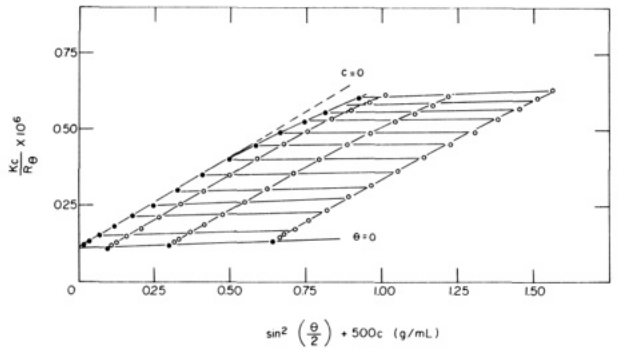

FIGURE 3: Zimm plot of total light-scattering intensity from solutions of myosin minifilaments in 5 mM sodium pyrophosphate (pH 7.0). K is the optical constant, c is protein concentration, R_θ is the reduced scattering intensity, and θ is the scattering angle. Protein concentrations ranged between 0.15 and 1.2 mg/mL, and the temperature was kept at 15.0 ± 0.1 °C.

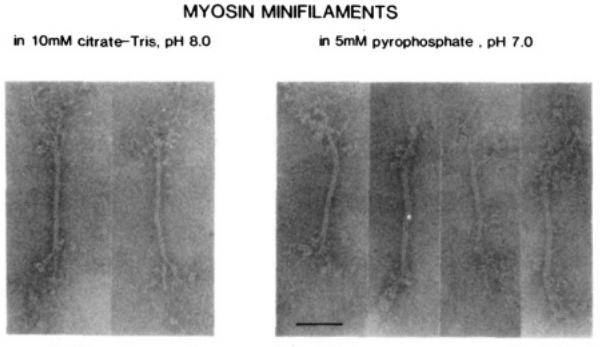

FIGURE 4: Representative myosin minifilaments prepared in 10 mM citrate–Tris at pH 8.0 (left panel) and in 5 mM sodium pyrophosphate at pH 7.0 (right panel). The minifilaments were cross-linked with dimethyl suberimidate prior to application onto the grid. All pictures were taken at the same magnification. The bar corresponds to 0.1 μm.

standard myosin minifilaments in citrate–Tris buffer (Figure 4). In fact, visual inspection of unlabeled fields containing the pyrophosphate and citrate–Tris minifilaments provides no clues as to their origin and conditions of preparation. Also, we do not detect any difference between minifilaments prepared from filamentous (in 0.13 M KCl) or monomeric (in 0.5 M KCl) myosin.

pH 8.0 and 7.5: From Myosin Monomers to Minifilaments. A decrease in the pH of the pyrophosphate buffer from 8.5 to 8.0 induces the association of myosin monomers to higher order species. At pH 8.0, the $s^0_{20,w}$ value of myosin in 5 mM pyrophosphate ranges between 6.5 and 7.2 S. Although these values are close to the intrinsic sedimentation coefficient of myosin at pH 8.5, the s vs. c dependence of protein solutions is significantly different under pH 8.0 and 8.5 conditions. Sedimentation velocity runs at pH 8.0 yield a family of $1/s$ vs. c curves with slopes varying from positive to negative values. The negative slopes are indicative of concentration-dependent association reaction, and the spread of $1/s$ curves attests to the sensitivity of myosin association at pH 8.0 to the exact experimental conditions. The extent of myosin polymerization appears to be rather limited, with the highest s values falling between 10 and 11 S in the concentration range of up to 3 mg/mL. Both the assembly of myosin and the spread of sedimentation coefficients at pH 8.0 are significantly reduced by increasing the concentration of pyrophosphate to 10 mM. Under these conditions, the value of $s_{20,w}$ is between 6.4 and 6.8 S.

An interesting feature of myosin assembly at pH 8.0 is best revealed in solutions buffered to pH 8.0 in the presence of 5 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (Bis-Tris) and 5 mM pyrophosphate. A characteristic $1/s$ vs. c dependence for such system is shown in Figure 5. Independent sets of experiments yield $1/s$ curves which are displaced by ±1 S from the solid curve in Figure 5. The dissociation of myosin into monomeric species at low protein concentrations is evidenced by the sharp upturn in the $1/s$ curve below 1.0 mg/mL. At the higher myosin concentrations, between 1.0 and 3.0 mg/mL, the sedimentation data can be fitted with a straight line which extrapolates to an $s^0_{20,w}$ value between 10 and 11 S (Figure 5). The positive slope of such straight line attests to minimal association of myosin beyond the 10S or 11S species in this range of protein concentrations. We reach the same conclusion on the basis of light-scattering experiments. Zimm plots constructed for scattering measurements on our best preparations (down to 1 mg/mL) extrapolate to $M_w = 1.1 \times 10^6$ (±5%) and R_g = 52 nm (±6%). The dimeric nature of the 10S and 11S particles is also indicated by their diffusion coefficient: $D_{20,w} = 0.92 \times 10^{-7}$ and 0.90×10^{-7} cm^2 s^{-1} at 1.3 and 1.0 mg/mL, respectively (Herbert & Carlson, 1975). Our attempts to stabilize the oligomeric myosin for the purpose of electron microscopy visualization were unsuccessful. Although dimethyl suberimidate and glutaraldehyde could cross-link myosin molecules, they induced much faster polymerization reactions and produced highly heterogeneous solutions.

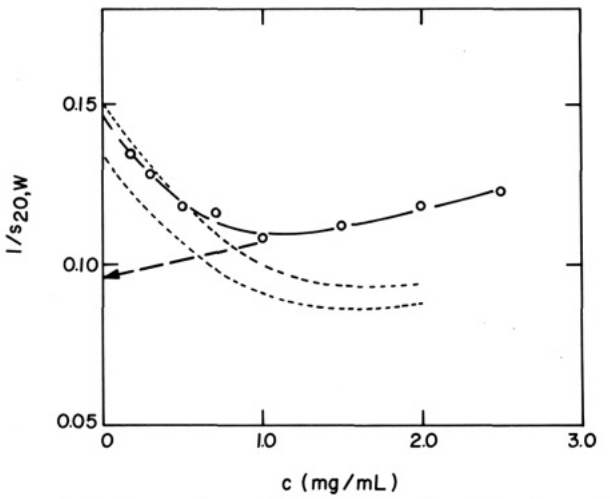

FIGURE 5: Sedimentation coefficients of myosin solutions in 5 mM sodium pyrophosphate and 5 mM Bis-Tris, pH 8.0 (○). Separate preparations of myosin in this solvent yield $1/s$ vs. c curves which are displaced by at most ± 1 S with respect to the solid curve. Extrapolation of the solid curve to $c = 0$ from measurements carried out in the concentration range between 1.0 and 3.0 mg/mL is indicated by an arrow and yields $s^0_{20,w} = 10.3$ S for the dimeric particles. In other experiments, similar extrapolations gave $s^0_{20,w}$ between 10 and 11 S. $1/s$ vs. c curves obtained for myosin solutions in 5 mM sodium pyrophosphate at pH 7.5 are confined between the two dashed curves. Each set of experiments produces a single $1/s$ vs. c curve, but different preparations produce a family of curves within the dashed area of the plot.

We were also unable to cross-link the 11S or 12S myosin species formed in 5 mM pyrophosphate at pH 7.5. The assembly of myosin at this pH resembles that observed at pH 8.0. Sedimentation coefficients of myosin solutions fall within the range indicated by dashed curves shown in Figure 5. The spread of sedimentation data reflects differences between separate preparations and sets of experiments, each one yielding a $1/s$ vs. c curve. The $1/s$ curves contained between the dashed lines show the dissociation of myosin into monomeric species at low protein concentrations and the preponderance of 11S or 12S particles between 1 and 2 mg/mL

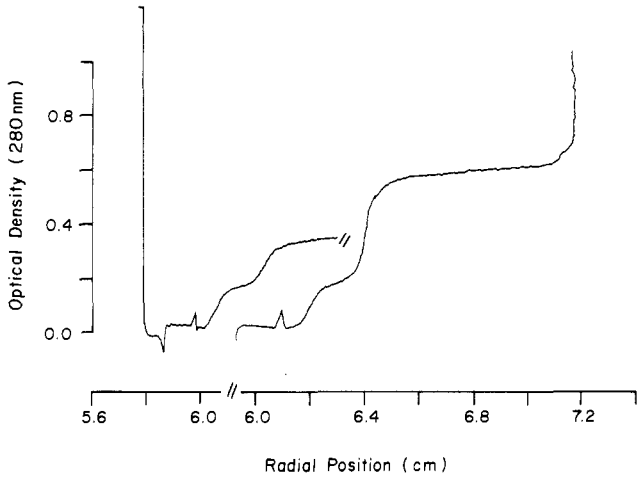

FIGURE 6: Sedimentation velocity scans of myosin in 10 mM sodium pyrophosphate at pH 7.5. The slowly sedimenting boundary corresponds to the monomer–dimer system and the fast boundary to myosin minifilaments. The sedimentation run was carried out at 20 °C at rotor speeds of 30 000 rpm.

concentrations. In contrast to pH 8.0 conditions, the association reaction at pH 7.5 proceeds to higher order species at higher protein concentrations. This is evident from sedimentation patterns of myosin solutions (for $c > 2$ mg/mL, not shown) and the absence of a distinct region of positive $1/s$ vs. c dependence in the pH 7.5 curves shown in Figure 5.

A striking effect on the association of myosin at pH 7.5 is produced by increasing the concentration of pyrophosphate to 10 mM. Sedimentation velocity patterns of such solutions reveal the presence of at least two components. The fast and slowly sedimenting materials appear to be in a dynamic, concentration-dependent equilibrium (Figure 6). The larger particles yield $s^0_{20,w} = 34$ S and thus correspond to myosin minifilaments (Figure 7). The smaller species sediment at rates corresponding to s values between 7.0 (at $c = 0$) and 8.5 S (Figure 7) and most likely represent a monomer–dimer equilibrium system. The concentration dependence of this last equilibrium is evident from the positive slope of the $1/s$ curve corresponding to the monomer–dimer material (upper curve in Figure 7). However, since the scale employed in Figure 7 refers to total protein concentration, and not that of monomer–dimer species, the s vs. c dependence is significantly underestimated in that plot. Although the monomer–dimer species appears to be in dynamic equilibrium with the minifilaments (Figure 6), we cannot exclude the existence of small quantities of other intermediate forms of assembly on the basis of the recorded sedimentation boundaries.

Discussion

Several recent studies described the dissociation of native and synthetic myosin filaments into stable, short, bipolar assemblies of myosin (Reisler et al., 1980; Ishiwata, 1981; Trinick & Cooper, 1980; Niederman & Peters, 1982). In spite of different experimental conditions employed in these studies, and the somewhat different final dissociation products observed by the various authors, there appears to be a common factor responsible for the "graded" dissociation–association of myosin. Such assembly behavior is consistent with the presence of different bonding interactions within the central and distal portions of the filament. A similar conclusion can be deduced from kinetic dissociation experiments (Davis, 1981), which distinguish between two separate, pressure-sensitive and -insensitive, phases of the assembly reaction.

Our present interest in the effects of PP_i on the assembly of myosin was stimulated by the reported partial dissociation

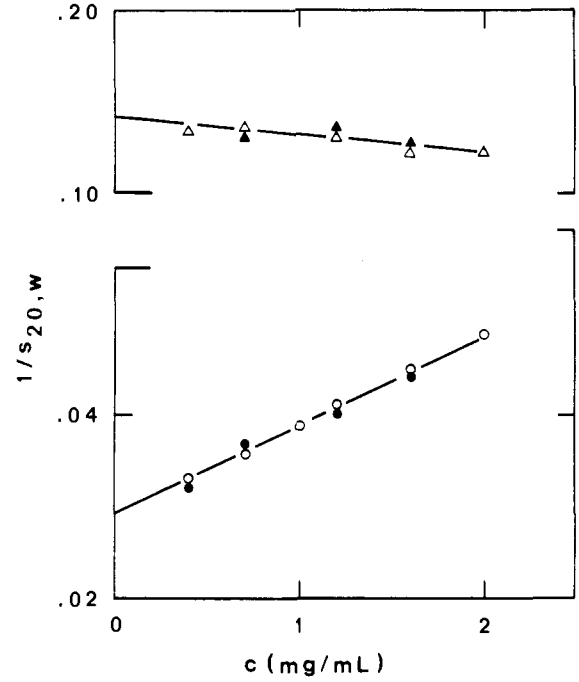

FIGURE 7: $1/s$ vs. c plots for the slow- (▲, △) and fast-sedimenting species (○, ●) in solutions of myosin in 10 mM sodium pyrophosphate at pH 7.5. The slowly sedimenting monomer–dimer system extrapolates to $s^0_{20,w} = 7.0$ S; the fast-sedimenting minifilaments yield $s^0_{20,w} = 34$ S. Total protein concentrations, and not those of the individual species, were used in this plot. Thus, the extrapolations to $c = 0$ do not provide an accurate estimate of the intrinsic coefficients of the different particles and are used only for their identification.

of thick filaments in myofibrils (Ishiwata, 1981) and muscle fibers (Ishiwata et al., 1985) in the presence of this ligand. One goal of the present study was to compare, under well-controlled in vitro conditions, the products of PP_i-induced filament dissociation with the previously described myosin minifilaments. The results of this work demonstrate that myosin forms the same minifilament structures in 5 mM pyrophosphate (pH 7.0) and 10 mM citrate–Tris (pH 8.0). Not only are the intrinsic parameters of myosin virtually identical in these two solvents but also the concentration dependence of the hydrodynamic coefficients (s, η) is very similar, in spite of different ionic conditions. The presence of myosin minifilaments in 5 mM PP_i (pH 7.0) adds considerable credence to a claim that the formation of these structures is an inherent property of the myosin assembly reaction, and not a peculiarity of a specific ion or solvent.

Although electron microscopy and hydrodynamic methods do not reveal any significant differences between the PP_i (pH 7.0) and the citrate–Tris (pH 8.0) minifilaments, light-scattering measurements indicate slightly greater size heterogeneity of the former minifilaments. This is expressed in the values of molecular weight (weight average) determined from Zimm plots, and to a greater extent in the radii of gyration (z average) for the two types of minifilaments. It should be noted that radii of gyration obtained from Zimm plots for structures of minifilament size have only a comparative value. For large structures, the theoretical scattering curves are model dependent, and the experimental data need to be fitted to curves calculated for thin rods, coils, spheres, etc. (Eisenberg, 1971). The data on citrate–Tris minifilaments produced a reasonably good fit (between 12° and 90°) to curves for thin rods with $R_g = 99$ nm, whereas PP_i showed a poorer fit to thin rods with $R_g = 120$ nm. The extent of such fit depends not only on the size heterogeneity of the sample but most of all on the validity

of the geometrical model chosen to represent the molecule. Thus, assuming that minifilaments are best represented by thin rods, the quality of each approximation may depend on the disposition of cross-bridges in each structure.

The second aim of this work was to characterize the dissociated myosin (in 5 mM PP_i, pH 8.0) used in preparations of minifilaments (Reisler et al., 1980) and in other conformational studies. We show in this work that myosin exists in a concentration- and pH-dependent monomer–dimer equilibrium around pH 8.0. Yet, it is possible that small amounts of higher order species are present in such monomer–dimer systems and that at higher protein concentrations (>3 mg/mL) the oligomeric material would be more abundant. At more alkaline pH conditions (pH 8.5), the dimerization reaction is suppressed, and all the measured parameters (molecular weight, s, R_g) are characteristic of monomeric myosin.

The molecular properties of myosin dimer are estimated by extrapolating to $c = 0$ the measurements carried out between 1.0 and 3.0 mg/mL protein. Such extrapolation is dictated by the specific association reaction of myosin in 5 mM PP_i and 5 mM Bis-Tris (pH 8.0). This includes the dissociation of dimers to monomers below 1 mg/mL and the apparent stability and preponderance of dimers in the chosen range of protein concentrations. On the basis of the concentration dependence of sedimentation and scattering data, we assume that, in contrast to pH 7.5 conditions, higher order species are either absent or contribute very little to the properties of myosin solutions in the PP_i/Bis-Tris solvent. Although the above extrapolation procedure is inherently imprecise, and in addition we note some preparation-dependent variations in polymerization of myosin (Figure 5), it is possible to assign an intrinsic sedimentation coefficient between 10 and 11 S for the dimeric particles. The best light-scattering results, obtained for solutions which conform to the $1/s$ vs. c dependence shown in Figure 6, yield R_g = 52 nm for the dimeric protein.

The experimental ratio $R_{g,dimer}/R_{g,monomer} = 1.15$ provides an indication of dimer geometry. Assuming that the radius of gyration of the myosin head is 3.3 nm (Mendleson & Kretzschmar, 1980) and the tail portion can be represented by a 144-nm-long thin rod (Sutoh et al., 1978), we calculate $R_{g,dimer}/R_{g,monomer} = 1.13$ and 1.38 for parallel (head to tail) and antiparallel dimers with a 43-nm stagger. The antiparallel, fully overlapping dimer cannot be distinguished on the basis of R_g ratios from the staggered parallel geometry. However, the bulk of literature data supports the parallel arrangement (Burke & Harrington, 1972; Herbert & Carlson, 1971; Reisler et al., 1973; Davis et al., 1982).

We can easily envisage that antiparallel assembly of parallel myosin dimers will lead to the formation of myosin minifilaments (Pepe, 1982). A dynamic equilibrium between the monomer–dimer system and the minifilament particles is established at pH 7.5 in the presence of 10 mM PP_i (Figure 6). This equilibrium suggests, but does not prove, that dimeric myosin is used as an assembly material. Unstable higher order species, such as myosin tetramers and octamers, which were found in a separate study in citrate–Tris buffer (Reisler et al., 1986), if present in small amounts in the monomer–dimer–minifilament equilibrium in PP_i (pH 7.5), escaped our detection. Notably, when higher order species are formed at pH 7.5 in the presence of reduced concentrations of PP_i (Figure 5), such assembly "overshoot" appears to occur at the expense of minifilament formation. Of course, these aggregates need not be the same as the tetramers and octamers mentioned above. Thus, the simplest interpretation of the myosin assembly into minifilaments in pyrophosphate is that of two main equilibrium polymerization reactions, both strongly pH dependent. The initial reaction leads to the formation of parallel myosin dimers, which are then used in a second reaction to yield myosin minifilaments. The involvement of transient, unstable assembly intermediates in the second reaction could not be substantiated in this work.

ACKNOWLEDGMENTS

We are grateful to Dr. H. Eisenberg for making his laboratory in the Polymer Department at the Weizmann Institute of Science (Rehovot, Israel) available to us for light-scattering experiments and for his help and hospitality to one of us (E.R.). We thank J. Washizaki for electron microscopy and photography.

Registry No. Sodium pyrophosphate, 10042-91-8.

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Active-Site Mutants of β-Lactamase: Use of an Inactive Double Mutant To Study Requirements for Catalysis†

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ABSTRACT: We have studied the catalytic activity and some other properties of mutants of *Escherichia coli* plasmid-encoded RTEM β-lactamase (EC 3.5.2.6) with all combinations of serine and threonine residues at the active-site positions 70 and 71. (All natural β-lactamases have conserved serine-70 and threonine-71.) From the inactive double mutant Ser-70 → Thr, Thr-71 → Ser [Dalbadie-McFarland, G., Cohen, L. W., Riggs, A. D., Morin, C., Itakura, K., & Richards, J. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6409–6413], an active revertant, Thr-71 → Ser (i.e., residue 70 in the double mutant had changed from threonine to the serine conserved at position 70 in the wild-type enzyme), was isolated by an approach that allows identification of active revertants in the absence of a background of wild-type enzyme. This mutant (Thr-71 → Ser) has about 15% of the catalytic activity of wild-type β-lactamase. The other possible mutant involving serine and threonine residues at positions 70 and 71 (Ser-70 → Thr) shows no catalytic activity. The primary nucleophiles of a serine or a cysteine residue [Sigal, I. S., Harwood, B. G., & Arentzen, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 7157–7160] at position 70 thus seem essential for enzymatic activity. Compared to wild-type enzyme, all three mutants show significantly reduced resistance to proteolysis; for the active revertant (Thr-71 → Ser), we have also observed reduced thermal stability and reduced resistance to denaturation by urea.

The application of in vitro mutagenesis techniques to problems in enzymology has recently acquired momentum as it allows study of diverse problems in protein biochemistry through the experimental tools of molecular biology. Such problems include studies of the role of specific residues in catalysis as in the case of trypsin and chymotrypsin (Craik et al., 1984), dihydrofolate reductase (Villafranca et al., 1983), tyrosyl-tRNA synthetase (Winter et al., 1982; Wilkinson et al., 1984), and β-lactamase (Dalbadie-McFarland et al., 1982; Sigal et al., 1982, 1984), the role of disulfide bridges in protein stability (Villafranca et al., 1983; Perry et al., 1984; Dalbadie-McFarland, 1985), and the structural requirements for functional leader sequences in the secretion of proteins such as β-lactamase (Kadonaga et al., 1984) and the outer membrane lipoprotein of *Escherichia coli* (Inouye et al., 1982). In this approach, the strategy of specific in vitro mutagenesis allows a systematic study of structural variants resulting from precise amino acid substitution(s) introduced within a protein in a rational manner. In a complementary strategy, requiring a selectable phenotype, one demands a particular function of a protein, generates structural variants by various techniques of random mutagenesis, and then screens the resultant variants for those that can perform the required function.

In a combination of these two strategies, specific in vitro mutagenesis can be used first to produce an inactive protein; then numerous methods can generate random mutants that can be screened for recovery of activity which can reflect either changes at the site of the original mutation(s) or changes at other sites. Implementation of this approach can be particularly effective if reversion from the inactive mutant to the wild-type enzyme is prevented as this avoids screening against a background of many active revertants which simply have regained the sequence, structure, and function of the parental protein. In achieving this objective, one can use, as the inactive progenitor, a mutant that requires at least two base changes to re-form the wild-type and allow random mutagenesis to occur under conditions in which only single base changes are likely in the mutants being screened.

An enzyme of considerable medical significance and

†Contribution No. 7264 from California Institute of Technology. This work was supported by a grant from the National Institutes of Health (GM 16424). G.D.-M. and J.J.N. were supported by NRSA Grant GM 07626.

0006-2960/86/0425-0332$01.50/0